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Sébastien This

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**Régulation des réponses immunitaires  
adaptatives par l'intégrine  $\alpha_v\beta_8$  -  
Implications pour l'immunité des  
muqueuses et la réponse humorale**

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Devant le jury composé de :

Espéli, Marion  
Agace, William  
Aloulou, Meryem  
Dalod, Marc  
Nancey, Stéphane  
Païdassi, Helena  
Defrance, Thierry

CRCN  
Professor  
CRCN  
DR1  
PU-PH  
CRCN  
DR2

Institut de Recherche Saint-Louis  
Lund University  
Centre de Physiopathologie Toulouse Purpan  
Centre d'immunologie de Marseille-Luminy  
Centre International de Recherche en Infectiologie  
Centre International de Recherche en Infectiologie  
Centre International de Recherche en Infectiologie

Rapporteure  
Rapporteur  
Examinatrice  
Examinateur  
Examinateur  
Directrice de thèse  
Membre Invité

# Abstract

The ability of a host to generate an appropriate immune response is critical to provide protection against a particular pathogen and to provide long-lasting memory against future reinfection. However, this immune response must be tightly regulated to prevent its persistence or inadequate activation which can lead to the development of immune pathologies. Mammalian immune system comprises a wide array of immune cells and molecules. In particular, the ability of immune cells to secrete and respond to cytokines is central to the orchestration of immune responses.

My PhD project has focused on the role of a particular cytokine named Transforming Growth Factor  $\beta$  (TGF $\beta$ ). Unlike most other cytokines, TGF $\beta$  is secreted in a latent form and must be activated to bind its receptor and induce response on target cell. Our team and others have shown that  $\alpha_v\beta_8$  integrin plays a critical role in TGF $\beta$  activation and thus the regulation of TGF $\beta$ -dependent immune responses.

More precisely, I investigated the role of  $\alpha_v\beta_8$  integrin in the regulation of intestinal immunity and humoral B cell responses. In particular, my work focused on three immune processes: 1/ the induction of T<sub>REG</sub> and T<sub>H17</sub> in Mucosal Associated Lymphoid Tissues and 2/ the regulation of intestinal IgA humoral responses and 3/ the regulation of T-dependent B cell responses during the germinal center reaction.

This PhD consists of 3 main parts:

1. Regulation of TGF $\beta$ -dependent intestinal immune responses by  $\alpha_v\beta_8$  expressed by conventional dendritic cells (cDC). Thanks to a new reporter mouse model for  $\beta_8$  expression, we establish the precise expression pattern of this integrin in cDC. Then we analyze the relative contribution of  $\alpha_v\beta_8$  expressed by type 1 (cDC1) and type 2 (cDC2) cDC in the regulation of intestinal T<sub>REG</sub> differentiation via TGF $\beta$  activation. We show that both cDC1 and cDC2 are partially involved in the induction of T<sub>REG</sub> differentiation through  $\alpha_v\beta_8$  expression. Our results indicate a cooperation between these two DC subsets for TGF $\beta$  activation and thus regulation of intestinal T cell responses.
2.  $\alpha_v\beta_8$  integrin-expression by BATF3-dependent dendritic cells facilitates early IgA responses to Rotavirus. In this study, published in *Mucosal Immunology*, we show that  $\alpha_v\beta_8$  expression by cDC1 is required for the optimal induction of IgA responses in the context of Rotavirus infection. We show that  $\alpha_v\beta_8$  expression by cDC1 and their ability to activate TGF $\beta$  is one of the mechanisms by which they are licenced to promote IgA B cell responses to rotavirus infection.
3. Investigating the relevance of  $\alpha_v\beta_8$  integrin expression by follicular T cell for the TGF $\beta$ -mediated control of B cell responses. In this part of the study, I report our surprising discovery that follicular T cells (T<sub>FH</sub> and T<sub>FR</sub>) express  $\alpha_v\beta_8$  integrin. This section describes the efforts made during my PhD to understand the functional relevance of  $\alpha_v\beta_8$  expression by T<sub>FH</sub> and T<sub>FR</sub> in the regulation of B cell response during the Germinal Center Reaction.

# Résumé Français

À la suite d'une infection, la capacité de l'hôte à produire une réponse immunitaire efficace et adaptée est cruciale pour se défendre et générer une réponse mémoire permettant de se protéger contre une future réinfection. En revanche, il est absolument nécessaire que cette réponse soit correctement régulée afin d'éviter sa persistance ou son activation incontrôlée pouvant mener au développement de maladies immunitaires. Le système immunitaire comprend un ensemble complexe de cellules et de molécules immunitaires. En particulier, la capacité des cellules immunitaires à produire et à répondre aux cytokines est absolument critique pour l'orchestration des réponses immunitaires.

Mon travail de thèse s'est intéressé à une molécule particulière, la cytokine Transforming Growth Factor  $\beta$  ou TGF $\beta$ . Contrairement à la plupart des autres cytokines, le TGF $\beta$  est produit sous une forme latente et doit être activé afin de pouvoir se lier à son récepteur et induire une réponse sur la cellule cible. Nous avons montré par le passé que l'intégrine  $\alpha_v\beta_8$  joue un rôle clé dans le processus d'activation du TGF $\beta$  et ainsi dans la régulation des réponses immunitaires dépendantes du TGF $\beta$ .

Plus précisément, j'ai cherché au cours de ma thèse à comprendre le rôle de l'intégrine  $\alpha_v\beta_8$  dans la régulation des réponses immunitaires intestinales et la régulation des réponses humorales. Mon travail s'est plus particulièrement concentré sur la compréhension de trois processus : 1/ l'induction de lymphocytes T<sub>REG</sub> et T<sub>H17</sub> dans l'intestin nécessaires au maintien de l'homéostasie immunitaire intestinale, 2/ la régulation des réponses humorales de classe A (IgA) intestinales et 3/ la régulation des réponses humorales T-dépendantes au cours de la réaction du Centre Germinatif.

Cette thèse peut être séparée en 3 grandes parties :

1. Régulation des réponses intestinales TGF $\beta$ -dépendantes par  $\alpha_v\beta_8$  exprimée par les cellules dendritiques conventionnelles (cDC). Grâce à un nouveau modèle murin rapporteur de l'expression de l'intégrine  $\beta_8$ , nous établissons le profil d'expression précis de cette intégrine dans les cDC. Puis, nous analysons la contribution relative de l'intégrine  $\alpha_v\beta_8$  exprimée par les DC de type 1 (cDC1) et de type 2 (cDC2) pour la différenciation des lymphocytes T<sub>REG</sub> dans l'intestin via l'activation du TGF $\beta$  par l'intégrine  $\alpha_v\beta_8$ . Nous montrons que les cDC1 et les cDC2 sont partiellement impliqués dans l'induction des T<sub>REG</sub> intestinaux grâce à leur expression de l'intégrine  $\alpha_v\beta_8$ . Nos résultats indiquent une coopération entre les sous-types de DC pour l'activation du TGF $\beta$  et ainsi la régulation des réponses T intestinales.
2. «  $\alpha_v\beta_8$  integrin-expression by BATF3-dependent dendritic cells facilitates early IgA responses to Rotavirus ». Dans cette étude, publiée dans 'Mucosal Immunology', nous montrons que l'expression de l'intégrine  $\alpha_v\beta_8$  par les cDC1 est nécessaire pour permettre l'induction optimale des réponses IgA suite à une infection par le Rotavirus.
3. L'intégrine  $\alpha_v\beta_8$  est exprimée par les lymphocytes T folliculaires. Dans cette partie du manuscrit, je relaie notre découverte surprenante que les lymphocytes T folliculaires expriment l'intégrine  $\alpha_v\beta_8$ . Je décris les efforts déployés pour comprendre l'importance fonctionnelle de l'expression d' $\alpha_v\beta_8$  pour la régulation des réponses B lors de la réaction du Centre Germinatif.

# Abbreviations

## Diseases

IBD	Inflammatory Bowel Disease
AHR	Airway HyperResponsiveness
GVHD	Graft-Versus-Host Disease

## Organs

GALT	Gut Associated Lymphoid Tissues
MALT	Mucosal Associated Lymphoid Tissues
MLN	Mesenteric Lymph Nodes
PP	Peyer's Patches
LP	<i>Lamina propria</i>
SILP	Small Intestine <i>Lamina propria</i>
CLP	Colon <i>Lamina propria</i>
SPL	Spleen
DLN	Draining Lymph Nodes
BM	Bone Marrow
SED	SubEpithelial Dome

## Cellular processes

GCR	Germinal Center Reaction
GC	Germinal Center
DZ	Dark Zone
LZ	Light Zone
CSR	Class Switch Recombination
SHM	Somatic HyperMutation

## Cells

APC	Antigen Presenting Cell
pDC	Plasmacytoid Dendritic Cell
FDC	Follicular Dendritic Cell
cDC	Conventional Dendritic Cell
moDC	Monocytes Derived Dendritic Cell
Tip-DC	TNF/iNOS Producing Dendritic Cell
LC	Langerhans Cell
T <sub>FH</sub>	Follicular Helper T cell
T <sub>FR</sub>	Follicular Regulatory T cell
T <sub>REG</sub>	Regulatory T cell
T <sub>H</sub>	Helper T cell
T <sub>CONV</sub>	Conventional T cell
PC	Plasma cell
mBC	Memory B cell
IEL	IntraEpithelial Lymphocyte

## Molecules

IgA	Class A Immunoglobulin
IgG	Class G Immunoglobulin
ECM	ExtraCellular Matrix
SLC	Small Latency Complex
LLC	Large Latency Complex
LAP	Latent Associated Peptide
LTBP	Latent TGF $\beta$ Binding Protein
GARP	Glycoprotein-A Repetitions Predominant
DAMP	Danger Associated Molecular Pattern
RA	Retinoic acid
TLR	Toll-Like Receptor
BAFF	B-cell Activating Factor
APRIL	A Proliferation-Inducing Ligand
FoxP3	Foxhead box P3
TGF $\beta$	Transforming Growth Factor $\beta$
TGF $\beta$ R	Transforming Growth Factor $\beta$ Receptor
BCR	B cell receptor
TCR	T cell receptor
MHC	Major Histocompatibility Complex
OVA	Ovalbumin
HDM	House Dust Mice

## Other

UTR	UnTranslated Region
ER	Endoplasmic Reticulum
KO	Knock-Out

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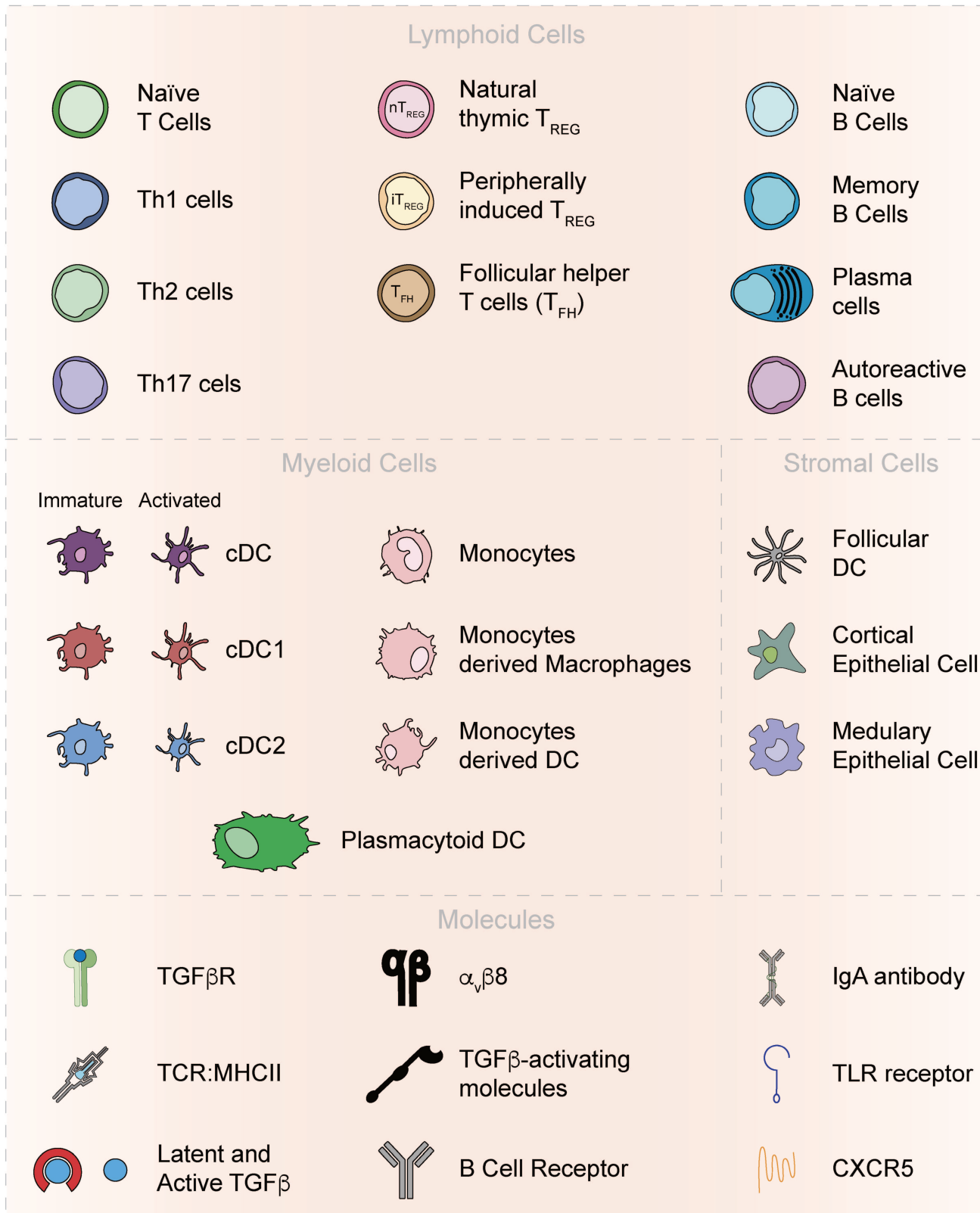


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# Introduction

Historically, the Transforming Growth Factor- $\beta$  (TGF $\beta$ ) was simultaneously identified by several teams in 1978 and 1981, investigating the ability of secreted molecules from virally transduced fibroblasts to promote cell transformation and formation of growth colonies into soft agar. In the early 1980's, the growth inhibitory functions of TGF $\beta$  had been well characterized. Large scale commercial purification of the TGF $\beta$  molecule and the cloning of the TGF $\beta$  gene have greatly participated in the identification of TGF $\beta$  structure and function. In the late 1980's to early 1990's, the three isoforms of the TGF $\beta$  molecules (TGF $\beta$ -1, TGF $\beta$ -2 and TGF $\beta$ -3) had been described, the TGF $\beta$  receptor (TGF $\beta$ R) had been cloned and the members of the TGF $\beta$  superfamily were starting to be identified. The study of TGF $\beta$  also started to expand to other fields of biology such as wound healing, fibrosis and carcinogenesis ever expanding the biological functions of TGF $\beta$ . The extent of the described functions of TGF $\beta$  to this day would be too large to list exhaustively. However, it has been shown that TGF $\beta$  is involved in the inhibition of proliferation, in the promotion of apoptosis, cell differentiation, chemotaxis and immune cell function, and in the accumulation of Extracellular Matrix (ECM). The historical aspects of TGF $\beta$  structure, family and function is extensively reviewed in (Moses, Roberts, and Derynck 2016a).

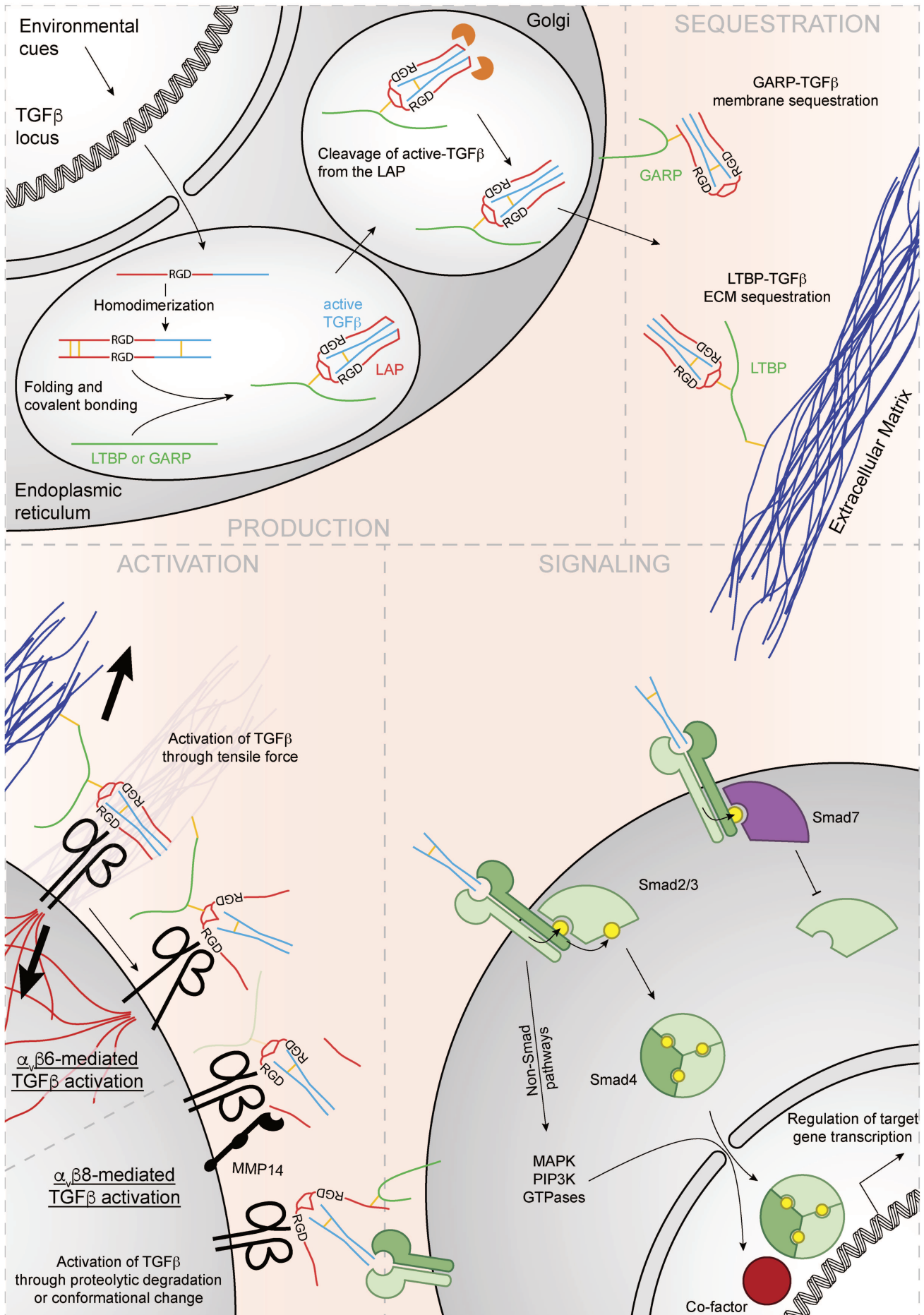
As early as 1984, the notion of TGF $\beta$  latency was suspected. It was shown that TGF $\beta$  was secreted from cells in an inactive form and required activation through acidification to induce any biological function. Other TGF $\beta$  activating mechanisms were rapidly identified (chaotropic agents, boiling, proteolysis, ...) (Moses, Roberts, and Derynck 2016a). It is now widely accepted that TGF $\beta$  is encapsulated in a latent peptide, and that the secreted protein requires activation through various mechanisms in order to allow TGF $\beta$  binding to TGF $\beta$ R and promote downstream signaling on target cells.

In this Introduction, I will dissect the mechanisms by which the TGF $\beta$  cytokine and its activation by integrins regulate immune responses. This part is subdivided in 3 sections which will focus on : 1/ the molecular mechanisms by which latent-TGF $\beta$  is secreted, activated and presented to target cells and its downstream signaling pathways, 2/ The importance of integrin-mediated TGF $\beta$  activation in the maintenance of intestinal homeostasis and 3/ the importance of TGF $\beta$  activation and signaling in the regulation of B cell responses.

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## **Figure 1 - TGF $\beta$ production, activation by integrins and signaling.**

Upon perception of the environmental cues, transcription of the TGF $\beta$  locus produces a proTGF $\beta$  peptide containing the active-TGF $\beta$  molecule and the LAP which dimerizes. In the ER, association with the LTBP or GARP enhances proper folding of the latent complex. In the Golgi, enzyme cleaves the active-TGF $\beta$  from the LAP. Upon secretion, the Large Latency Complex is sequestered to the ECM through binding of the LTBP or anchored in the plasma membrane by GARP. Activation of the TGF $\beta$  molecule is realized by  $\alpha_v$  integrins such as  $\alpha_v\beta_6$  and  $\alpha_v\beta_8$  among other mechanism.  $\alpha_v\beta_6$  activation mechanisms relies on the tensile force created by contraction of the cytoskeleton and anchoring of TGF $\beta$  in the ECM.  $\alpha_v\beta_8$  activation mechanisms relies on the recruitment of metalloproteases such as MMP14. Alternatively, conformational changes of the latent TGF $\beta$  molecule can allow the presentation of the active-TGF $\beta$  molecule to the TGF $\beta$ R without release of the active molecule from the latent complex. Upon binding to the TGF $\beta$ R, the Smad, MAPK, Small GTPases and PIP3K pathways are activated to instruct regulation of TGF $\beta$  target genes.



# 1. Physiological activation of the latent-TGF $\beta$ cytokine is required for downstream signaling.

Because of its important immunomodulatory effects and importance for immune cell differentiation, TGF $\beta$  is a widely studied cytokine. However, studies on the regulation of immune responses by TGF $\beta$  have often been limited to the analysis of its secretion and its downstream effects. The latent nature of this cytokine is often overlooked, which can lead to some misunderstanding of the potential role of TGF $\beta$  and its regulation.

Because of its latent properties, it is very important to properly understand the four important steps of TGF $\beta$  biology which are as follow: production, sequestration and localization, activation and signaling. All these 4 steps involve several particular biological processes that I will develop in this section of the manuscript.

## 1.1. TGF $\beta$ is produced and secreted in a latent form.

Like most cytokines, production of the TGF $\beta$  cytokine, is tightly regulated and results from the integration of various external signals, resulting in the induction of TGF $\beta$  transcription, translation and secretion. Because TGF $\beta$  is secreted by a very wide variety of immune and non-immune cells, in a lot of different contexts, the pathways by which its expression is regulated are likely diverse. Furthermore, this aspect of TGF $\beta$  biology has not been very well documented. A few studies in restricted experimental conditions however suggest that some canonical pathway for TGF $\beta$  expression are conserved such as the p38, Erk and Jnk MAP Kinase pathways (M. Zhang, Fraser, and Phillips 2006; Xiao et al. 2008). This is not very surprising given the importance of these pathways in the downstream signaling of most cytokine receptors. Furthermore, a few mechanisms for post-transcriptional regulation of TGF $\beta$  expression through micro-RNA or 5' UTR loop motif have been described (R. H. Jenkins et al. 2010; Martin et al. 2011).

TGF $\beta$  transcription and translation produce a large proprotein containing both the active portion of the TGF $\beta$  molecule and the Latency-Associated Peptide (LAP) (Fig. 1). In the endoplasmic reticulum (ER), this proprotein homodimerizes hence forming the proTGF $\beta$  molecule. In the Golgi, the mature TGF $\beta$  molecule is cleaved from the LAP by furin-type enzymes but remains non-covalently associated with the LAP until activation. Together, the active-TGF $\beta$  and the LAP forms the Small-Latency Complex (SLC) also called latent-TGF $\beta$ . Early association of the LAP with the Latent-TGF $\beta$  Binding Protein (LTBP) through disulfide bonds allows proper folding of the proTGF $\beta$  molecule. Altogether the TGF $\beta$ , LAP and LTBP form the Large-Latency Complex (LLC). The LTBP proteins (4 distinct proteins) have specialized cysteine-rich domains which form multiple protein-protein bonds. The protein binding ability of the LTBP allows the binding of LLC to the extracellular matrix (ECM) and its sequestration in tissues in the ECM (Reviewed in (Annes, Munger, and Rifkin 2003; Robertson and Rifkin 2016)).

In the last 10 years, the Glycoprotein-A repetitions predominant protein (GARP) has emerged as another TGF $\beta$  binding molecule, replacing LTBP in the LLC. GARP, encoded by the *Lrrc32* gene, is a transmembrane protein with a large extracellular domain. This domain contains multiple Leucine Rich Repeat domains which can form disulfide bonds with the LAP of the SLC. Much like for the LTBP, GARP facilitates the maturation of the SLC in the ER/Golgi by enhancing the cleavage of proTGF $\beta$  in a furin-independent manner. Furthermore, GARP can

compete with LTBP for SLC binding thanks to its higher affinity to the LAP. Because GARP is a trans-membrane protein, binding to the SLC allows presentation of latent-TGF $\beta$  at the cell surface, as opposed to LTBP, which sequesters TGF $\beta$  in the ECM. In the immune system, GARP is expressed by activated T<sub>REG</sub> and B cells (Stockis et al. 2009; Dedobbeleer et al. 2017). Through its ability to present latent-TGF $\beta$  at the cell surface for activation, GARP has a large role in maintaining peripheral tolerance and participates to the promotion of oncogenesis. GARP biology is extensively reviewed in (Metelli et al. 2018).

These observations imply that large quantities of latent-TGF $\beta$  can be found in tissues. However, the sequestration of large quantity of latent-TGF $\beta$ , in the ECM or at the cell surface does not mean that it is bioavailable. Activation of latent-TGF $\beta$  through mechanisms described below is mandatory before signaling on their target cells.

## 1.2. Latent TGF $\beta$ is activated by the RGD-binding $\alpha_v$ integrins.

A large number of biological and chemical mechanisms of TGF $\beta$  activation have been described over the years. These mechanisms include but are not restricted to 1/ proteolytic degradation by proteases, such as metalloproteases, serine proteases, calpain, cathepsin D and many other. 2/ Deglycosylation of TGF $\beta$  N-glycosylation sites, potentially revealing protease-sensitive sites. 3/ Other protein factors such as TSP-1, F-spondin, Neuropilins and PSG-1 although the molecular mechanisms are not always known. 4/ Physicochemical factors, such as heat, ROS, low pH condition, UV radiation. These means of activation are however not all biologically relevant and have not been found *in vivo* so far. Finally, and most relevant to this dissertation is 5/ the ability of alpha-v ( $\alpha_v$ ) integrins to bind the LAP and activate TGF $\beta$ . The ability and mechanisms by which integrins can activate TGF $\beta$  is detailed in the paragraph below. The various mechanisms of TGF $\beta$  activation described above are extensively detailed in (Robertson and Rifkin 2016).

### 1.2.1. $\alpha_v$ Integrins bind to an RGD motif in the LAP to mediate TGF $\beta$ activation.

Integrins are heterodimeric transmembrane receptors comprised of an alpha ( $\alpha$ ) and a beta ( $\beta$ ) subunit. It is generally accepted that the  $\alpha$  subunit determines the ligand specificity while the  $\beta$  subunit transduces information bidirectionally between the extracellular and the intracellular spaces (Fig. 2A). The integrin family is composed of 18  $\alpha$  subunits which can be non-covalently associated with one or several of the 8  $\beta$  subunits. The integrin family and its ligand binding ability are represented in Fig. 2B. A general review of the integrin family can be found in (Barczyk, Carracedo, and Gullberg 2010).

$\alpha\beta$  integrin dimer can be found in different conformational states: 1/ a folded conformation in which integrins are inactive and cannot bind their ligand, 2/ an extended conformation in which integrins are 'primed' for ligand binding and 3/ a fully extended conformation with an open headpiece in which the affinity for the ligand is highest. The transition between these states is mediated by intracellular signaling which culminates in the binding of Talin to the intracellular tail of the  $\beta$ -subunit. This process is commonly called 'inside-out signaling' and leads to the activation of the surface integrins. Furthermore, binding of the integrin to its ligand and the tension applied to the integrin headpiece also promotes the recruitment of signaling proteins to the integrin

intracellular tail inducing an 'outside-in' signaling and the regulation of gene expression supporting survival, activation or differentiation. The activation of integrin is detailed in (Legate, Wickström, and Fässler 2009; Askari et al. 2009)

In the integrin family, the subfamily of  $\alpha_v$  integrins which can bind to RGD (Arginine-Glycine-Aspartate peptide) is of particular interest in the biology of TGF $\beta$  activation. This RGD amino acid sequence is present in many ECM proteins such as fibronectin, vitronectin, fibrillin or thrombospondin. Their ability to bind RGD therefore allow  $\alpha_v$  integrins to mediate cell binding to ECM (Humphries, Byron, and Humphries 2006). An RGD motif is also present at the surface of the LAP of latent-TGF $\beta$ . Binding to LAP RGD by integrin is crucial for TGF $\beta$  activation. This is best illustrated in the drastic phenotype of mice bearing a mutated RGD binding site on LAP. Transgenic mice in which LAP RGD motif is converted to a RGE motif (*Tgfb1*<sup>RGE/RGE</sup>), develop multiorgan inflammation and defect in vascular development, a phenotype closely resembling that of *Tgfb1*<sup>-/-</sup> KO mice (Yang et al. 2007). Similarly, mice lacking all  $\alpha_v$  integrins (*Itgav*<sup>-/-</sup> mice) develop developmental defects phenocopying TGF $\beta$ 1 and TGF $\beta$ 3 KO mice (Bader et al. 1998).

The phenotype of KO mice in which individual  $\alpha_v$ -associated  $\beta$  subunits are deleted reveals the specific and nonredundant functions and importance of each  $\alpha_v$  integrin for the activation of TGF $\beta$  (Fig. 2C).

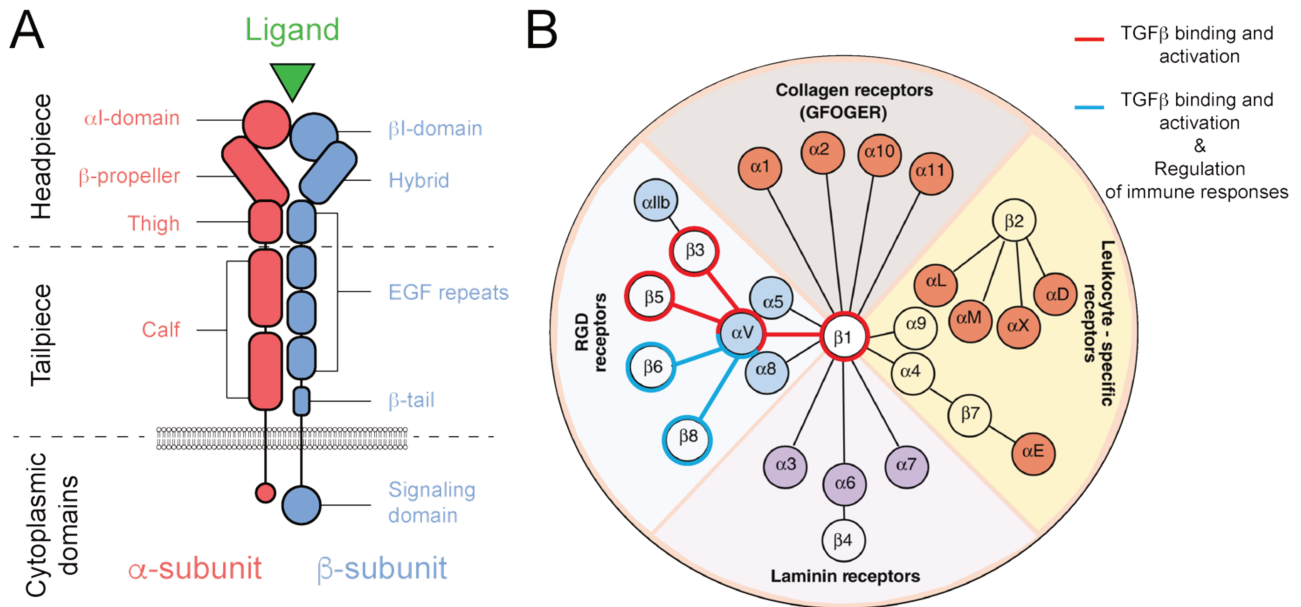
*Itgb1* KO mice display an embryonically lethal phenotype, probably due to its association with multiple other  $\alpha$ -integrins. *Itgb3* and *Itgb5* KO mice however only display mild developmental defects ( $\beta$ 3 and  $\beta$ 5) (Worthington, Klementowicz, and Travis 2011). However, these phenotypes do not appear to be related to TGF $\beta$  activation defects. These integrins have been involved in numerous processes, not directly related to TGF $\beta$  such as phagocytosis, migration or autophagy (Dupuy and Caron 2008; Yoshioka et al. 2010; Raso et al. 2018). However, several studies have shown that  $\alpha_v\beta$ 1,  $\alpha_v\beta$ 3 and  $\alpha_v\beta$ 5 can indeed bind and activate TGF $\beta$  to regulate TGF $\beta$ -dependent responses in the context of fibrosis and fibroblast differentiation and function (J. S. Munger et al. 1998; Ludbrook et al. 2003; Asano et al. 2006; Tatler et al. 2011; Sarrazy et al. 2014; N. I. Reed et al. 2015). However, the importance of these integrins for the regulation of TGF $\beta$ -dependent immune responses has not been demonstrated to this day.

The *Itgb6*<sup>-/-</sup> mice display a mild inflammatory phenotype which is restricted to the lung and skin. These symptoms include infiltration of macrophages into the skin, and accumulation of activated lymphocytes in the lung (Huang et al. 1996). Furthermore, it has been shown in the late 1990's that  $\alpha_v\beta$ 6 is able to bind and activate TGF $\beta$  through RGD specific binding and with the help of other activating molecules (John S Munger et al. 1999). These studies first suggested the importance of  $\alpha_v\beta$ 6 in the control of TGF $\beta$ -dependent immune homeostasis. Since then,  $\alpha_v\beta$ 6 has been shown to be expressed by stromal and epithelial cells of the skin, lung and gut.  $\alpha_v\beta$ 6 expression by these cells is required for TGF $\beta$  activation and maintenance of tissue-resident memory (T<sub>RM</sub>) cells, Langerhans Cells (LC) residency and preventing lung emphysema (McEntee, Gunaltay, and Travis 2019). Lastly, *Itgb8*<sup>-/-</sup> mice, like *Itgav*<sup>-/-</sup> mice, display large developmental defects and often die before birth from defects in brain vascular development (Zhu et al. 2002). The few mice that are born have cleft palate, such as *Tgfb3*<sup>-/-</sup> mice and abnormal vascular morphogenesis such as the *Tgfb1*<sup>RGE/RGE</sup>.*Tgfb3*<sup>-/-</sup> mice and die soon after birth. More details on the *Itgb8*<sup>-/-</sup> mice can be found in (Worthington, Klementowicz, and Travis 2011). *In vitro* studies

further demonstrated that  $\alpha_v\beta_8$  integrin can bind and activate TGF $\beta$  through the RGD peptide (Mu et al. 2002). Because *Itgb8*<sup>-/-</sup> mice have such important developmental defects, the study of the role of  $\alpha_v\beta_8$  in the immune system has relied on conditional KO mouse allowing targeted deletion of  $\beta_8$  using the Cre-Lox system. These models and the importance of  $\alpha_v\beta_8$  for the control of immune responses will be detailed in the next sections. The biology of  $\alpha_v\beta_6$  and  $\alpha_v\beta_8$  on the regulation of immune responses and homeostasis are extensively reviewed in (McEntee, Gunaltay, and Travis 2019; Nolte and Margadant 2020)

Overall, these studies have largely contributed to understanding the importance of  $\alpha_v$  integrin-mediated TGF $\beta$  activation for the control of immune and non-immune responses. The next paragraph focuses on the mechanisms by which  $\alpha_v$  integrins can activate TGF $\beta$ .





**C**

Phenotype	Deleted gene						
	<i>Itgav</i> <sup>-/-</sup>	<i>Itgb1</i> <sup>-/-</sup>	<i>Itgb3</i> <sup>-/-</sup> , <i>Itgb5</i> <sup>-/-</sup>	<i>Itgb6</i> <sup>-/-</sup>	<i>Itgb8</i> <sup>-/-</sup>	<i>Tgfb1</i> <sup>-/-</sup> , <i>Tgfb1</i> <sup>RGE/RGE</sup>	<i>Tgfb3</i> <sup>-/-</sup>
Embryonically lethal	Yes	Yes	No	No	Yes	Yes	Yes
Autoimmune syndrome	NT	NT	No	Mild inflammation (lung and skin)	NT	Lethal autoimmune syndrome	NT
Abnormal vasculogenesis	80%	NT	Enhanced pathological angiogenesis	0%	60%	50%	0%
Abnormal CNS development	100%	NT	0%	0%	100%	0%	0%
Cleft palate	100%	NT	0%	0%	10%	0%	100%

NT = not tested because of early lethality

**Figure 2 - Integrin can bind and activate latent-TGFβ**

(A) – Structure of a prototypical αβ-integrin dimer in the extended conformation. The Headpiece allows the binding of the ligand while the Tailpiece conformation determines the overall conformation of the integrin. The β-subunit intracellular domain allows the transmission of signal 'inside-out' or 'outside-in'.

(B) - Schematic representation of the integrin family detailing the α and β integrin couple and their specific ligand class. Highlighted in red is their ability to bind and activate TGFβ. α<sub>v</sub>β<sub>6</sub> and α<sub>v</sub>β<sub>8</sub> integrins have also been shown to be critical in the control of immune responses.

(C) – Summary of the major phenotype α<sub>v</sub>-integrin KO mice.

Figure adapted from (Barczyk, Carracedo, and Gullberg 2010; Yang et al. 2007).

### 1.2.2. Mechanism of TGF $\beta$ activation by $\alpha_v$ integrins.

There are two main pathways by which  $\alpha_v$  integrins can activate TGF $\beta$  after binding the RGD motif on the LAP: 1/  $\alpha_v\beta6$ -mediated conformational changes of the SLC through mechanical forces and 2/  $\alpha_v\beta8$ -mediated recruitment of metalloproteases.

It has been shown that the orientation in which TGF $\beta$  binds  $\alpha_v\beta6$  and the integrin tri-dimensional structure of the  $\alpha_v\beta6$  integrin have evolved to support the transmission of tensile force through the  $\beta6$  subunit to the TGF $\beta$  molecule (Dong et al. 2017). Contraction of the cytoskeleton, attached to the intracellular domain of  $\alpha_v\beta6$ , enhanced by PAR1 signaling, and the binding of latent-TGF $\beta$  to the extracellular matrix through the LTBP induce a force which ultimately leads to the release of the active-TGF $\beta$  molecule (Annes et al. 2004; R. G. Jenkins et al. 2006). This mechanism is well reviewed in (Robertson and Rifkin 2016)

The activation of TGF $\beta$  by the  $\alpha_v\beta8$  integrin is different. Contrarily to most  $\beta$  chains,  $\beta8$  integrin has a short intracellular domain and hence limited connection to cytoskeleton and is thus not subjected to inside-out signaling. It has recently been shown that the  $\alpha_v\beta8$  integrin displays a constitutionally 'extended' active form and binding of TGF $\beta$  does not affect its conformation (Minagawa et al. 2014; J. Wang et al. 2017). Furthermore, it has previously been demonstrated that the intracytoplasmic sequence of  $\beta8$  integrin is not required for TGF $\beta$  activation, hence excluding the possibility of a contractility-dependent mechanism. This same study however showed that the MT1-MMP (or MMP14) metalloprotease is required for the activation of TGF $\beta$  (Mu et al. 2002). In addition, a few studies have demonstrated the importance of the GARP molecule in 'chaperoning' the latent-TGF $\beta$  molecule for  $\alpha_v\beta8$ -mediated activation (R. Wang et al. 2012; Edwards, Thornton, and Shevach 2014). The crystal structure of the GARP-TGF $\beta$  complex was recently elucidated and showed the binding of the LAP by GARP in a manner that allows further binding on  $\alpha_v\beta8$  on the RDG sites (Liénart et al. 2018). Further Cryo-Electron Microscopy of the  $\alpha_v\beta8$ -latent-TGF $\beta$  complex has elegantly shown the flexibility of the  $\alpha_v\beta8$ -bound latent-TGF $\beta$ . The authors suggest that presentation of latent-TGF $\beta$  by GARP to  $\alpha_v\beta8$  integrin might allow for active-TGF $\beta$  to be presented to the TGF $\beta$ R without the need of active-TGF $\beta$  release (Campbell et al. 2020).

Whether released in the extracellular milieu for paracrine signaling or directly presented to target cell by cell-cell contact, TGF $\beta$  binds to its receptor for intracellular signaling.

### 1.3. TGF $\beta$ signaling involves different pathways and induces a wide range of target genes.

The signaling pathway of TGF $\beta$ R starts with the binding of the TGF $\beta$  to the tetrameric TGF $\beta$  receptor (TGF $\beta$ R). The TGF $\beta$ R is composed of two subunits, TGF $\beta$ RI and TGF $\beta$ RII, which homo-dimerize and further form a hetero-tetrameric receptor. Binding of TGF $\beta$  induces the phosphorylation of TGF $\beta$ RI by TGF $\beta$ RII which will in turn phosphorylate other members of the signaling pathway.

The canonical Smad intracellular signaling cascade is composed of 3 main components: the Receptor-regulated Smads (R-Smad), the Common mediator Smad (Co-Smad) and the Inhibitory Smads (I-Smads). In the case of TGF $\beta$ , Smad2/3 (R-Smad), Smad 4 (Co-Smad) and Smad6/7 (I-Smad) compose the signaling cascade. TGF $\beta$ RI phosphorylates the Smad2/3 molecule which heterotrimerizes with Smad4. Smad2/Smad4 complex then translocates into the nucleus where, in coordination with several co-factors, it regulates target gene transcription. Smad6/7 exerts a negative regulation on the TGF $\beta$  signaling pathway by competing for TGF $\beta$ RI binding site with Smad2/3.

A lot of other Smad-independent signaling pathways have been described for the TGF $\beta$ R, many of them well known for their importance in surface receptor signaling: the MAPK pathways (Erk, Jnk and p38), the small GTPases pathway and the Phosphatidylinositol 3-kinases (PI3K) pathway. These signaling pathway can both reinforce or attenuate Smad signaling pathway and participate to the regulation of TGF $\beta$  target genes. The Smad-dependent and Smad-independent pathways for TGF $\beta$ R signaling are extensively reviewed in (A. Weiss and Attisano 2013; Y. E. Zhang 2009).

The translocation of phosphorylated Smad in the nucleus participates in the regulation of a very large quantity of target genes, which are highly dependent on the experimental setup. However, it is generally accepted that the post-translational modifications of the Smad proteins along with the co-factor they are associated to are critical to determine the target and effects of Smad on chromatin accessibility and gene transcription (reviewed in (Massagué, Seoane, and Wotton 2005; Gaarenstroom and Hill 2014)).

#### 1.4. Spatial and temporal regulation of TGF $\beta$ -dependent response via regulation of its latency, activation and signaling.

In their review of TGF $\beta$  activation, Annes, Munger and Rifkin propose a 3-part model for TGF $\beta$  Large Latency Complex (LLC). They propose that TGF $\beta$  activity can be understood through the 3 components of the LLC (Annes, Munger, and Rifkin 2003).

1. The sequestration and localization of the TGF $\beta$  molecule is determined by the TGF $\beta$  binding molecule – LTBP or GARP – which they refer to as the ‘localizator’. LTBP binding of TGF $\beta$  favors the ECM sequestration while GARP binding favors the cell-bound localization.
2. The latency of the TGF $\beta$  molecule and its mean of activation are determined by the LAP. The protection of the TGF $\beta$  molecule by the LAP and the ability of integrin to bind the RGD motif of the LAP allow the activation of the molecule. They refer to the LAP as the ‘detector’.
3. The signaling cascade and downstream effector function rely on the active-TGF $\beta$  molecule, or the ‘effector’.

The latent nature of the TGF $\beta$  molecule creates a particular situation where the regulation of TGF $\beta$  availability is not regulated at the level of its production (i.e. transcription, translation and secretion) by secreting cells but rather at the level of the activation of TGF $\beta$ . Regulation of the availability of bioactive TGF $\beta$  at a particular time

and at a particular place, creates a very local increase in TGF $\beta$  concentration and for a length of time which are all regulated by the availability and activity of TGF $\beta$  activating molecules.

If the secretion, activation, canonical and non-canonical signaling pathways have been well identified, the mechanisms by which TGF $\beta$  signaling can generate widely different physiological responses depending on the context is still poorly understood. Attempts at reconciling these signaling pathway with the physiological responses observed *in vivo* have shifted towards systematic approaches taking into account a wide number of parameters for mathematical modelling of TGF $\beta$  signaling. In particular, Zi and colleagues point out the importance of considering the high sensitivity of TGF $\beta$  signaling to the variation of ligand concentration (or rather TGF $\beta$  molecule per cell) and in the duration of the stimulation (Zi, Chapnick, and Liu 2012). They insist on the fact that at a particular concentration of TGF $\beta$  per cell, transient or sustained TGF $\beta$  signaling can be critical for deciding cell fate.

An overall model of TGF $\beta$  production, sequestration, activation and signaling is shown in Figure 1. This model allows to describe the functional relevance of each component of the LLC in the biology of TGF $\beta$ . The rest of the manuscript will focus on the regulation of TGF $\beta$ -dependent immune responses by the  $\alpha_v\beta_8$  integrin. I will in particular focus on the expression of  $\alpha_v\beta_8$  for the regulation of intestinal T cell responses and in the control of various aspects of B cell responses.

## 2. The regulation of intestinal homeostasis is dependent on TGF $\beta$ activation by $\alpha_v\beta_8$ integrins.

The gastrointestinal tract harbors a large diversity of commensal microorganisms and dietary antigens which are important to provide metabolic help and protection against potential pathogenic infection. The mucosal immune system therefore faces a complex challenge and it must be able to mount effective proinflammatory responses to protect the host against such infections and at the same time to tolerate and/or ignore the multitude of innocuous antigens it is constantly exposed to. The balance of anti- and pro-inflammatory CD4<sup>+</sup> T cell responses is critical to maintain intestinal immune homeostasis. In particular, intestinal T<sub>REG</sub> and T<sub>H17</sub> are important components of intestinal immune responses. However, dysregulation of intestinal T cell responses can lead to immune pathologies such as autoimmunity or chronic infections among many others.

Interestingly, TGF $\beta$  has been shown to be required both for the development of T<sub>REG</sub> and T<sub>H17</sub> and for T<sub>REG</sub> immunomodulatory functions. This section of the manuscript reviews the importance of TGF $\beta$  and TGF $\beta$  activation in the biology of CD4<sup>+</sup> T cell responses through:

- A brief overview of the importance of T<sub>REG</sub> and T<sub>H17</sub> in the maintenance of intestinal homeostasis
- A review of the pathways for T<sub>REG</sub> and T<sub>H17</sub> differentiation in Gut Associated Lymphoid Tissues (GALT)
- A review of the role TGF $\beta$  activation by  $\alpha_v\beta_8$  integrin in the regulation of T cell differentiation by DC and T cell activation by T<sub>REG</sub>.

This section will be concluded with an assessment of the open questions remaining in the field of TGF $\beta$  activation by  $\alpha_v\beta_8$  for the control of T cell responses.

## 2.1. Dendritic cells are critical to the maintenance of mucosal homeostasis and the regulation of the intestinal T cell fate.

T cell differentiation, activation and the function of CD4<sup>+</sup> T<sub>REG</sub> and T<sub>H</sub>17 have been extensively studied over the years, especially in the context of intestinal immune responses. Detailed reviews can be found in (Littman and Rudensky 2010; Josefowicz, Lu, and Rudensky 2012; Weaver et al. 2013; Chewing and Weaver 2014; Omenetti and Pizarro 2015; Plitas and Rudensky 2016; Whibley, Tucci, and Powrie 2019).

### 2.1.1. Mucosal T cells control intestinal immune responses through cytokine release.

Mouse model and human deficient for T<sub>REG</sub> have massive inflammatory disorder, clearly showing the requirement of T<sub>REG</sub> to maintain immune homeostasis in the gut. In particular, T<sub>REG</sub> are involved by various mechanisms in the maintenance of tolerance to the microbiota and food antigen and inhibiting aberrant immune responses.

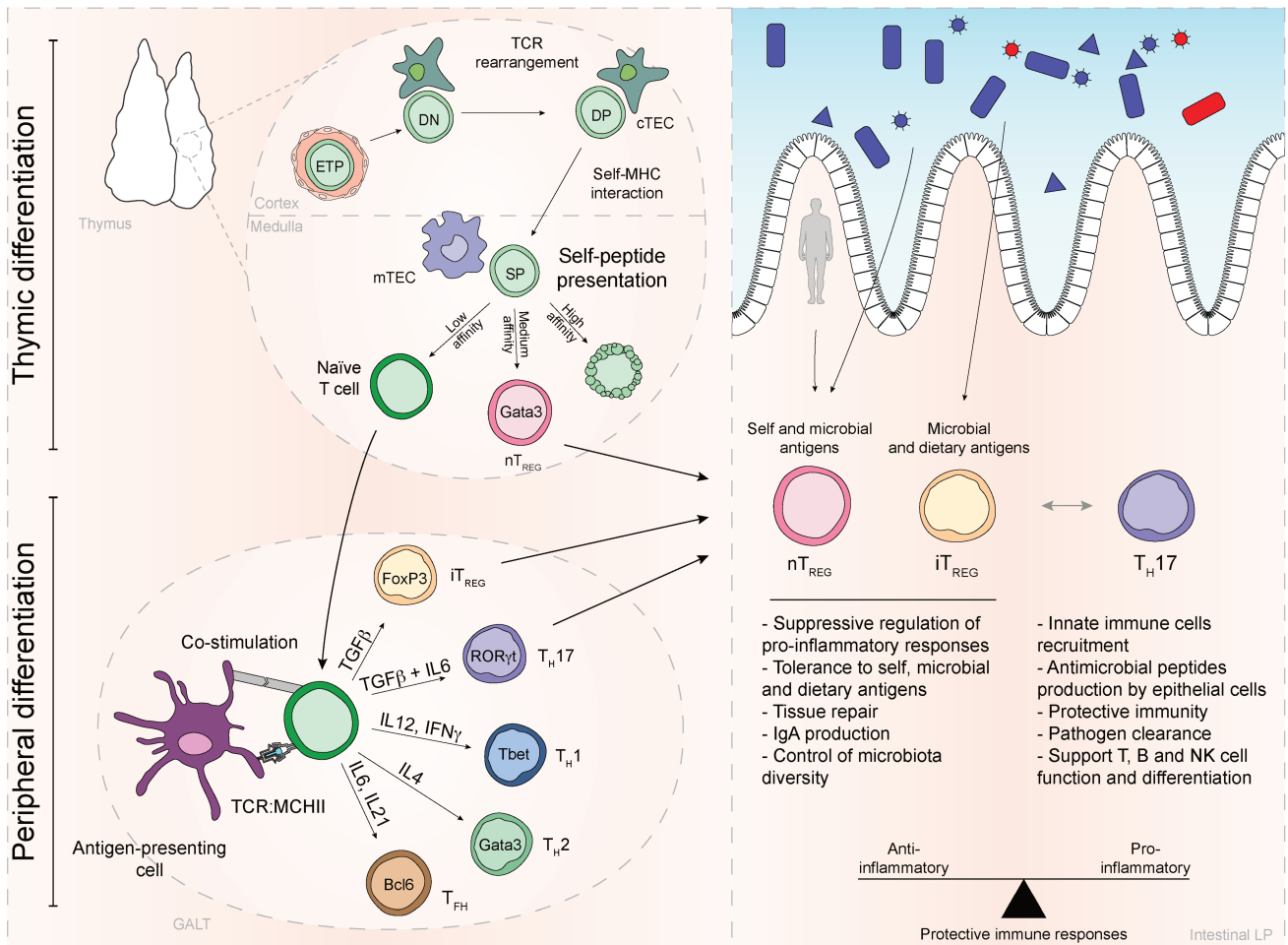
T<sub>REG</sub> in the intestinal *lamina propria* (LP) can be subdivided into two different subsets: natural T<sub>REG</sub> (nT<sub>REG</sub>) and induced T<sub>REG</sub> (iT<sub>REG</sub>). These two populations arise from two different processes. nT<sub>REG</sub> are generated during normal T cell development in the thymus and then migrate to the periphery, where they regulate activation of self-reactive T cells and prevent autoimmunity. On the contrary, iT<sub>REG</sub> are generated from mature naïve T cells in the periphery where they suppress immune responses to both self and non-self-antigens including those from food and commensal microbes (Whibley, Tucci, and Powrie 2019).

In the Gut Associated lymphoid Tissues (GALT) and intestinal LP, iT<sub>REG</sub> and nT<sub>REG</sub> can be identified by their expression of Neuropilin 1 (Nrp1). nT<sub>REG</sub> express Nrp1 while iT<sub>REG</sub> do not. Alternatively, these two subsets can be recognized by their expression of different transcription factors. In particular, iT<sub>REG</sub> induced in the GALT express the ROR $\gamma$ t transcription factor while a large proportion of nT<sub>REG</sub> express the GATA3 transcription factor (Whibley, Tucci, and Powrie 2019).

T<sub>REG</sub> can exert their immune-suppressive function through various mechanisms including expression of surface checkpoint inhibitors (CTLA4, TIGIT, LAG-3, ...) and secretion of cytokines and proteases (IL10, Granzyme, TGF $\beta$ , ...). The biology of TGF $\beta$  on T<sub>REG</sub> function is discussed later in the manuscript.

In contrast, T<sub>H</sub>17 cells are generated in response to bacterial and fungal pathogens and stimulate innate immune and inflammatory defense. T<sub>H</sub>17 function relies on their ability to secrete IL17A, IL17F and IL22. T<sub>H</sub>17 have been implicated in autoimmune and inflammatory diseases including IBD (O'Connor, Zenewicz, and Flavell 2010). However, T<sub>H</sub>17 are also present in non-inflamed intestine in the presence of certain microbes and are thought to contribute to the recruitment of effector cells in the intestine to promote immune responses to bacterial and fungal infection and to the enhancement of epithelial barrier integrity and anti-microbial activity.

The functions of T<sub>REG</sub> and T<sub>H</sub>17 in the intestinal LP are summarized in Fig. 3.



**Figure 3 - Overview of T cell differentiation and function.**

Upon exit from the Bone Marrow, Early T cell Precursor (ETP) enter the cortex of the thymus and begin differentiation into mature T cells. In the cortex, rearrangement of the VDJ region of the TCR locus in CD4<sup>+</sup>CD8<sup>-</sup> Double Negative (DN) thymocytes leads to the production of a functional TCR. CD4<sup>+</sup>CD8<sup>+</sup> Double Positive (DP) cells expressing a functional TCR can interact with Self-MHC molecule. Their ability to interact with MHC I or MHC II governs their differentiation into CD4<sup>+</sup> or CD8<sup>+</sup> Single Positive (SP) thymocytes. This process of positive selection is mediated by the interaction of thymocytes with stromal cortical Thymic Epithelial Cells (cTEC) and cDC presenting self-MHC. SP cells migrate to the thymus cortex and undergo of negative selection. MHC molecules containing self-peptide are presented by medullary Thymic Epithelial Cells (mTEC). High affinity interaction with MHC presenting self-peptides leads to the apoptosis of thymocytes. In contrast, low affinity to these MHC molecule leads to the generation of mature naïve T cells which can migrate out of the thymus and into the peripheral compartment. Furthermore, thymocytes bearing a TCR with medium self-reactivity are oriented towards the natural T<sub>REG</sub> lineage (nT<sub>REG</sub>).

In the SLO, presentation of foreign peptides associated with co-stimulatory molecules by antigen-presenting cells leads to the peripheral activation of T cells. The cytokine combination perceived by T cell during this process orients naïve T cells towards the different effector T cell subsets, characterized by various specific transcription factors.

T cells activated in the Gut Associated Lymphoid Tissues (GALT) can migrate toward the intestinal *Lamina Propria* (LP) in a CCR9 and  $\alpha 4\beta 7$ -dependent fashion. T<sub>REG</sub> and T<sub>H17</sub> are an important population of the intestinal LP and are crucial to maintain tissue homeostasis. T<sub>H17</sub> and T<sub>REG</sub> are very plastic in their nature and upon stimulation with the proper cytokines, can change their differentiation program towards another T cell subset. In the intestinal LP, T<sub>REG</sub> induce tolerance to intestinal antigens, suppress aberrant immune responses and promote tissue repair. T<sub>H17</sub> are pro-inflammatory T cells orchestrating the response toward invading pathogen by recruitment of effector cells and maintenance of the epithelial integrity and immune mechanisms. Although this response is often overly activated in inflammatory syndromes, T<sub>H17</sub> responses are central to protecting tissue integrity at steady state.

### 2.1.2. Self-reactive T cells give rise to natural nT<sub>REG</sub> in the thymus

The process of T cell selection in the thymus is a critical process to ensure that newly generated naïve T cells have a functional and non-self-reactive TCR. In the process of CD4<sup>+</sup> T cell negative selection, T cells interact with medullary Thymic Epithelial Cells (mTEC) presenting MHCII loaded with self-antigen. Strong affinity of the TCR with these MHCII molecules results in the deletion of these cells, thus preventing the development of autoreactive T cell clones. However, interaction of a lower affinity with these MHC molecules promotes the development of thymic derived natural regulatory T cells (nT<sub>REG</sub> or tT<sub>REG</sub>). Non self-reacting T cells further differentiate into naïve conventional T cells (T<sub>CONV</sub>) which migrate to the Secondary Lymphoid Organs (SLO) (Josefowicz, Lu, and Rudensky 2012). nT<sub>REG</sub> expression of CCR7 and  $\alpha 4\beta 7$  allow them to migrate to the intestine.

In the process of nT<sub>REG</sub> differentiation, cytokine signaling and in particular IL2 and TGF $\beta$  signaling is critical for the proper development of nT<sub>REG</sub>, promoting T cell survival rather than *Foxp3* induction as this is the case for peripheral T<sub>REG</sub> generation (see 2.1.3.b).

The process of nT<sub>REG</sub> differentiation is summarized in Fig. 3.

### 2.1.3. Intestinal T cell differentiation heavily relies on DC cell help

Dendritic cells (DC) are integral to the differentiation of helper T cell differentiation. Indeed, DC have the unique ability to sense their environment, sample and present local antigens to naïve T cells, integrate these signals and forward this information to the adaptive immune system by antigen presentation. DC will thus induce adaptive immunity according to tissue micro-environment and context information. cDC comprise different sub-populations defined by their lineage of differentiation, their tissue location and activation status. In this section we review the different subsets present in the intestinal mucosa and MALT and their specific role in the differentiation of T<sub>REG</sub> and T<sub>H17</sub>.

#### *a. Mucosal myeloid lineage differentiation and migration.*

Dendritic cell differentiation begins in the bone marrow with the commitment of Hematopoietic Stem Cells (HSC) to the common myeloid progenitor giving rise to monocytes and a common DC progenitor (CDP). CDP gives rise to plasmacytoid DC (pDC) and circulating conventional DC (cDC) precursors (pre-DC) (Merad et al. 2013). Pre-DC will then seed peripheral tissues to produce cDC. cDC have been shown to differentiate into two lineages based on their expression of distinct transcription factors: cDC1 and cDC2. In the intestinal LP these cells can mainly be distinguished by their expression of the CD103 ( $\alpha_E$ ) and CD11b ( $\alpha_M$ ) integrins (Bekiaris, Persson, and Agace 2014; Stagg 2018; Tezuka and Ohteki 2019).

A recent publication by Brown and colleagues further proposed that cDC2 could be subdivided into cDC2A and cDC2B depending on their expression of ROR $\gamma$ t and T-bet (C. C. Brown et al. 2019). Further studies would be required to determine the functional relevance of both these subsets in mucosal immunity.

Upon activation, these cells can migrate through CCR7-mediated mechanisms to the MLN where they can interact with T cells and B cells to activate the adaptive immune response. These migratory cells are characterized by their high expression of activation markers and MHC expression.

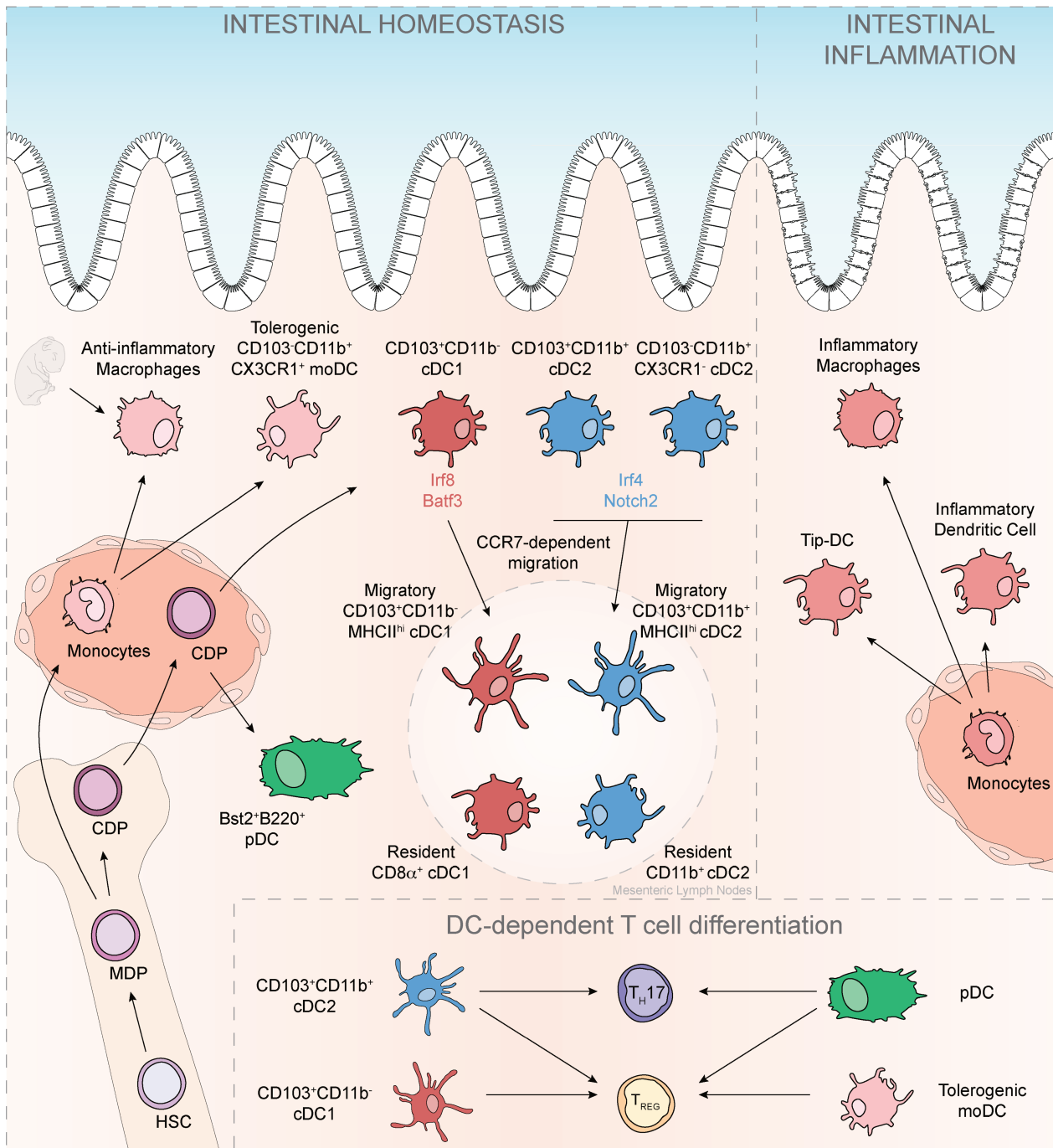
The common Myeloid progenitor also gives rise to monocytes, which upon entering in intestinal tissue can differentiate into monocyte-derived DC (moDC) or macrophages. This differentiation can occur in the homeostatic and inflamed gut. These cells give rise to a wide variety of moDC, characterized by CX3CR1 expression (Gross, Salame, and Jung 2015). These moDC and macrophages can be both tolerogenic or pro-inflammatory depending on the inflammatory context in which they have been generated (Dominguez-Villar and Hafler 2018).

It is important to note that pDC and embryonically-derived or monocyte-derived macrophages are other myeloid cells present in the intestinal LP and the GALT. pDC have been shown to support many aspects of intestinal immunity (IgA B cell, T cell differentiation, DC migration, ...). They have been involved in the control of intestinal infection and found increased in intestinal pathologies such as Inflammatory Bowel Disease (IBD) and colorectal cancer (Won et al. 2019). Intestinal macrophages perform a wide range of functions participating in intestinal homeostasis from antigen sampling in the LP, clearance of apoptotic bodies, and maintenance of epithelial integrity to the promotion of DC and T cell differentiation. Their functions are extensively reviewed in (Bain and Schridde 2018).

The importance of TGF $\beta$  on the development of DC populations is multifold. TGF $\beta$  has been shown to promote the expression of cDC-specific and cDC lineage-specific transcription factors such as IRF4 and IFR8 in myeloid progenitor. Furthermore, decrease or abrogation of TGF $\beta$  signaling on DC leads to important inflammatory disorder. This suggests that TGF $\beta$  induces an anti-inflammatory phenotype on DC, further allowing to induce T<sub>REG</sub>. Additionally, TGF $\beta$  has been shown to regulated DC migration by modulating chemokine receptor expression. The effect of TGF $\beta$  on cDC and other DC populations is reviewed in (Kelly et al. 2017).

The surface marker and the differentiation factors required for intestinal myeloid cell differentiation are summarized on Fig. 4.





**Figure 4 - Dendritic cell (DC) subset in the intestinal immune system**

Arising from Hematopoietic Stem Cells (HSC) through the Macrophage and DC Progenitor (MDP) and Common DC progenitor (CDP), the intestinal LP is comprised of 3 main lineages of phagocytes: plasmacytoid DC (pDC), monocyte derived DC (moDC) of macrophages and conventional DC (cDC). Alternatively, tissue macrophages can arise from fetal liver progenitor at a very early age.

Monocyte derived DC are generated at mucosal homeostasis but more particularly upon inflammation. The inflammatory context in which they are generated defines their tolerogenic or inflammatory phenotype and their effector functions (Domínguez and Ardavin 2010).

pDC seed the LP and the GALT and are not only responsible for response to intestinal infection but also play an integral part in maintaining intestinal immune homeostasis (Won et al. 2019)

cDC can be separated into cDC1 and cDC2, two distinct lineages of DC characterized by the differential expression of the CD103 and CD11b integrins. Upon activation in the LP, DC can migrate to the mesenteric Lymph Nodes (MLN), fully mature, and activate adaptive immune responses.

A schematic representation of the involvement of the various intestinal DC populations in the TGF $\beta$  dependent induction of T<sub>REG</sub> and T<sub>H</sub>17 is represented in the bottom part of the figure. The role for T cell differentiation represented here does not always depends on TCR:MHCII-peptide interaction with naïve T cell.

*b. iT<sub>REG</sub> and T<sub>H</sub>17 generation in the periphery is dependent on APC.*

In the periphery, naïve T cell activation and differentiation critically relies on three distinct signals.

- TCR signaling upon recognition of MHCII:peptide complex for which the T cell is specific.
- Interaction of CD80/CD86 on APC with T cell CD28 or CTLA4. These costimulatory molecules synergize with TCR signaling to promote or inhibit T cell activation (review in (Chen and Flies 2013)).
- Cytokine stimulation of the activated T cell allows to direct the differentiation of T cell into one of the helper T cell subsets (T<sub>H</sub>1, T<sub>H</sub>2, T<sub>H</sub>17, T<sub>REG</sub>, T<sub>FH</sub>, etc.)

In particular, T<sub>REG</sub> and T<sub>H</sub>17 cell differentiation critically depends on TGFβ signaling. The current paradigm states that TGFβ alone drives induced T<sub>REG</sub> (iT<sub>REG</sub>) generation while TGFβ and IL6 synergize to induce T<sub>H</sub>17. In naïve T cell, TGFβ signaling induces the expression of the *Foxp3* or *Rorc* genes. Furthermore, it has been demonstrated that high concentration of TGFβ will orientate toward T<sub>REG</sub> development while lower concentration favor T<sub>H</sub>17 differentiation (Chewning and Weaver 2014). The specifics of TGFβ activation and signaling for T<sub>REG</sub> and T<sub>H</sub>17 differentiation are detailed later in the manuscript.

Upon activation, T cells can seed the different tissues depending on their expression of homing receptors. In particular, expression of CCR9 and α4β7 allows homing of differentiated T cells to the intestinal LP.

CD4<sup>+</sup> T cell subsets are plastic and modification of the cell environment and cytokine stimulation can induce modification of the cell subset commitment.

This has been well described in the commitment between T<sub>H</sub>1 and T<sub>H</sub>2 subsets. IL12 or IL4 stimulation of T<sub>H</sub>2 or T<sub>H</sub>1 respectively can induce the switch of T cell to the other subset. Similarly, the T<sub>H</sub>17 subset is relatively instable and reports of T<sub>H</sub>17 switching to other cell subsets is common. In particular, the balance between T<sub>REG</sub> and T<sub>H</sub>17 intestine is reliant on the plasticity of these subsets. It has been described that T<sub>H</sub>17 can switch to a T<sub>REG</sub> phenotype and reversely. In this context, TGFβ stimulation of T<sub>H</sub>17 has been shown to induce their switch to the T<sub>REG</sub> phenotype. In addition, in mice, TGFβ also prevents the development of “pathogenic” IL17 & IFNγ-producing T<sub>H</sub>17 cells (Ghoreschi et al. 2010).

The process of peripheral T cell differentiation is summarized in Fig. 3.

*c. Relative contribution of the different DC lineages in iT<sub>REG</sub> and T<sub>H</sub>17 differentiation.*

Several populations of DC are present in the SI and colon LP, and in the GALT (PP, MLN). Several studies have highlighted the specific functional properties of these different DC subsets in particular for the differentiation of T cells in the intestinal mucosa. Studies that established the progeny of intestinal DC subsets have allowed the development of mouse models allowing to specifically deplete cDC1 or cDC2 in the intestine by targeting the transcription factor responsible for each subset development. In particular, *Batf3*<sup>-/-</sup> or *Cd11c*<sup>Cre</sup>.*Irf8*<sup>flox/flox</sup> mouse models present a complete deletion of cDC1 and in particular intestinal CD103<sup>+</sup> cDC1. On the other hand, *Cd11c*<sup>Cre</sup>.*Irf4*<sup>flox/flox</sup> or human-Langerin-DTA (huLang-DTA) mice impairs the development on intestinal cDC2.

These models have allowed to show that depletion of CD103<sup>+</sup>CD11b<sup>+</sup> cDC2 was sufficient to abrogate T<sub>H</sub>17 generation in the gut (Persson et al. 2013; Welty et al. 2013; Schlitzer et al. 2013). Similarly, TLR5 expression

by CD11b<sup>hi</sup> mucosal DC, believed to be cDC2 is essential for T<sub>H</sub>17 generation (Uematsu et al. 2008). On the other hand, *Cd11c*<sup>Cre</sup>.*Irf8*<sup>flox/flox</sup> mice did not exhibit any modification of T<sub>H</sub>17 responses (Luda et al. 2016a). Many other studies have confirmed the idea that the generation of T<sub>H</sub>17 responses in the intestine or in other context is orchestrated by signals provided by cDC2.

The investigation of the DC subset responsible for T<sub>REG</sub> induction has however not been so successful. In 2007, two studies showed that intestinal CD103<sup>+</sup> DC were able to generate T<sub>REG</sub> in a TGFβ and RA dependent fashion (Coombes et al. 2007; C.-M. Sun et al. 2007). However, these studies did not distinguish between CD103<sup>+</sup>CD11b<sup>-</sup> cDC1 and CD103<sup>+</sup>CD11b<sup>+</sup> cDC2. Since then, no clear evidence has determined that cDC1 or cDC2 were solely responsible for T<sub>REG</sub> induction. This is best exemplified by the absence of T<sub>REG</sub> deficiency in the LP and MLN of both cDC1-specific and cDC2-specific ablation. Indeed, neither *Batf3*<sup>-/-</sup> or *Cd11c*<sup>Cre</sup>.*Irf8*<sup>flox/flox</sup> mice nor *Cd11c*<sup>Cre</sup>.*Irf4*<sup>flox/flox</sup> or huLangerin-DTA mice have altered intestinal T<sub>REG</sub> (Edelson et al. 2010; Welty et al. 2013; Persson et al. 2013; Luda et al. 2016a). These results are in favor of a complementary and/or redundant function of cDC1 and cDC2 for the induction of iT<sub>REG</sub>, where the loss of one subset can be compensated by the other. Some evidence for such a mechanism can be found in Welty et al. The authors generate a model where huLang-DTA mice are crossed with *Batf3*<sup>-/-</sup> mice to delete both CD103<sup>+</sup>CD11b<sup>-</sup> cDC1 and CD103<sup>+</sup>CD11b<sup>+</sup> cDC2. These mice have a strong reduction of T<sub>REG</sub> in the gut and a complete loss of CCR9 expression by T<sub>REG</sub> (Welty et al. 2013). These results suggest that both CD103<sup>+</sup> subsets are mutually redundant for the generation of intestinal T<sub>REG</sub>.

A few example of the cDC1 or cDC2 specific induction of T<sub>REG</sub> can nevertheless be found in a few studies but reflect the generation of T<sub>REG</sub> in a very particular setting such as oral tolerance and do not study steady state T<sub>REG</sub> generation in the intestinal LP (Esterházy et al. 2016; Veenbergen et al. 2015).

The study of integrin-dependent activation of latent-TGFβ for the generation of T cell response is still recent with some of the first description dating from 2007. In the next paragraph, I review the research efforts developed to understand the importance of the α<sub>v</sub>β8 integrin in the generation of T<sub>REG</sub> and T<sub>H</sub>17 responses in the mucosal compartment and in other contexts where appropriate.

The ability of intestinal DC to induce T<sub>H</sub>17 and T<sub>REG</sub> are summarized on Fig. 4.

## 2.2. Expression of α<sub>v</sub>β8 by CD11c-expressing phagocytes is critical to generate T<sub>REG</sub> and T<sub>H</sub>17 and maintain immune homeostasis.

### 2.2.1. Conditional deletion of α<sub>v</sub> or β8 integrin in myeloid cells leads to the development of severe spontaneous colitis.

As mentioned previously, *Itgav*<sup>-/-</sup> and *Itgb8*<sup>-/-</sup> KO in mice is embryonically lethal. To circumvent this problem, studies on the implication of α<sub>v</sub>β8 integrin in the control of immune responses have relied on conditional KO mice using the Cre-Lox targeting system. In 2007, two studies developed mice models in which α<sub>v</sub> or β8 were deleted in the immune system (*Tie2*<sup>Cre</sup>.*Itgav*<sup>flox/flox</sup> and *Vav1*<sup>Cre</sup>.*Itgb8*<sup>flox/flox</sup> mice). These models allowed to target

the deletion of  $\alpha_v$  or  $\beta_8$  to the hematopoietic (Tie2 and Vav1) and the endothelial (Tie2 only) compartments (Boer et al. 2003; Constien et al. 2001).

Both *Tie2*<sup>Cre</sup>.*Itgav*<sup>flox/flox</sup> and *Vav1*<sup>Cre</sup>.*Itgb8*<sup>flox/flox</sup> mice develop severe spontaneous colitis, as shown by an early wasting syndrome, an important immune infiltrate in the liver and colon and recruitment of immune cells in the spleen and mesenteric lymph nodes (MLN). T cell phenotyping in these mouse models revealed an increase in IFN $\gamma$ - and IL4-producing cells suggesting the development of an important T<sub>H1</sub> and T<sub>H2</sub> responses in these mice. In parallel, the authors observed a decrease in the frequency of T<sub>REG</sub> in the intestinal LP (Travis et al. 2007; Lacy-Hulbert et al. 2007). Later studies also showed that these mice also lack IL17-producing ROR $\gamma$ <sup>+</sup> T<sub>H17</sub> cells in the intestinal and peripheral compartment (Acharya et al. 2010; Melton et al. 2010). Using other Cre-expressing mouse model, the authors demonstrated that deletion of  $\alpha_v$  or  $\beta_8$  on myeloid (*Lysm*<sup>Cre</sup>.*Itgav*<sup>flox/flox</sup>) and dendritic cells (*Cd11c*<sup>Cre</sup>.*Itgb8*<sup>flox/flox</sup>) developed similar inflammatory symptoms and T cell dysregulation (Lacy-Hulbert et al. 2007; Travis et al. 2007). Furthermore, they also demonstrated that total sorted CD11c<sup>+</sup> DC were able to generate T<sub>REG</sub> and T<sub>H17</sub> cells from naïve T cells *in vitro* through an  $\alpha_v\beta_8$ -dependent mechanism (Lacy-Hulbert et al. 2007; Travis et al. 2007; Melton et al. 2010; Acharya et al. 2010).

Altogether, these studies demonstrated for the first time the importance of  $\alpha_v\beta_8$  expression by CD11c-expressing phagocytes in the maintenance of intestinal immune homeostasis and the generation of intestinal T<sub>H17</sub> and T<sub>REG</sub>.

Since then, multiple studies have further characterized the ability of each DC subset for the induction of T<sub>REG</sub> and T<sub>H17</sub> by  $\alpha_v\beta_8$ -dependent mechanisms. Additionally, these studies have dissected the mechanisms by which  $\beta_8$  integrin expression is regulated in DC.

### 2.2.2. CD103<sup>+</sup>CD11b<sup>-</sup> MLN cDC1 preferentially express $\beta_8$ integrin and can generate T<sub>REG</sub> and IEL by $\alpha_v\beta_8$ -mediated TGF $\beta$ activation.

Subsequent studies from these teams and ours investigated the expression pattern of *Itgb8* at the DC subset level, their ability and the mechanisms involved in the generation of T<sub>REG</sub> in a  $\alpha_v\beta_8$ -dependent manner.

With the discovery that CD103<sup>+</sup> DC were able to induce T<sub>REG</sub> generation in an retinoic acid (RA)-dependent manner (Coombes et al. 2007; C.-M. Sun et al. 2007), two independent studies investigated the requirement of  $\alpha_v\beta_8$ -mediated TGF $\beta$  activation by these DC for this particular function (Païdassi et al. 2011; Worthington et al. 2011). The studies formally demonstrated *in vitro* that MLN, Small Intestine LP and to some extent Colon LP CD103<sup>+</sup> DC are able to activate TGF $\beta$  and generate T<sub>REG</sub> through  $\alpha_v\beta_8$ -activated TGF $\beta$  and in a RA dependent manner. These studies also show that CD103<sup>+</sup> DC express higher level of *Itgb8* than their CD103<sup>-</sup> counterparts which specializes them for T<sub>REG</sub> generation. They also provide the evidence that  $\alpha_v$  or  $\beta_8$  deficiency *in vivo* prevents the *de novo* generation of intestinal T<sub>REG</sub> to oral antigens. However, as previously stated, CD103<sup>+</sup> DC regroups both CD103<sup>+</sup>CD11b<sup>-</sup> migratory cDC1 and CD103<sup>+</sup>CD11b<sup>+</sup> migratory cDC2. Further studies have shown that CD103<sup>+</sup>CD11b<sup>-</sup> cDC1 preferentially expressed the  $\beta_8$  integrin subunit (Boucard-Jourdin et al. 2016; Shiokawa et al. 2017; Esterházy et al. 2016). The study by Boucard-Jourdin and colleagues has further

demonstrated that cDC1 preferential expression of *Itgb8* specialized them for  $\alpha_v\beta_8$ -mediated TGF $\beta$  activation and T<sub>REG</sub> generation (Boucard-Jourdin et al. 2016).

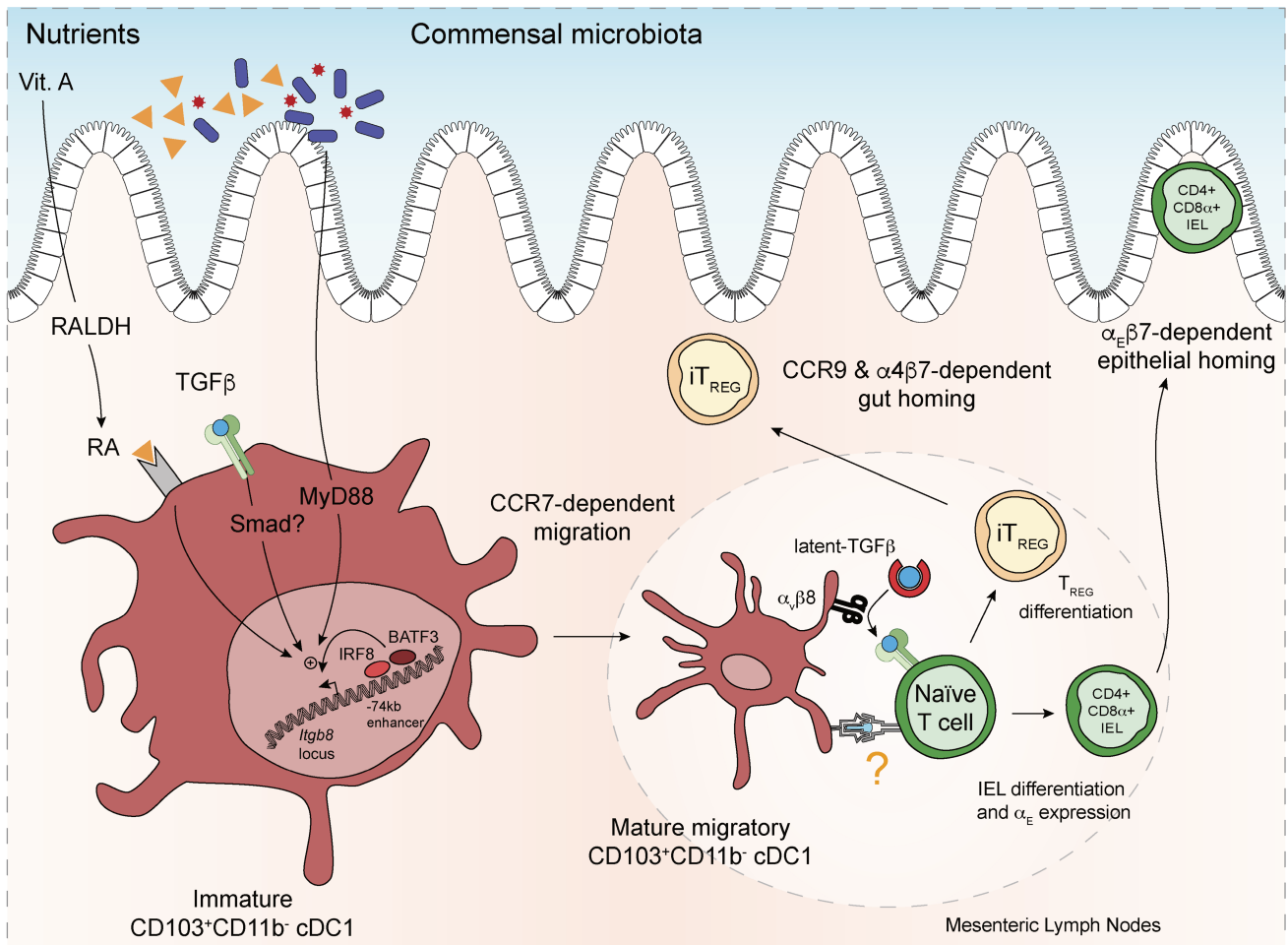
These studies have also investigated the mechanisms that dictate *Itgb8* expression by migratory cDC1. Boucard-Jourdin and colleagues show that *Itgb8* expression by MLN migratory cDC1 is dependent on 1/ TGF $\beta$  itself and RA signaling, 2/ commensal microbiota sensing by the MyD88 pathway and 3/ TLR and especially TLR9 (CpG) signaling (Boucard-Jourdin et al. 2016). These results highlight the fact that cDC1 integrates the signals detected in its environment to induce *Itgb8*.

This study further highlights that expression of *Itgb8* by DC is restricted to the cDC1 lineage. Indeed, we showed that *Itgb8* can be induced on peripheral (Spleen and BM derived DC) cDC1 (CD8 $\alpha^+$ ) but not cDC2 (CD11b $^+$ ) through the presentation of appropriate signals (TGF $\beta$ +RA+CpG). We further demonstrate the existence of a regulatory enhancer for  $\beta_8$  located 74kb upstream of *Itgb8* which is characterized by the enrichment of IRF8 and BATF3 binding site, cDC1 specific transcription factor, and the enrichment of H3K4me1 and H3K27ac histone modification, indicating an active enhancer. Furthermore, enhanced chromatin accessibility and BATF3 and IRF8 binding was restricted to cDC1 and not cDC2. We postulated that this -74kb enhancer could be responsible for the cDC1 restricted expression of *Itgb8*.

Additionally, another independent study has shown that the cDC1-specific IRF8 transcription factor is required for the expression of *Itgb8* by DC and macrophages (Yoshida et al. 2014).

$\beta_8$  expression by CD103 $^+$ CD11b $^-$  cDC1 has been shown to be critical in the generation of IntraEpithelial Lymphocytes (IEL). In particular, Luda and colleagues have demonstrated that *Cd11c<sup>Cre</sup>.Itgb8<sup>fllox/fllox</sup>* mice had a drastically reduced number of CD4 $^+$ CD8 $\alpha\alpha^+$  IEL and large decrease of the TGF $\beta$ -dependent (Grueter et al. 2005) CD103 expression by CD4 $^+$ TCR $\alpha\beta^+$  IEL (Luda et al. 2016a). This further highlight the importance of  $\alpha_v\beta_8$  expression by cDC1 in control of intestinal immune responses.

The function of TGF $\beta$  activated by  $\alpha_v\beta_8$  on cDC1 is summarized in Fig. 5.



**Figure 5 -  $\alpha_v\beta_8$  integrin on MLN cDC1 is critical for the generation of intestinal T cell responses**

LP DC can sense their environment through various receptors. In the study of  $\alpha_v\beta_8$  biology, it has been shown that *Itgb8* expression depends on RA, TGF $\beta$  and MyD88 signaling in LP DC. Upon migration into the LP, MLN migratory cDC1 express the  $\alpha_v\beta_8$  integrin which allows them to activate TGF $\beta$ .  $\alpha_v\beta_8$ -mediated TGF $\beta$  activation and presentation to naïve T cells by intestinal DC has been shown to be critical for the generation of peripheral iT<sub>REG</sub> and differentiation and migration of Intra-Epithelial CD4<sup>+</sup>CD8 $\alpha$ <sup>+</sup> Lymphocytes (IEL). Upon activation in the MLN, these cells can migrate to the LP in a CCR9 and  $\alpha_4\beta_7$ -dependent fashion and home to the epithelial layer thanks to their expression of CD103 and  $\alpha_E\beta_7$ .

### 2.2.3. Expression of $\alpha_v\beta 8$ by DC is required for the generation of $T_H17$ .

In parallel, multiple studies have investigated the requirement of  $\alpha_v\beta 8$  expressed by CD11c-expressing phagocytes for the generation of  $T_H17$ . This was first highlighted by the loss of  $T_H17$  in the LP, MLN, PP and Spleen of *Tie2<sup>Cre</sup>.Itgav<sup>fllox/fllox</sup>* mice (Acharya et al. 2010) and in the Colon LP of *Cd11c<sup>Cre</sup>.Itgb8<sup>fllox/fllox</sup>* (Melton et al. 2010).

In the gut, Steel and colleagues have demonstrated the importance of  $\alpha_v\beta 8$  expression by DC for the protection against *Trichinella spiralis* parasitic infection (Steel et al. 2019). The authors show that *Cd11c<sup>Cre</sup>.Itgb8<sup>fllox/fllox</sup>* mice are more sensitive to the infection and have a reduced  $T_H17$  frequencies. Interestingly, Worthington and colleagues have demonstrated that  $\beta 8$  expression by intestinal DC had deleterious consequences on the clearing of *Trichuris muris* parasitic infection by inhibiting type 2 responses (Worthington et al. 2013).

The importance of  $\alpha_v\beta 8$  for  $T_H17$  generation has however mostly been studied in the context of Experimental Autoimmune Encephalomyelitis (EAE), modelling the human multiple sclerosis (MS). Upon induction self-antigen-specific  $T_H17$  (among other) are generated and participate in disease initiation and progression. Two study in 2010, demonstrated that *Lysm<sup>Cre</sup>.Itgav<sup>fllox/fllox</sup>* and *Cd11c<sup>Cre</sup>.Itgb8<sup>fllox/fllox</sup>* mice, lacking expression on myeloid cells were protected against the development of the disease. These mice also had severely impaired  $T_H17$  generation following disease induction (Melton et al. 2010; Acharya et al. 2010). As indicated earlier, these studies also showed that  $\alpha_v$ - or  $\beta 8$ -deficient DC had a decreased ability to generate  $T_H17$  from naïve T cells *in vitro*.

A recent study investigating the Proteasome activator complex subunit 3-deficient (*REG $\gamma$ <sup>-/-</sup>*) mice showed that the worsened outcome of these mice to EAE was due to an increase in  $T_H17$  frequency. The author went on to show that *REG $\gamma$ <sup>-/-</sup>* DC expressed higher *Itgb8* transcript and had an increased ability to generate  $T_H17$  through  $\alpha_v\beta 8$ -dependent mechanisms (Zhou et al. 2019). The authors showed that *REG $\gamma$*  (encoded by *Lrrc32*) can promote IRF8 degradation and propose that *REG $\gamma$* -deficiency allows accumulation of IRF8, promoting *Itgb8* expression by DC in turn increasing  $T_H17$  generation.

Similarly, Kudo and colleagues demonstrated that  $\beta 8$  expression by CD11c-expressing phagocytes is required for  $T_H17$  generation in a model of airway hyperresponsiveness (AHR). *Cd11c<sup>Cre</sup>.Itgb8<sup>fllox/fllox</sup>* were therefore protected from both Ovalbumin (OVA) and House Dust Mice (HDM) induced AHR (Kudo et al. 2012).

Although these studies have demonstrated a novel function of the TGF $\beta$ -activating  $\alpha_v\beta 8$  integrin for the control of T cell differentiation, which DC subset is involved in this process still needs to be determined. The high specialization of cDC2 for the induction of  $T_H17$  irrespectively of  $\alpha_v\beta 8$ -mediated TGF $\beta$ -activation would suggest that these cells could also participate in the  $\alpha_v\beta 8$ -mediated  $T_H17$  induction. While it has been shown that steady state expression of *Itgb8* by cDC2 is very low, evidence point towards a possible upregulation of *Itgb8* on cDC2 following activation. Atarashi and colleagues demonstrated that *Itgb8* is upregulated on LP cDC2 upon ATP stimulation either secreted by the microbiota or released from apoptotic epithelial cells (Atarashi et al. 2008). Furthermore, investigation of human LP cDC in reveals that IBD patients display higher frequency of  $\alpha_v\beta 8^+$  cDC2 than non IBD patients. LPS stimulation also induces the increase of the frequency of  $\alpha_v\beta 8^+$  cDC2 but not cDC1 (Fenton et al. 2016).

These results show that  $\alpha_v\beta_8$  is critical for the generation of  $T_H17$  in inflammatory conditions. cDC2 specialization for  $T_H17$  induction and their ability to express  $\alpha_v\beta_8$  upon stimulation by inflammatory signals makes them likely candidates for  $T_H17$  generation in this context. A formal demonstration of the involvement of cDC2 is still required.

A schematic summary of the function of  $\alpha_v\beta_8$  on the generation of  $T_H17$  is presented in Fig. 6

### 2.3. $\alpha_v\beta_8$ expression by blood monocytes and tissue macrophages regulates immune homeostasis.

In their recent study, Kelly and colleagues have demonstrated a novel, human restricted, function for  $\alpha_v\beta_8$  on monocytes and macrophages for the control of immune homeostasis (Kelly et al. 2018).

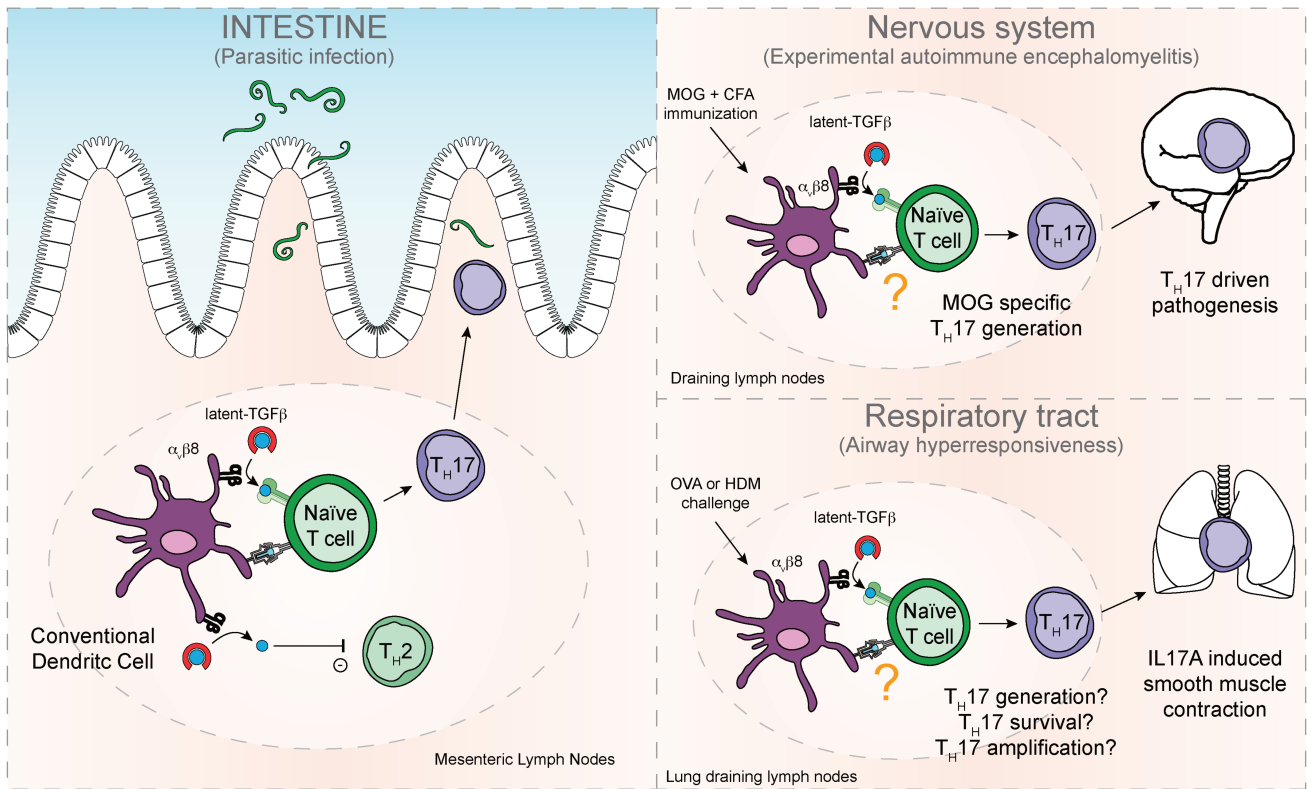
The authors demonstrate that around 40 to 90% of human  $CD16^+$  and  $CD14^+$  monocytes express the  $\beta_8$  integrin. However,  $CD14^+$  but not  $CD16^+$  blood monocytes can activate latent-TGF $\beta$  *in vitro* in a  $\beta_8$ -dependent manner. The preferential expression of MMP14 by the former explains this restriction of TGF $\beta$ -activation to  $CD14^+$  classical monocytes. This  $\alpha_v\beta_8$ -mediated activation of TGF $\beta$  by monocytes allows the autocrine regulation of monocyte responsiveness to LPS and decreases their expression of the pro-inflammatory TNF $\alpha$  cytokine. Furthermore, upon *in vitro* differentiation into pro-inflammatory but not anti-inflammatory macrophages, the expression of  $\beta_8$  integrin by monocytes and their ability to activate TGF $\beta$  is lost. Furthermore, expression of  $\alpha_v\beta_8$  by monocytes and macrophages can be found in the healthy human *lamina propria* but is lost in patient with active Inflammatory Bowel Disease (IBD) symptoms. It is interesting to note that this is the opposite effect as the one observed on cDC2 expression of  $\alpha_v\beta_8$  in IBD patients (Fenton et al. 2016), suggesting a different regulation of the *Itgb8* locus.

Although we have not investigated the function of  $\alpha_v\beta_8$  expressing tissue resident macrophages, it is likely that these cells participate in the regulation of TGF $\beta$ -dependent immune responses and the promotion of intestinal homeostasis. Whether the loss of  $\alpha_v\beta_8$  expression in IBD patients is a cause or a consequence of the disease would be a very interesting point to investigate. Furthermore, although mouse monocytes do not express the  $\alpha_v\beta_8$  integrin, the expression by tissue resident macrophages would be very interesting. In particular, it is interesting to note that macrophages express the CD11c integrin and  $\alpha_v\beta_8$  deletion on these cells could participate in the inflammatory phenotype observed in *Cd11c<sup>Cre</sup>.Itgb8<sup>fllox/fllox</sup>* mice.

A schematic summary of  $\alpha_v\beta_8$  role on monocytes and macrophages in the homeostatic and inflamed intestine is presented in Fig. 7.

Overall the studies presented in these sections have highlighted the importance of the ability of mononuclear phagocytes to sense their environment (metabolites, cytokines, TLR ligands or inflammation) and to shape the adaptive immune response accordingly, sometimes to the detriment of homeostasis. The importance of TGF $\beta$  and its activation by the  $\alpha_v\beta_8$  integrin appears to be important in the regulation of intestinal homeostasis and T cell differentiation.





**Figure 6 - Described function for  $\alpha_v\beta_8$  integrin on cDC for the generation of T<sub>H</sub>17 responses**

In the context of steady state immunity, parasitic intestinal infection, experimental Autoimmune Encephalomyelitis (EAE) or Airway Hyper Responsiveness (AHR), it has been shown that  $\alpha_v\beta_8$  expression by cDC is critical for the generation of protective (*Trichinella spiralis* infection) or harmful (EAE and AHR) T<sub>H</sub>17 generation. The requirement for cognate contact and the exact mechanisms by which cDC present active TGF $\beta$  to T cells have however not been fully elucidated.

## 2.4. $\alpha_V\beta_8$ expression by T<sub>REG</sub> is required to control intestinal inflammation.

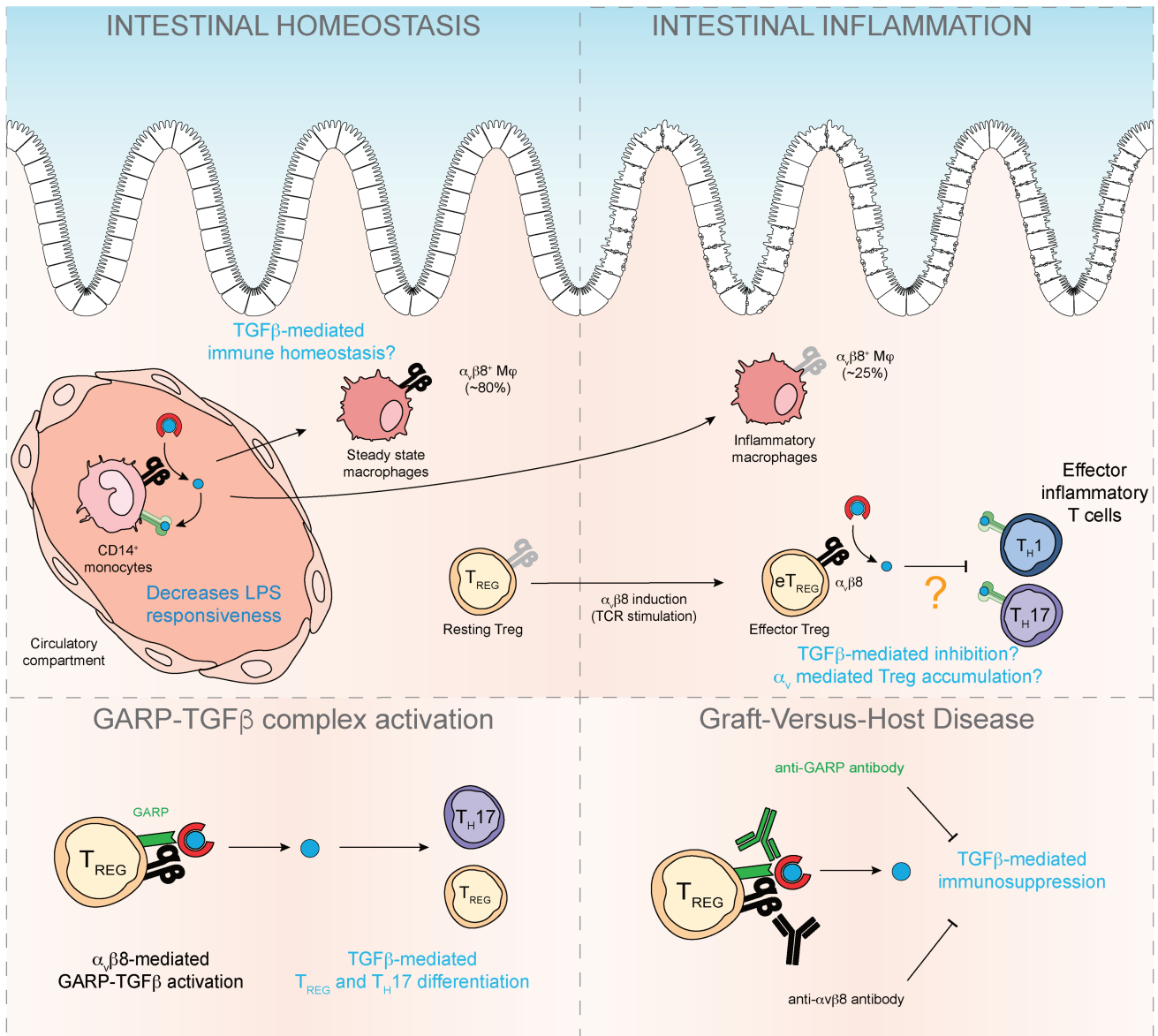
Initial characterization of *Lck<sup>Cre</sup>.Itgav<sup>flox/flox</sup>* or *Cd4<sup>Cre</sup>.Itgb8<sup>flox/flox</sup>* mice revealed that  $\alpha_V\beta_8$  expression was not required for the maintenance of T cell homeostasis in resting mice. However, a study by Worthington and colleagues revealed that T<sub>REG</sub> and in particular activated KLRG1<sup>+</sup> T<sub>REG</sub> expressed high levels of the *Itgb8* transcript. Furthermore, T<sub>REG</sub> from *Cd4<sup>Cre</sup>.Itgb8<sup>flox/flox</sup>* had a reduced ability to suppress T cell proliferation in a model of naïve T cell transfer into *Rag<sup>-/-</sup>* mice and in a model of T cell mediated colitis. Additionally, *Foxp3<sup>Cre</sup>.Itgb8<sup>flox/flox</sup>* display a worsen outcome to DSS-mediated colitis due to excessive intestinal and peripheral cell activation. The authors further demonstrated that  $\beta_8$ -deficiency of T<sub>REG</sub> did not affect T<sub>REG</sub> maintenance but rather decrease pSmad signaling in colitic activated cells (Worthington et al. 2015).

However, previous publications by Edwards and colleagues suggested that conditionally deleting *Itgb8* on T<sub>REG</sub> did not lead to any deterioration of their *in vivo* and *in vitro* inhibitory proficiency (Edwards, Thornton, and Shevach 2014). Recently published data further suggest that  $\alpha_V$ <sup>-/-</sup> T<sub>REG</sub> can suppress T cell responses in a similar way than  $\alpha_V$ -competent T<sub>REG</sub> (Mair et al. 2018). This study rather suggests that  $\alpha_V$ -mediated control of T cell response in a colitis model rather depends on the capacity of T<sub>REG</sub> to accumulate at the inflammatory site. There is, at this moment, no clear explanation to explain the discrepancies between these studies.

Edwards and colleagues have demonstrated through several publications that  $\beta_8$  expression on T<sub>REG</sub> was required for the release of GARP-bound latent-TGF $\beta$ . They further demonstrated that GARP-TGF $\beta$  was not required for T<sub>REG</sub> inhibitory functions as stated above but rather that active-TGF $\beta$  released from this  $\beta_8$ -mediated activation can induce T<sub>H17</sub> and T<sub>REG</sub> generation in the presence of exogenous IL6 or IL2 respectively (Edwards et al. 2013; Edwards, Thornton, and Shevach 2014). Independent work by Stockis and colleagues have shown that  $\beta_8$  blocking antibodies inhibits T<sub>REG</sub> TGF $\beta$ -activation and in turn worsen Graft-Versus-Host Disease (GVHD) outcome (Stockis et al. 2017). The authors further show that  $\beta_8$  interacts with the GARP-TGF $\beta$  complex and suggest that  $\beta_8$ -mediated activation of GARP-bound latent-TGF $\beta$  is the main mechanism behind  $\beta_8$ -mediated T<sub>REG</sub> inhibition in the context of GVHD. Similarly, Cuende and colleagues, have also suggested that GARP-TGF $\beta$  monoclonal antibody, inhibiting the immunosuppressive activity of human T<sub>REG</sub> could function by disrupting the interaction between GARP-TGF $\beta$  complexes and the  $\alpha_V\beta_8$  integrin (Cuende et al. 2015).

These previous studies have demonstrated that TCR stimulation of T<sub>REG</sub> was a potent inducer of *Itgb8* expression (Worthington et al. 2015; Edwards, Thornton, and Shevach 2014; Stockis et al. 2017). Furthermore, Siede and colleagues demonstrated that activated ST2<sup>+</sup> T<sub>REG</sub> expressed higher levels of the *Itgb8* transcript than ST2<sup>-</sup> cells, and suggested that this was responsible for their enhanced TGF $\beta$ -mediated suppressive ability (Siede et al. 2016). ST2 is the newly discovered receptor for the IL33 cytokine, released upon cell damage of the epithelium of the endothelium. This suggest that *Itgb8* expression by T<sub>REG</sub> also depends on their sensing of the cytokine environment and inflammation. The mechanisms underlying the specific expression of *Itgb8* by T<sub>REG</sub> are however unknown.

A schematic summary of  $\alpha_V\beta_8$  function on T<sub>REG</sub> in the regulation of TGF $\beta$ -mediate immune responses is presented in Fig. 7.



**Figure 7 -  $\alpha_v\beta_8$  expression by T<sub>REG</sub>, monocyte and monocyte-derived macrophages regulates intestinal homeostasis.**

In the context of intestinal homeostasis, it has been shown that  $\alpha_v\beta_8$  expression by human monocytes is critical for preventing excessive immune responses to LPS. In the LP,  $\alpha_v\beta_8$  expression by human monocyte-derived macrophages is lost in inflammatory conditions such as IBD (Kelly et al. 2018). Furthermore,  $\alpha_v\beta_8$  expression by T<sub>REG</sub> is important for inhibition of effector inflammatory T cell responses in the context of T cell induced colitis although the underlying mechanisms are still not entirely understood (Worthington et al. 2015; Edwards, Thornton, and Shevach 2014; Mair et al. 2018) More generally,  $\alpha_v\beta_8$  and the latent-TGF $\beta$  binding GARP protein expression by T<sub>REG</sub> has been shown to be critical for the activation of TGF $\beta$  and regulating TGF $\beta$  dependent responses. Furthermore, anti-GARP or anti- $\beta_8$  antibodies have been shown to inhibit TGF $\beta$  mediated T<sub>REG</sub> functions (Edwards et al. 2013; Edwards, Thornton, and Shevach 2014; Stockis et al. 2017; Cuende et al. 2015).

## 2.5. Open questions on the role of $\alpha_v\beta_8$ expression in the control of T cell responses.

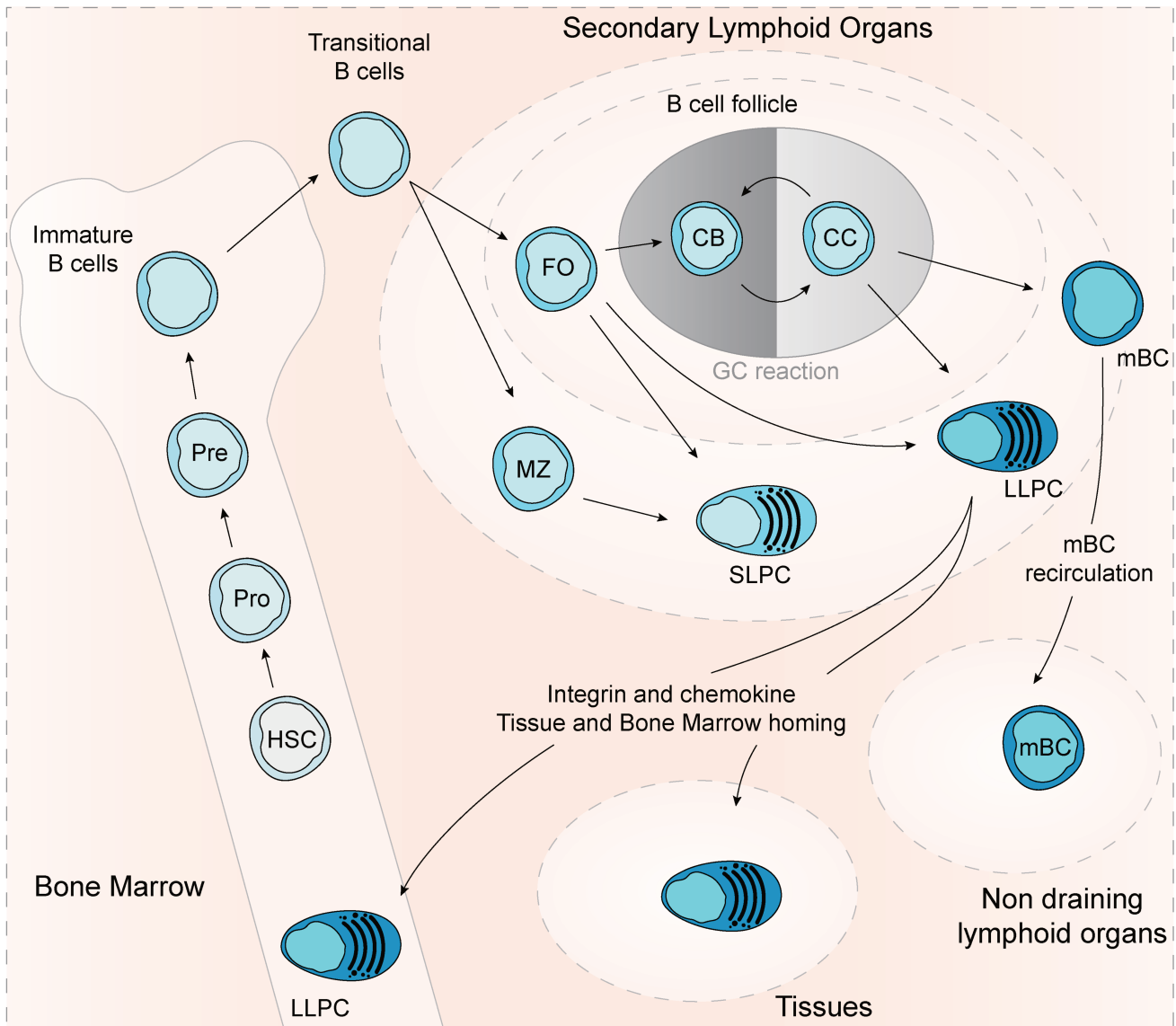
All the studies presented above have sketched an interesting model, summarized across the various figures (Fig. 5 to 7), for  $\alpha_v\beta_8$  expression by DC and T<sub>REG</sub> for the control of immune responses. However, a few key data are missing to complete the existing models. In this paragraph, I will attempt to highlight the remaining open questions that we have investigated in the upcoming “Results” or commented in the “Discussion” section.

Concerning the ability of MLN DC to generate T<sub>REG</sub> and T<sub>H17</sub> by  $\alpha_v\beta_8$ -activated TGF $\beta$ , it would be interesting to have an insight on:

- The precise *in vivo* contribution of cDC1 and cDC2 in the  $\alpha_v\beta_8$ -mediated generation of T<sub>REG</sub> and T<sub>H17</sub> in the intestine, both at steady state and in an inflammatory or tolerance setting. To this day, no real *in vivo* evidence that cDC1 or cDC2 restricted expression of *Itgb8* is necessary and sufficient to induce T<sub>REG</sub> or T<sub>H17</sub>.
- The contribution of  $\alpha_v\beta_8$  expression by cDC1 and cDC2 and their ability to generate T<sub>REG</sub> or T<sub>H17</sub> in the very strong inflammatory phenotype of *Cd11c<sup>Cre</sup>.Itgb8<sup>flox/flox</sup>* mice. To this day, it is still unknown whether the inflammation is a consequence of an imbalance in the T<sub>REG</sub>/T<sub>H17</sub> ratio or related to other mechanisms.
- Discrepancies between studies have been found regarding *Itgb8* expression by the various subsets of DC in the various immune compartment. The ability to investigate single-cell expression of the  $\beta_8$  subunit by the various DC subsets would be greatly useful.

The precise mechanisms controlling the subsets specific, tissue restricted and the temporal control through activation of *Itgb8* expression by DC and T<sub>REG</sub> is still obscure in the field. Whether determined genetically by the lineage or epigenetically through conditioning by the environment, the mechanisms underlying the ability for a particular DC to express the  $\beta_8$  integrin is still unknown.

The requirement of cell:cell contact for the presentation of active-TGF $\beta$  by integrins and in particular  $\alpha_v\beta_8$  is still unknown. Interestingly, the study by Acharya and colleagues has highlighted the importance of MHC-mediated cognate contact between DC and T cells for the proper generation of  $\alpha_v\beta_8$  mediated T<sub>H17</sub> generation (Acharya et al. 2010). However, in the rest of the immune synapses, the mechanism is unknown. In particular, the  $\alpha_v\beta_8$ -mediated immunomodulatory function of T<sub>REG</sub> and its inhibition of inflammatory T cell responses probably does not involve such a cognate synapse. The very recent study by Campbell and colleagues, proposes a molecular model allowing for the close-contact activation of latent-TGF $\beta$  by  $\alpha_v\beta_8$ . However, the precise spatiotemporal regulation of TGF $\beta$  presentation to DC, monocytes, macrophages or T<sub>REG</sub> is still unknown.



**Figure 8 - Overview of B cell differentiation and activation mechanisms.**

B cell differentiation in the BM compartment from Hematopoietic Stem Cells (HSC) to mature B cells is a multi-step process. During this process Pro-B cells (Pro) and Pre-B cells (Pre) undergo genetic rearrangement of the VDJ region of the BCR locus gives rise to immature B cell bearing a functional BCR. Migration of Immature B cells to the peripheral Secondary Lymph Organs (SLO) finishes the maturation of naïve B cells. B cell seeding the B cell follicle (Follicular B cells - FO) or the spleen marginal zone (MZ B cells) can be activated by recognition of an antigen by their BCR. BCR activation of B cells first generates Short Lived Plasma Cells (SLPC) to produce a rapid low-affinity B cell response. FO B cells can be activated through T-dependent (Germinal Center dependent) or T-independent mechanisms (extrafollicular), to provide long lasting memory. In the GC, B cell realize multiple cycle between dark zone Centroblasts (CB) and light zone Centrocytes (CC) allowing proliferation, diversification and selection of novel B cell clones. Upon exit from the GC or at the end of the T-independent activation, Long Lived Plasma Cells (LLPC) and memory B cells (mBC) are generated. LLPC home to the Tissues or the Bone Marrow for antibody production. mBC recirculate and seed other SLO. Upon antigen reencounter the mBC can be reactivated and generate faster and higher affinity immune responses.

### 3. TGF $\beta$ is involved in the regulation of B cell responses.

Pathogen clearance and formation of long-lasting protection requires the activation of B lymphocytes and subsequent differentiation into antibody (Ab)- secreting plasma cells (PC) and memory B cells (mBC). A few studies have demonstrated the importance of TGF $\beta$  signaling in the regulation of B cell responses, in particular during B cell lymphopoiesis, B cell proliferation and differentiation, and class-switch recombination. However, TGF $\beta$  signaling on B cell is a classic example where the importance of TGF $\beta$  activation has not been properly investigated.

In this section of the manuscript I will address the importance of TGF $\beta$  and of TGF $\beta$  activation on B cells responses:

- A brief overview of B cell responses, from mature B cell to their differentiation into plasma cells or memory B cells.
- The importance of TGF $\beta$  and TGF $\beta$  activation on B cell Class-Switch to IgA
- The importance of TGF $\beta$  signaling in the regulation of the Germinal Center Response

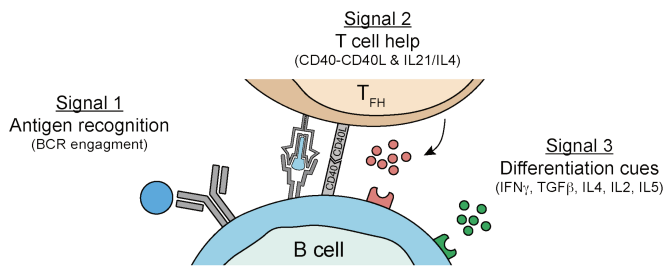
#### 3.1. The multiple pathways of B cell activation.

B cells development is initiated in the Bone Marrow (BM) from hematopoietic stem cells. The succession of different developmental stages allows the generation of B cells with a functional non-self-reactive B cell receptor (BCR). Upon egress from the BM, immature B cells migrate into secondary lymphoid organs (SLO), localize in B cell follicles (Follicular B cells) or spleen marginal zone (Marginal Zone B cells) where they complete their development into mature naïve B cells. A brief overview of B cell differentiation and activation processes is shown in Fig. 8.

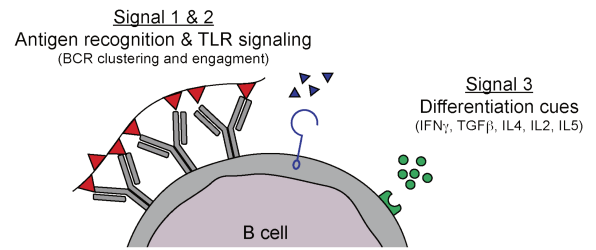
##### 3.1.1. B cell responses can be categorized into T cell-dependent or -independent responses.

B cell activation begins upon encounter with an antigen specific for its BCR. However, additional signals are required for B cells to complete activation and allow differentiation into effector and memory cells, namely Plasma Cells (PC) and memory B cells (mBC). There are two main pathways by which B cell responses can be generated which depend on the nature of the antigen and whether T cell help is required: the T-dependent and T-independent pathways. The mechanisms involved in both these pathways are presented in this section, summarized in Fig. 9 and are extensively reviewed in (Murphy and Weaver 2016).

## T-DEPENDENT RESPONSES



## T-INDEPENDENT RESPONSES



**Figure 9 - Schematic representation of B cell activation through T cell-dependent and -independent pathways.**

T-dependent activation of B cell relies on the recognition of antigen by the B cell BCR, the help signals provided by follicular T cells (CD40L and IL21 among other) and cytokine stimulation for differentiation into the proper isotype and memory cell. Long reticulated antigen, sometime associated with TLR ligand, can bypass the requirement for T cell help by cross-linking large amount of BCR, allowing for B cell activation and survival independently of T cell help. Similarly, cytokine stimulation provides differentiation cues to B cells.

In the classical T cell-dependent pathway, binding of the antigen, usually small protein antigens, induce downstream BCR signaling through the Syk and the phosphatidylinositol (PIP) signaling pathway. This signal alone is however insufficient to fully activate B cells. Antigen binding to the BCR leads to uptake, degradation, and presentation on MHCII molecules. This is associated with the upregulation of CCR7, and subsequent migration of activated B cells to the T:B cell border. There, interaction with T cells, through TCR:MHCII-peptide binding, allows co-stimulation of B cells by CD40L and cytokines presented by T cells. Upon encounter with T cells, B cells will continue their maturation and differentiation either in follicles where they form GC or in extrafollicular foci where they develop into short-lived plasmablasts for a rapid, low affinity short burst of antibody delivery (Fig. 8). The delivery T cell help by CD40/CD40L and cytokines throughout the GC response promotes B cell survival.

In the T-independent pathway, binding of the antigen, including polysaccharides, will activate B cell without the requirement of T cell help. On one hand, TI-1 antigens, are able to activate B cells by the simultaneous presentation of TLR ligands, while on the other hand, TI-2 antigens can bind and cluster a large number of BCR at once allowing to bypass the requirement of T cell help. The activation of B cells through this pathway will not allow formation of GC but will allow production of both short-lived and long-lived PC, thus providing memory. Regardless of the activation pathway, the differentiation of B cell into effector cells (PC or mBC) and of the appropriate class depend on signals delivered by helper cell. In particular, cytokine signaling is thought to be critical to determine the appropriate class-switch depending on the context.

### 3.1.2. The GC reaction is a coordinated cascade of events during which the amplitude, affinity and class of T-dependent B cell responses are determined.

The germinal center (GC) is a micro-anatomical specialized structure found in B cell follicles upon T-dependent B cell activation. This structure is generally composed of a dense, B cell rich area called the 'Dark Zone' (DZ) and a lighter zone, rich in B cells but also follicular helper T cells ( $T_{FH}$ ) and follicular dendritic cells (FDC) called the 'Light Zone' (LZ). These two zones are sites for two distinct processes of the GC reaction (GCR) namely B cell proliferation and selection respectively. The GCR is a highly coordinated response that I will try to succinctly describe in the following paragraph. A schematic representation can also be found in Fig. 10. Additionally, the GCR is extensively reviewed in (Gatto and Brink 2010; Stebegg et al. 2018; Victora and Nussenzweig 2012; Mesin, Ersching, and Victora 2016).

The GCR consists of the cycling of B cells between two phases of B cell activation in the LZ and in the DZ. B cells will successively cycle between the DZ where they proliferate and acquire germline mutation and the LZ where they are selected for their affinity towards the antigen. This selection determines the ability of DZ B cells to re-enter the LZ, die or differentiate into effector or memory cells. Iterative cycles of proliferation, mutation and selection will allow the generation of high affinity B cell clones.

In the DZ, upon interaction with T cell, B cells are instructed for survival and proliferation. This proliferation is the main determinant of the breadth of the antibody response mounted against a particular antigen. Concomitantly, B cells undergo important AID-mediated germline mutation of the VDJ-CR3 region of their BCR.



This phenomenon, called 'Somatic Hyper Mutation' (SHM), induces mutations in the antigen recognition site of the BCR allowing to slightly change the antigen specificity and affinity of the B cell. Downregulation of CXCR4 on DZ B cells (also called Centroblast) induces the migration of these cells to the LZ (William et al. 2002).

In the LZ, these newly generated B cell clones are selected for high antigen affinity as well as low self-reactivity preventing the development of autoreactive B cell clones potentially generated through random mutagenesis during SHM. The selection of B cell clones relies on the presentation of the antigen to B cells by FDC cells and the presentation of survival signals by FDC and T<sub>FH</sub>. The absence of BCR stimulation by the antigen and the survival signals provided by T cell induce B cell apoptosis. In that sense, the competition of B cells for the limited T<sub>FH</sub> cell help, based on their affinity for the antigen and the density of their peptide-presenting MHC, is thought to be the main mechanism for positive selection of B cell clones.

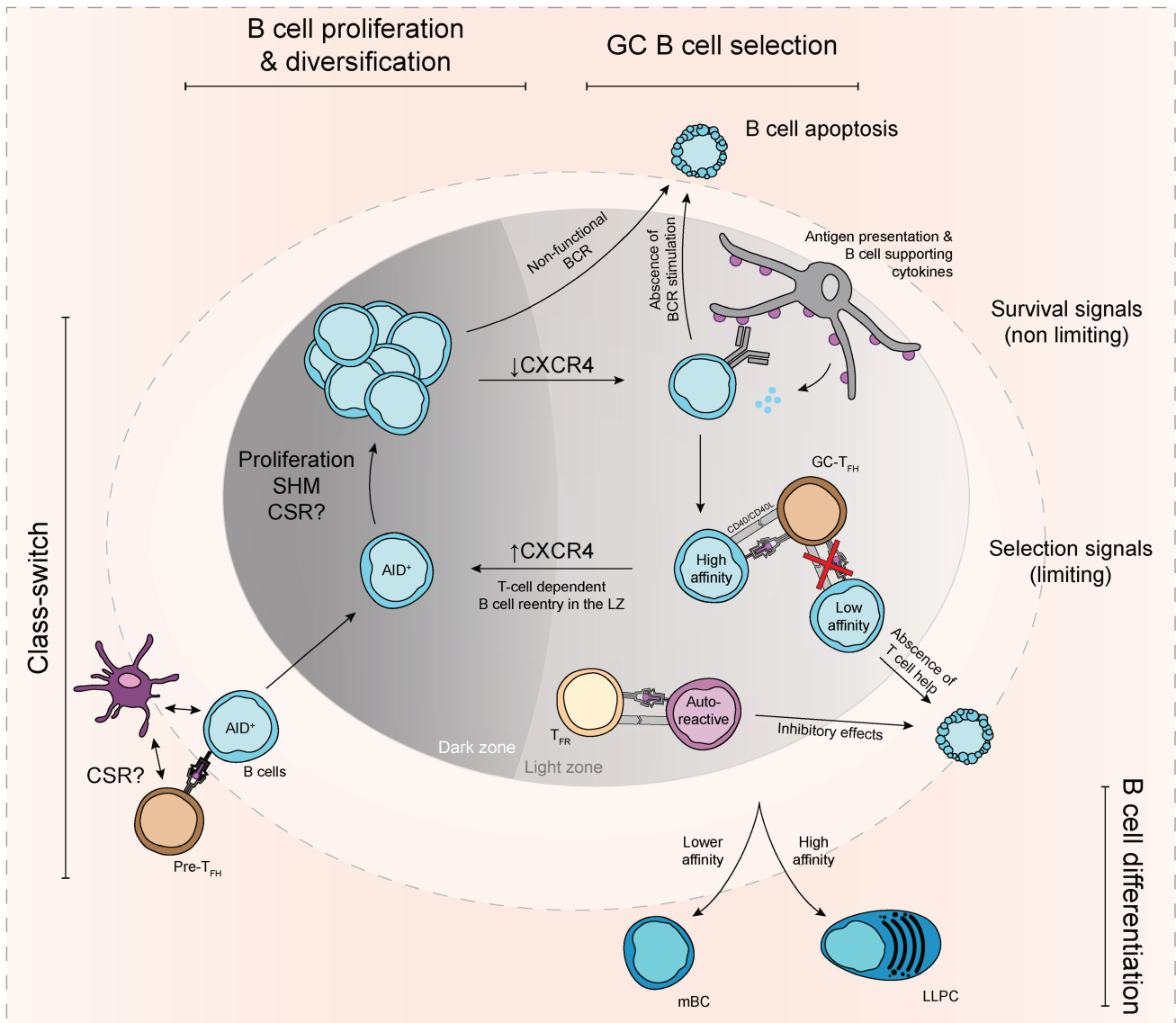
In contrast the negative selection of auto-reactive clones is poorly understood. The recent description of follicular regulatory T cells (T<sub>FR</sub>) generated from thymic T<sub>REG</sub> with self-reactive TCR is thought to be one of the mechanisms for negative GC B cell selection. Interaction of self-antigen presenting B cells with T<sub>FR</sub> and presentation of inhibitory molecule such and CTLA4 and PD1 might be one of the mechanisms for B cell apoptosis. Interaction with T<sub>FH</sub> in the LZ provides survival and proliferation signals which allow B cell to upregulate CXCR4, migrate to the DZ and go through a new round of proliferation, mutation and selection.

The strength of B cell:T cell interaction in the LZ is critical as it has been shown to be a major determinant for the intensity of B cell proliferation but also of the exit of B cell from the GC and differentiation into mBC or PC. B cell fate decision is detailed later in the manuscript.

During T-dependent responses, AID-mediated mutations in the constant region of the BCR locus allows the change of Ig class from IgM to other class such as IgG, IgA or IgE by recombination between different locus of the Ig locus. Recent publications have however challenged the fact that this process occurs during the GCR but rather suggest that it occurs prior to B cell entry in the GCR after initial interaction with T cells and DC (Roco et al. 2019; Reboldi et al. 2016). Cytokine stimulation of B cells is a major determinant of the CSR process. In particular, TGF $\beta$  has been shown to be critical for the class-switch to IgA class, while other stimulation such as IFN $\gamma$  or IL4 promotes IgG or IgE responses respectively.

Overall, the GCR is a key process for B cell responses, and its regulation is critical to provide an adapted humoral immune response. In particular, the GC is critical to determine the amplitude, the affinity and the class of the generated antibodies and the amount and quality of the mBC and long-lived PC (LLPC) generated, while preventing the development of inappropriate B cell responses (lymphomas, auto-immunity). It is however important to note that the generation of humoral memory and of class-switched B cell response is not unique to the GCR as extrafollicular B cell responses and T-independent B cell responses are also able to generate class-switched memory B cells.

In the rest of this manuscript we will focus on describing the regulation of the B cell CSR and GC B cell responses by the TGF $\beta$  cytokine.



**Figure 10 - Overview of B cell selection during the Germinal Center Reaction (GCR)**

Upon interaction by pre-GC  $T_{FH}$  at the T:B border, B cells enter the GC. The GCR is composed of two geographically distinct phases occurring in the Light Zone (LZ) and Dark Zone (DZ) of the GC.

In the Dark Zone, Centroblast (CB) undergo massive proliferation and AID-mediated Somatic Hyper Mutations (SHM) generating clones with new specificity. Downregulation of CXCR4 allows the migration of CB to the Light zone for T cell selection.

In the light zone, interaction of Centrocytes with the dense network of Follicular Dendritic cells (FDC) carrying the antigen allows to deliver non-specific and non-limiting survival signals. If the BCR is unable to recognize the antigen or does not allow functional signalization, B cell undergo apoptosis. Interaction of B cells with  $T_{FH}$  and the presentation of survival co-stimulatory molecule such as CD40L allows to promote survival of B cell, promotes reentry into the GC and proliferation of B cells. The number of antigen specific B cell being limited, competition between low affinity and high affinity B cell clones for T cell help allows to promote survival of high affinity clones. Absence or lower T cell help also induces B cell apoptosis. It has been proposed that self-reactive B cell clones could also be actively negatively selected by interaction with  $T_{FR}$ .

Finally, T cell help is also associated with the selection of B cell differentiation. High affinity B cells preferentially differentiate into Long Lived PC (LLPC) while lower affinity B cell rather differentiate into memory B cells (mBC) (Stebegg et al. 2018). The GCR also promotes B cell class-switch recombination (CSR). However, it is still unclear whether CSR occurs during the GCR or at earlier stage during the interaction of pre-GC B cells with T cells or DC (Reboldi and Cyster 2016; Roco et al. 2019).

### 3.2. IgA CSR is critically dependent on TGF $\beta$

One of the most well-known and investigated function of TGF $\beta$  in B cell biology is its ability to promote CSR of B cells. It is well accepted that TGF $\beta$  is a critical cytokine in the regulation of mucosal IgA<sup>+</sup> responses. However, it is important to note that, although not discussed further in this manuscript, TGF $\beta$  has also been described to promote IgG2b CSR and IgG3 secretion *in vitro* (McIntyre et al. 1993; Snapper et al. 1993).

In this part of the manuscript, I will briefly introduce the importance of intestinal IgA responses in the control of intestinal homeostasis and the generation of IgA responses before detailing the importance of TGF $\beta$  and TGF $\beta$  activation in the control of IgA<sup>+</sup> B cell responses.

The generation and function of IgA responses in the intestine has been extensively studied over the years. In this section, a brief overview of the mechanisms will be presented but more details can be found in (Bunker and Bendelac 2018; Woof and Kerr 2006; Brandtzaeg and Johansen 2005; Cerutti and Rescigno 2008; Feng, Elson, and Cong 2011)

#### 3.2.1. IgA B cell responses are critical for the control of commensal microbiota and host defense.

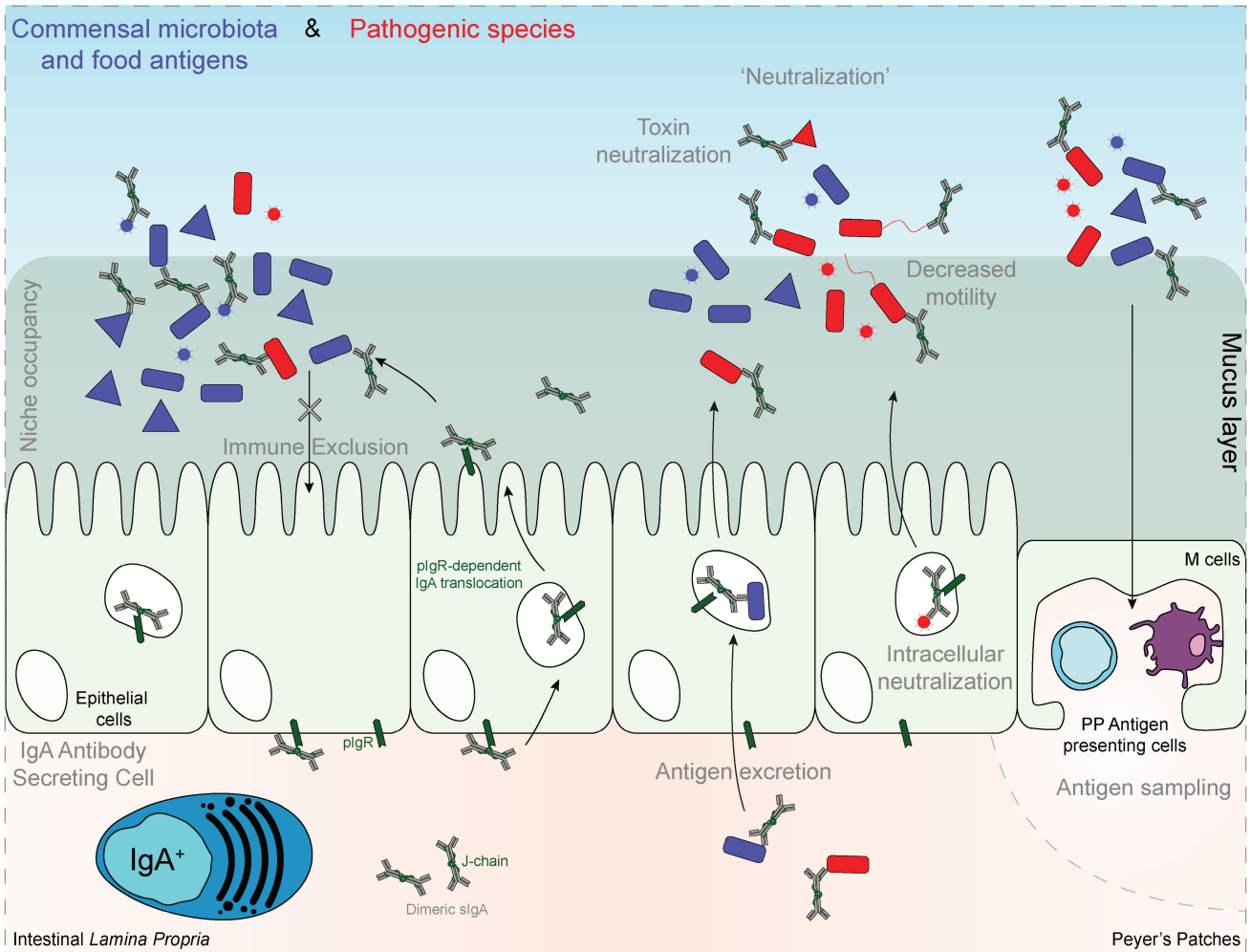
The intestinal *lamina propria* harbors a large population of PC, 70-80% of which are IgA<sup>+</sup>. These PC secrete large quantities of dimeric secretory IgA (sIgA) antibodies linked to the polypeptidic 'joining' chain (J-chain). These sIgA antibodies have the ability to bind to the polymeric Immunoglobulin receptor (pIgR) expressed by intestinal epithelial cells. Binding to pIgR promotes the endocytosis and transcytosis of the sIgA through the epithelial barrier and their secretion into the intestinal lumen.

Numerous functions for sIgA have been described or hypothesized. These functions are summarized in Fig. 11. These functions include:

- Neutralization of intestinal bacteria or viruses by binding toxins, preventing their binding and entry into the *lamina propria*, by altering their mobility, modulating their gene expression and by excluding by pathogenic entities present in the *lamina propria* by transcytosis.
- Promotion of antigen uptake
- Retention of intestinal flora to the mucus layer of the intestinal lumen.

Overall, sIgA promote the tolerance of the intestinal commensal flora, protecting against bacterial infection of the *lamina propria*, while maintaining and shaping a healthy bacterial flora at a close distance to benefit from their metabolic functions. The close interaction between the intestinal flora and environment and IgA responses has been the focus of numerous studies.

In the context of pathogenic infection, in particular viral infection, it has been shown that IgA responses can play an important role in limiting infection, helping for viral clearance and providing immune memory to prevent further reinfection. In the particular case of Rotavirus (RV) infection, it has been shown that IgA response to RV is important to clear RV infection and protect against reinfection (Blutt et al. 2012).



**Figure 11 - Functions of sIgA on intestinal homeostasis.**

Intestinal LP antibody secreting cells secrete large amount of secretory IgA (sIgA) containing a J-chain. Binding of the J-chain by polymeric Immunoglobulin Receptor (pIgR) on intestinal epithelial cell. Transcytosis of sIgA across the epithelial barrier allows the secretion of antibody in the intestinal lumen.

IgA antibody have been shown and hypothesized to exert a large array a function. Overall, sIgA promote the tolerance of the intestinal commensal flora, protecting against bacterial infection of the lamina propria, while maintaining and shaping a healthy bacterial flora at a close distance to benefit from their metabolic functions.

### 3.2.2. Generation of IgA responses to the commensal flora and food antigen.

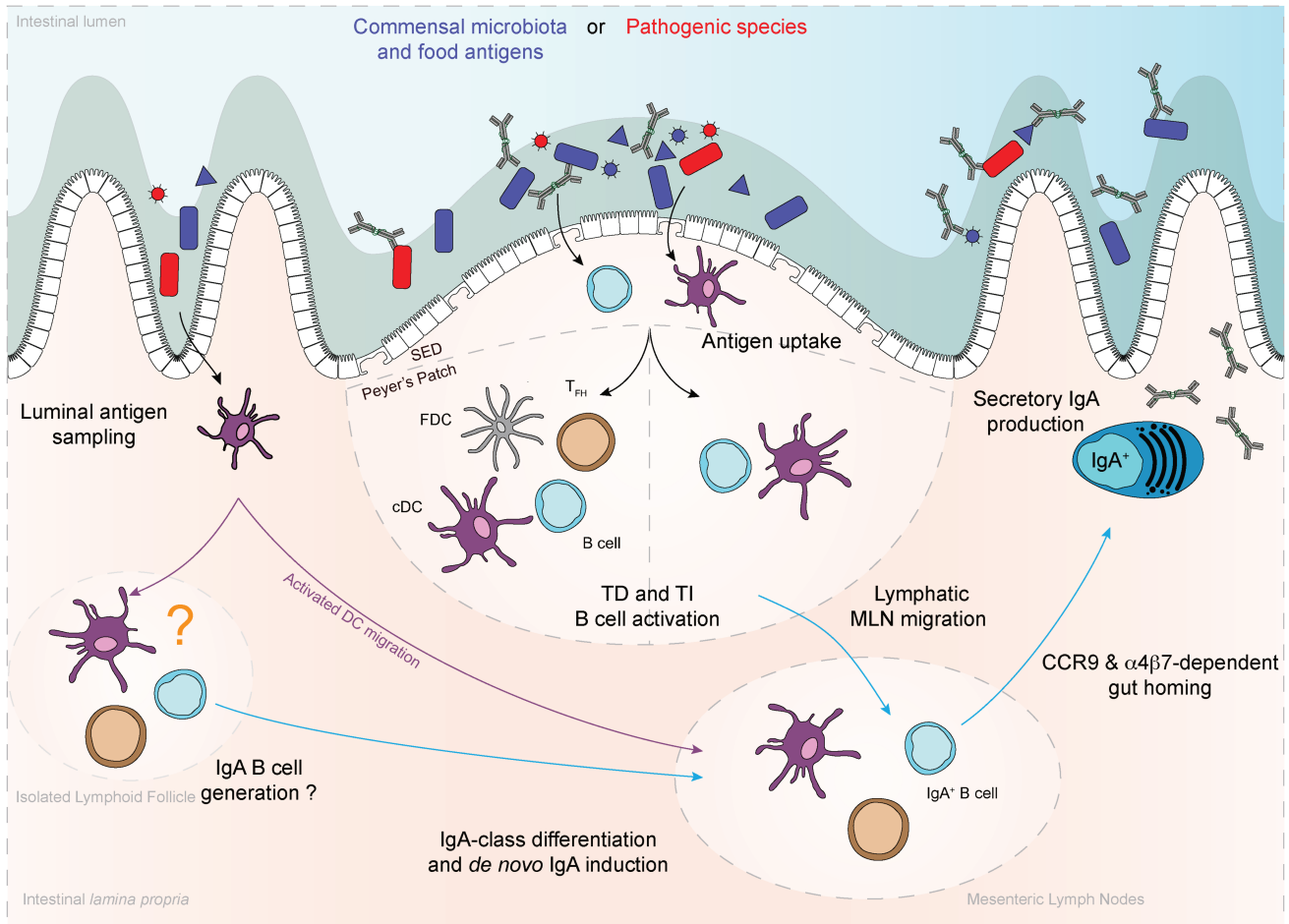
The overall IgA response is a well-coordinated response from the first induction of B cell CSR to IgA in the 'induction sites' to the differentiation of B cells to IgA-secreting PC, migration and seeding of the 'effector' sites. This process involves a lot of cellular and molecular factors that have been extensively studied over the years. I will however only give a brief overview of the whole IgA differentiation process (Fig. 12) and further detail the importance of DC in this process (Fig. 13).

Initiation of intestinal IgA responses mostly occurs in specialized secondary lymphoid organs called Peyer's Patches (PP), located in the intestinal wall. In PP, luminal antigens are sampled through specialized cells called M-cells. Upon translocation through M-cells, antigens are presented to antigen presenting cells (APC), notably DC and B cells, located in the Sub-Epithelial Dome (SED) of PP. Antigen recognition by B cells initiates the activation of IgA B cell responses. Alternative sites of IgA B cell induction have been described: intestinal draining Mesenteric Lymph Nodes (MLN), and Isolated Lymphoid Follicle (ILF), scattered along the intestinal lamina-propria. Sampling of intestinal antigens by DC extending dendrites through the epithelial layer, passive diffusion of antigens or migration of invasive pathogenic species, allows the generation of IgA responses in these organs (Brandtzaeg and Johansen 2005).

B cell activation in the GALT is mediated both by T cell-dependent and -independent pathways although a large majority of the response is T-dependent. Activation of B cells in both pathways relies on help signals provided by T cells, FDC (T-dependent pathway) and/or DC. These cells provide critical factors for the survival, proliferation and differentiation of B cells. TGF $\beta$  is absolutely required for IgA CSR, as TGF $\beta$ R deficient B cells totally lack IgA responses (Stavnezer and Kang 2009). Additionally, other cytokines such as BAFF, APRIL and retinoic acid (RA) also promote IgA B cell responses. More precisely, TGF $\beta$  and RA synergically act on B cell to induce IgA CSR on mucosal B cells while BAFF and APRIL provide non-IgA specific survival signals (Seo et al. 2013). The importance of TGF $\beta$  is detailed in the following paragraphs.

Following activation, B cells migrate from the 'induction site' through the MLN circulating in the lymph and enter the blood circulation on their way to the 'effector site'. During this migratory process, B cells will differentiate into plasmablasts before seeding the intestinal *lamina propria* and fully differentiating into IgA-producing plasma cells. This migration is mediated by the expression of the homing receptors  $\alpha 4\beta 7$ , CCR9 and CCR10.

Regardless of the activation pathway, the significance of DC help for promoting IgA responses has been well documented and is reviewed in (Tezuka and Ohteki 2019). As indicated previously, intestinal DC can be divided into three main categories: conventional DC (cDC), monocyte derived DC (moDC) and plasmacytoid DC (pDC). All three subtypes of DC have been implicated in the regulation of IgA responses as follows.



**Figure 12 - Generation of IgA B cell response in the intestine**

IgA response induction occurs in the Gut Associated Lymphoid tissues (GALT), namely Peyer's Patches (PP), Mesenteric Lymph Nodes (MLN) and Isolated Lymphoid Follicle (ILF) although the latest is still controversial.

In the PP, sampling of luminal antigens through the epithelium by the specialized M cells allows to present antigens to Sub Epithelial Dome (SED) B cells and Phagocytes. Alternatively, antigen sampling in the LP by phagocytes and subsequent migration to MLN of ILF allows to activate B cell responses.

B cell activation can occur through both T-dependent and T-independent B cell responses in these various sites depending on antigen biochemical nature. B cell activation induces migration of these B cell through the lymph to the MLN and then to the LP via the blood circulation. Exposition to the intestinal environment and the signals provided by the supporting cells allows the preferential CSR to IgA in mucosal B cells. CCR9 and  $\alpha 4\beta 7$  migration of IgA<sup>+</sup> plasmablast and final differentiation in the LP generates large amount sIgA producing cells seeding the intestinal LP.

### *a. Conventional DC*

Multiple pathways of IgA promotion by cDC have been described depending on the organ and context in which IgA induction was studied:

- In the Small Intestinal LP, Uematsu and colleagues have shown *in vitro* that TLR5-expressing CD11b<sup>+</sup> cDC can induce IgA PC differentiation from naïve cells following stimulation by flagellin. These cells promote IgA responses by their secretion of retinoic acid (RA) (Uematsu et al. 2008). More recently, Flores-Langarica and colleagues demonstrated that CD103<sup>+</sup>CD11b<sup>+</sup> MLN cDC2 can induce IgA responses to flagellin, a TLR5 agonist (Adriana Flores-Langarica et al. 2012; A. Flores-Langarica et al. 2018).
- In PP, work by Reboldi *et al.* elegantly demonstrated the role of SED CD11b<sup>+</sup>LTβR<sup>+</sup> DC in promoting IgA CSR. The LTβR ligand LTα1β2 was shown to be produced by RORγt<sup>+</sup> ILC3 cells promoting IgA CSR. Furthermore, the author suggested a role for α<sub>v</sub>β8-activated TGFβ which is detailed later in the manuscript (Reboldi et al. 2016). Additionally, Sato and colleagues proposed that CD11b<sup>+</sup> PP cDC express IL6 allowing to promote IgA<sup>+</sup> B cell responses (Sato et al. 2003).
- In the lung and lung draining lymph nodes, Ruane and colleagues described that both CD103<sup>+</sup> cDC1 and CD24<sup>+</sup> cDC2 can induce IgA<sup>+</sup> B cell responses in a TGFβ and RA dependent manner in responses to the microbiota. (Ruane et al. 2016). Furthermore, in the context of nasal Poly(I:C) immunization, Takaki et al. demonstrated that BATF3<sup>+</sup>TLR3<sup>+</sup>CD103<sup>+</sup> cDC1 are involved in IgA production. Their action is mediated by BAFF, APRIL and TGFβ.

### *b. Monocyte-derived DC*

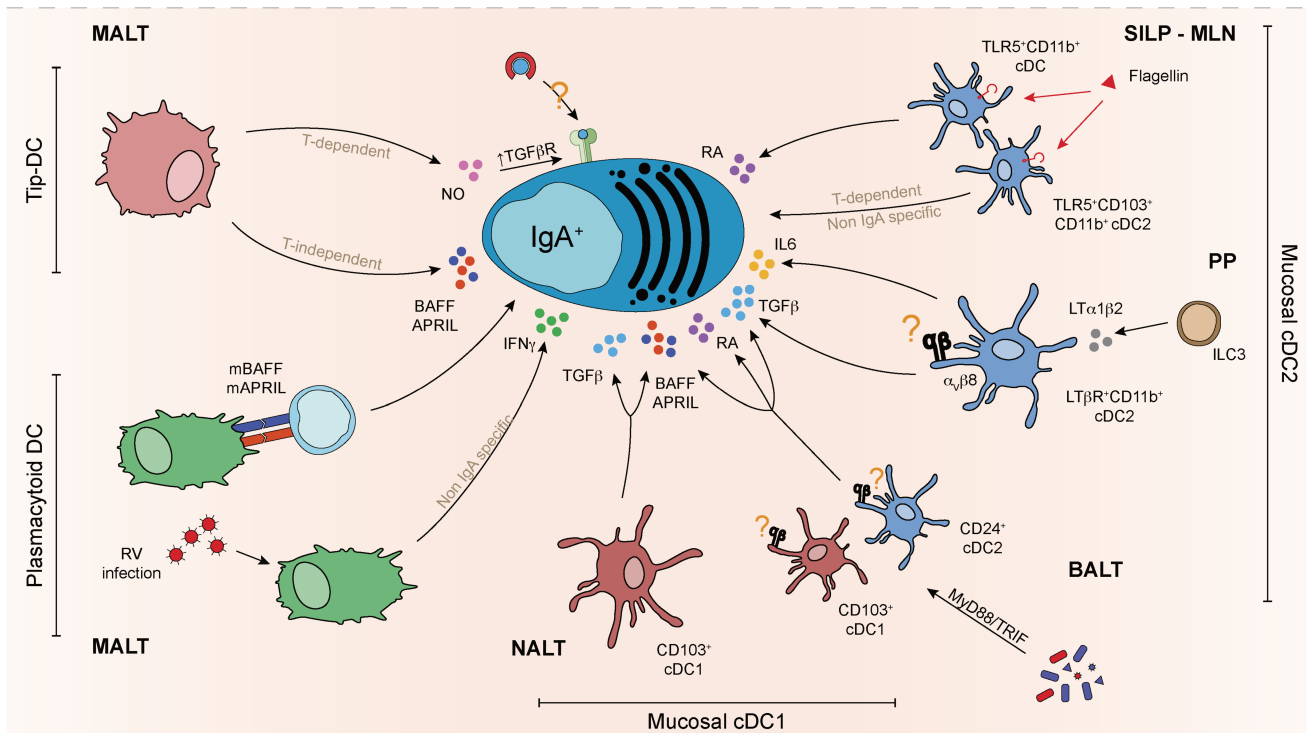
In 2007, Tezuka et al. showed that monocyte-derived TNF/iNOS-producing DC (Tip-DC) can support IgA CSR in PP. The authors further claim that Tip-DC can impact both T-dependent and T-independent IgA CSR (Tezuka et al. 2007). The authors showed that INOS is required for the B cell expression of TGFβR, which licenses their response to TGFβ, resulting in T-dependent IgA induction. They also show that INOS is critical to the production of BAFF and APRIL and the promotion of T-independent IgA responses. The authors further showed that INOS is required on monocyte derived DC (Tip-DC) for the promotion of these IgA responses.

### *c. Plasmacytoid DC*

pDC are key mediators of anti-viral responses, not only as the major producer of Type-I IFN but also display non-conventional function in intestinal immunity thanks to their ability to link innate and adaptive immunity (Reviewed in (Won et al. 2019).

In 2011, Tezuka et al. described that pDC can also induce IgA CSR. The authors further detailed that IFN<sub>γ</sub> produced by stromal cells cooperates with NO produced by Tip-DC to induce membrane bound BAFF and APRIL on pDC and promote T-independent IgA CSR (Tezuka et al. 2011).

In the context of Rotavirus infection, Deal *et al.* have demonstrated that pDC promote human and mouse B cell activation (Deal et al. 2013). We have however shown that pDC are dispensable for inducing steady state and post-immunization IgA responses (Moro-Sibilot et al. 2015).



**Figure 13 - IgA promoting factors secreted by the various mucosal DC populations**

Across the literature, numerous DC population have been involved in the promotion of IgA responses. This figure summarizes the cells, context and molecular factors involved in IgA promotion. Although  $\alpha_v\beta_8$  has been shown to be expressed preferentially expressed by PP cDC2 and lung draining Lymph Nodes DC, a proper formal demonstration that cDC1 or cDC2 expression of  $\alpha_v\beta_8$  is required for generation IgA responses is still lacking.



### 3.2.3. IgA CSR is critically dependent on TGF $\beta$ signaling in B cells.

The first observations that TGF $\beta$  signaling could induce IgA CSR came from the *in vitro* studies of LPS-stimulated mouse B cells (Coffman, Lebman, and Shrader 1989; Sonoda et al. 1989). These studies have shown that TGF $\beta$ , in collaboration with other cytokines such as IL5 and IL2, could specifically enhance IgA production by B cells while inhibiting the production of other immunoglobulin classes. These studies also showed that TGF $\beta$  could enhance IgA production from IgA<sup>-</sup> B cells but not IgA<sup>+</sup> B cells further suggesting that TGF $\beta$  could induce CSR rather than stimulate antibody production or secretion.

The importance for TGF $\beta$  signaling in the generation of IgA responses *in vivo* was demonstrated by Cazac and Roes using a transgenic mouse model with conditional deletion of the TGF $\beta$ RII molecule on CD19<sup>+</sup> B cells (*Cd19<sup>Cre</sup>.Tgfb2<sup>flox/flox</sup>*). These mice display a strong decrease of IgA responses (and to some extent IgG2b), whether looking at B cell, PC or immunoglobulin concentration in naïve mice or following immunization by multiple antigens and routes of administration (Cazac and Roes 2000; Borsutzky et al. 2004).

Further studies have characterized the molecular pathway leading to IgA CSR following TGF $\beta$  signaling. Smad signaling downstream of the TGF $\beta$ R, in cooperation with the RUNX3 and CREB proteins will bind TGF $\beta$ -responsive elements (TGF $\beta$ RE) in the I $\alpha$  promoter of the heavy chain locus. Fixation of these different transcription factors will lead to the transcription of the C $\alpha$   $\alpha$ -germline transcript ultimately leading to IgA CSR. The regulation of IgA CSR by TGF $\beta$  and other factors is extensively reviewed in (Cerutti 2008; Stavnezer and Kang 2009)

It is important note that TGF $\beta$  is absolutely critical to induce IgA CSR and cooperate with numerous other factors to promote IgA responses. The most commonly described include CD40L, BAFF and APRIL and TLR signaling. These factors provide additional signal to induce  $\alpha$ -germline transcription but also induces AID transcription therefore promoting the CSR process. Furthermore, Retinoic Acid (RA) has been shown to be a specific IgA inducing factor cooperating with TGF $\beta$ 1 to enhance IgA responses (Seo et al. 2013).

### 3.2.4. Active-TGF $\beta$ delivery for the control of IgA.

As stated previously, TGF $\beta$  is produced in a latent form and needs to be activated to bind to its receptor and signal. However, TGF $\beta$  activation pathways for the induction of IgA responses has only been poorly investigated. TGF $\beta$  is abundantly produced in the intestinal compartment. It has been suggested that there are four relevant sources for TGF $\beta$  in the gut: B cells, T cells, APCs, and stromal cells. Whether these cells or other can activate latent-TGF $\beta$  and by which mechanisms is still under investigation. In this paragraph, I review the different studies that have contributed to understanding the mechanisms of TGF $\beta$  activation for the regulation of IgA responses (Fig. 14).

**B cell** have been shown to secrete TGF $\beta$  upon activation through TLR ligand stimulation or CD40 engagement which is able to trigger IgA CSR (Snapper et al. 1993; Zan et al. 1998). However, it is important to note that these *in vitro* studies have not investigated the mechanisms by which TGF $\beta$  is activated. However, recent studies have shown that activated human B cells express the GARP-TGF $\beta$  complex (Dedobbeleer et al. 2017;

Wallace et al. 2018). These studies have shown that antibody-mediated blocking of GARP activity or overexpression of GARP respectively decreases IgA CSR or promotes IgA responses.

T cells and especially T<sub>REG</sub> are also major producers of TGF $\beta$ . Furthermore, numerous studies have shown that T<sub>REG</sub> can participate to IgA CSR through TGF $\beta$  dependent mechanisms (Cong et al. 2009; L. Wang et al. 2015). It is still unclear by which mechanisms latent-TGF $\beta$  is activated by T<sub>REG</sub> for the promotion of IgA CSR. However, given the expression of  $\alpha_v\beta_8$  by activated T<sub>REG</sub> and its ability to activate GARP-bound latent-TGF $\beta$  (Worthington et al. 2015, 201; Edwards, Thornton, and Shevach 2014; Stockis et al. 2017), it would be reasonable to assume that  $\alpha_v\beta_8$  on T<sub>REG</sub> could activate GARP-TGF $\beta$  presented by activated B cells or T<sub>REG</sub> themselves and induce IgA CSR. However, the mechanisms by which T<sub>REG</sub> can interact with activated B cells *in vivo* in the context of T-dependent or T-independent B cell activation is still not known. Whether this could be mediated by T<sub>FR</sub> would be interesting to consider, especially given their importance in regulating the composition of the microbiota through IgA diversification and selection (Kawamoto et al. 2014).

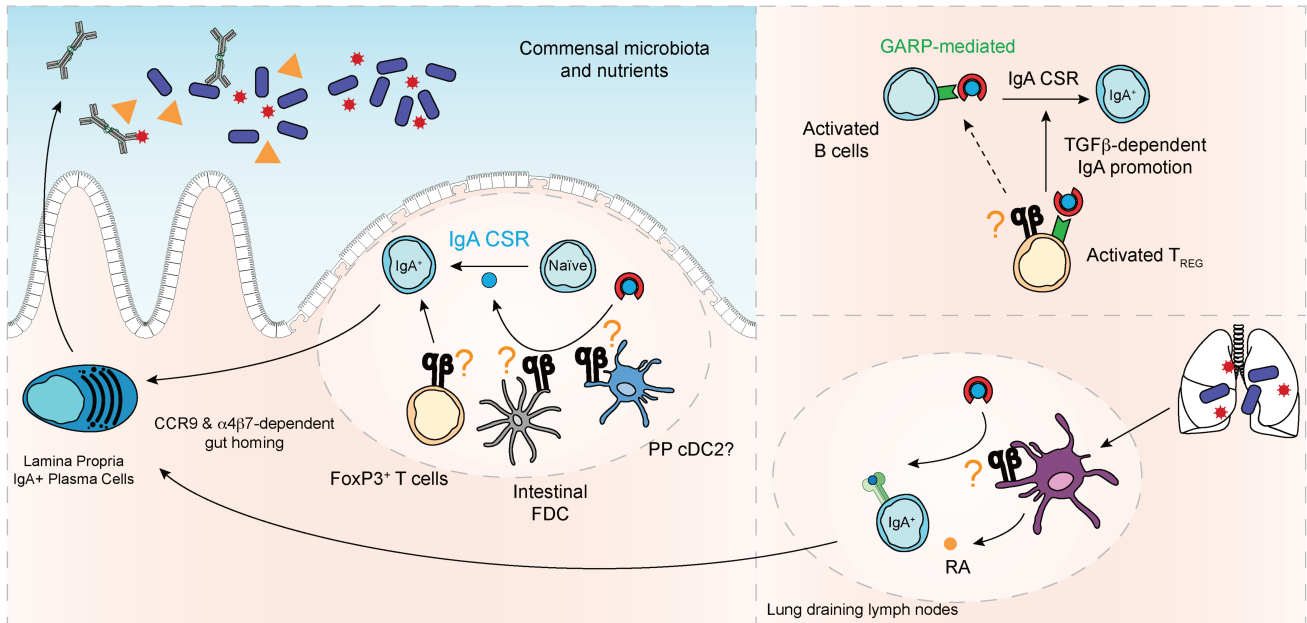
The ability of **DC (cDC or pDC)** to induce IgA responses is now well established (see section 3.2.2). However, the importance of these cells in presenting activated TGF $\beta$  has not been properly explored so far.

In their study, Reboldi and colleagues suggest that PP cDC2 express  $\alpha_v\beta_8$  integrin. *In vitro* co-culture models and observation of PP IgA<sup>+</sup> GC response in *Cd11c<sup>Cre</sup>.Itgb8<sup>flox/flox</sup>* mice suggest that this integrin is involved in the promotion of IgA CSR by PP cDC through the activation of TGF $\beta$  (Reboldi et al. 2016).

Ruane et al. describe that both lung CD103<sup>+</sup> and CD24<sup>+</sup> cDC but not lung macrophages are able to induce IgA CSR in response to the microbiota stimulation and through TGF $\beta$  production. Furthermore the authors also demonstrated that both cDC subsets express the *Itgb8* transcript although formal proof that it is required for IgA induction is still missing (Ruane et al. 2016). Additionally, Takaki and colleagues have demonstrated that Batf3-dependent cDC1 could induce IgA responses in a TGF $\beta$  mediated fashion. Their results suggest that TGF $\beta$  might be activated by MMP9 which is upregulated following Poly(I:C) immunization.

**Stromal cells, and especially Follicular Dendritic Cells (FDC)** have been shown to secrete TGF $\beta$  which could activate TGF $\beta$  signaling in B cells (Munoz et al. 2004). Furthermore, additional studies have demonstrated that TLR and RA stimulation of FDC in the intestinal compartment induces maturation and imprints a gut signature. The intestinal microenvironment of the gut therefore specializes them for IgA induction through TGF $\beta$  and BAFF dependent mechanisms (Suzuki et al. 2010; Garin et al. 2010). In their study, Suzuki and colleagues also demonstrate that gut FDC express high quantity of TGF $\beta$  activating molecules ( $\alpha_v$  integrin subunit, Matrix Metalloproteases, CD36). This suggest that  $\alpha_v$  integrins expression by FDC can participate in the IgA CSR by activating latent-TGF $\beta$ . However, it is interesting to question how these finding relate to the description that CSR occurs outside of the GC.

These studies suggest multiple candidates that could activate and present TGF $\beta$  to B cells for the promotion of IgA CSR (Fig. 14). Importantly, these proposed mechanisms for TGF $\beta$  activation are non-mutually exclusive and the relative requirement for one or the other could depend on the context (homeostatic or infectious environment) and of the site of induction (PP, MLN or ILF).



**Figure 14 - TGFβ activation for the control of IgA responses**

In the GALT, it has been suggested that FoxP3<sup>+</sup> T<sub>REG</sub>, FDC and cDC2 could promote IgA responses by TGFβ dependent mechanisms (Cong et al. 2009; Suzuki et al. 2010; Reboldi et al. 2016). In the case of FDC and PP cDC2, the requirement for TGFβ activating integrin has been suggested by not formally demonstrated.

Activated B cells produce TGFβ and express GARP allowing them to bind latent TGFβ. Given the requirement for T<sub>REG</sub> in promoting IgA CSR (Cong et al. 2009), their expression of α<sub>v</sub>β8 in the mucosal compartment (Worthington et al. 2015) and their ability to activate membrane bound TGFβ (Edwards, Thornton, and Shevach 2014), α<sub>v</sub>β8 activation of B cell bound TGFβ could be one of the mechanisms for TGFβ activation.

Ruane and colleagues have also demonstrated that lung draining cDC could promote intestinal IgA responses. Furthermore, this is associated with their expression of the α<sub>v</sub>β8 integrin. Although no formal proof has been made so far, α<sub>v</sub>β8-mediated TGFβ activation could be a mechanism for IgA induction in the lung draining system.

### 3.3. TGF $\beta$ activation and signaling during the Germinal Center Reaction.

If the induction of IgA responses is the best described role for TGF $\beta$  signaling on B cells, other functions of the cytokine have been described. In this paragraph, I will attempt to summarize the other processes of B cell responses regulated by TGF $\beta$ , focusing particularly on GC B cell responses

The role of TGF $\beta$  on GC B cell responses can be approached from two different perspectives : 1/ The direct effect of TGF $\beta$  signaling on B cells and 2/ The importance of TGF $\beta$  signaling on cells supporting the GCR such as follicular dendritic cells and follicular T cells and their ability to present activated-TGF $\beta$  to B cells.

#### 3.3.1. TGF $\beta$ signaling affects several aspects of B cell biology.

On one hand, early *in vitro* studies have highlighted the importance of TGF $\beta$  signaling for the inhibition of B cell proliferation and B cell or PC apoptosis (Reviewed in (Letterio and Roberts 1998)). On the other hand, the first *in vivo* demonstration of the role of TGF $\beta$  in the regulation of B cell responses was published 20 years ago by Cazac and colleagues using the transgenic mouse model with a conditional KO of TGF $\beta$ RII on CD19<sup>+</sup> B cells (*Cd19<sup>Cre</sup>.Tgfbr2<sup>flox/flox</sup>*). Besides a complete lack of IgA B cell responses (see 3.2.3 above), these mice displayed reduced lifespan of B2 B cells, expansion of B1-peritoneal B cells, elevated IgG3 responses to a weakly immunogenic antigen and B cell hyperresponsiveness (Cazac and Roes 2000). These results have highlighted the immunomodulatory functions of TGF $\beta$  in the prevention of overt and uncontrolled B cell activation.

A recent study has more precisely investigated the importance of specific TGF $\beta$  signaling in the GC B cells *in vivo*. Using several mouse model to conditionally delete TGF $\beta$ R in GC B cells (*Cr2<sup>Cre</sup>.Tgfbr1<sup>flox/flox</sup>*, *Aicda<sup>Cre</sup>.Tgfbr1<sup>flox/flox</sup>*- they have demonstrated that TGF $\beta$  signaling in activated B cells is not responsible for IgA CSR but rather promoted apoptosis and the transition of GC B cells from the Light Zone (LZ) to the Dark Zone (DZ) of the GC. TGF $\beta$ R KO in GC B cells induced accumulation of cells in the LZ, decreased apoptosis and in turn decreased the antibody affinity (Albright et al. 2019).

Once again, it is critical to consider TGF $\beta$  activation in the regulation of these processes.

As indicated previously, activated B cells express GARP and therefore present latent-TGF $\beta$  for activation (Wallace et al. 2018; Dedobbeleer et al. 2017). The model by Campbell and colleagues proposes that  $\alpha_v\beta_8$  expression can activate GARP-bound latent-TGF $\beta$  and present them directly on the TGF $\beta$ R of this same TGF $\beta$ -presenting cells. With this in mind, it is possible that cells supporting B cells responses during the GCR can activate B cell-bound latent-TGF $\beta$  to modulate B cell responses. Formal demonstration that  $\alpha_v\beta_8$ , other integrins or any other mechanisms of TGF $\beta$  activation might be important in the control of the GCR has not been made so far. Other source of latent-TGF $\beta$  is also likely as T cells and FDC, supporting the GCR, have been shown to secrete TGF $\beta$  (Li and Flavell 2008; Aguzzi, Kranich, and Krautler 2014).

Similarly, in the hypothesis that TGF $\beta$  activation is mediated by integrins, the cell type responsible for TGF $\beta$ -activation and presentation to B cells and/or GC B cells has not been identified yet. FDC have been suggested to present active-TGF $\beta$  to B cells for the control of IgA CSR. It is thus likely that these cells or other, such as

follicular T cells, could also present active-TGF $\beta$  to GC B cells to promote antigen responsiveness, apoptosis or LZ to DZ transition by integrin-dependent or -independent mechanisms.

In the next few paragraphs I will try to review the function of these GC supporting cells, the importance of TGF $\beta$  signaling on these cells and their ability to present activated TGF $\beta$  to GC B cells.

### 3.3.2. Follicular helper T cells ( $T_{FH}$ ) interact with B cells during the GCR and influence B cell fate.

#### *a. $T_{FH}$ differentiation relies on cell-cell interaction and cytokine stimulation.*

T cell help to B cells was one of the first function attributed T cell when they were first discovered in the late 1960's. First named T helper cells, a succession of discovery led to the coining of the term follicular helper T cells ( $T_{FH}$ ) in the early 2000's (Crotty 2015). Since then, the molecular mechanisms by which  $T_{FH}$  are differentiated and can help B cell responses have been extensively studied and summarized in Fig. 15.

$T_{FH}$  differentiation is under the strict control of the Bcl6 transcription factor. Expression of Bcl6 by T cells inhibits other T cell differentiation pathways, namely  $T_H1$ ,  $T_H2$  and  $T_H17$  by repressing respectively T-bet, GATA3 or ROR $\gamma$ t expression and/or function. Nevertheless,  $T_{FH}$  have been shown to produce low levels of  $T_H1$ ,  $T_H2$  or  $T_H17$  cytokines such as IFN $\gamma$ , IL4 or IL17 to control B cell fate.

Over the years, the study of the cellular and molecular factors involved in the commitment and differentiation of  $T_{FH}$  have been well characterized. Multiple models have been put forward to summarize all these studies. An integrated multistage and multifactorial model of  $T_{FH}$  differentiation has been developed by Dr. Shane Crotty over the years to (Crotty 2011; 2014; 2019). He proposes that  $T_{FH}$  differentiation relies on three important steps.

- **Priming** of naïve T cells by DC in the T cell zone of secondary Lymphoid organs through TCR:MHCII-peptide interaction with DC. Activation of ICOS on naïve T cell by DC-presented ICOSL and simultaneous IL21 and IL6 stimulation are critical for Bcl6 and CXCR5 expression on T cells. The study of the relative requirement of both cDC subset for the differentiation of  $T_{FH}$  has shown that cDC2 play a dominant role for  $T_{FH}$  priming. Indeed, deletion of cDC2 using the *Itgax<sup>Cre</sup>.Ifr4<sup>flox/flox</sup>* mouse but not cDC1 using the *Batf3<sup>-/-</sup>* mouse prevents the development of  $T_{FH}$  responses (Krishnaswamy et al. 2017). cDC1, moDC or Langerhans cells have however also been shown to promote  $T_{FH}$  development. The APC and molecular factors involved in the priming of  $T_{FH}$  is reviewed in (Krishnaswamy et al. 2018)
- **Commitment** to  $T_{FH}$  identity through their interaction with B cells. Induction of CXCR5 expression together with downregulation of CCR7 by pre-GC  $T_{FH}$  allow them to migrate towards the CXCL13 rich B cell follicle. There, presentation of ICOSL by activated B cells is critical for  $T_{FH}$  maintenance.
- **Final differentiation** of pre-GC  $T_{FH}$  into GC- $T_{FH}$  occurs in the GC. Upon B cell interaction, SAP and IL6-receptor signaling, T cells upregulate expression of Bcl6, CXCR5, PD1 and other surface molecules.

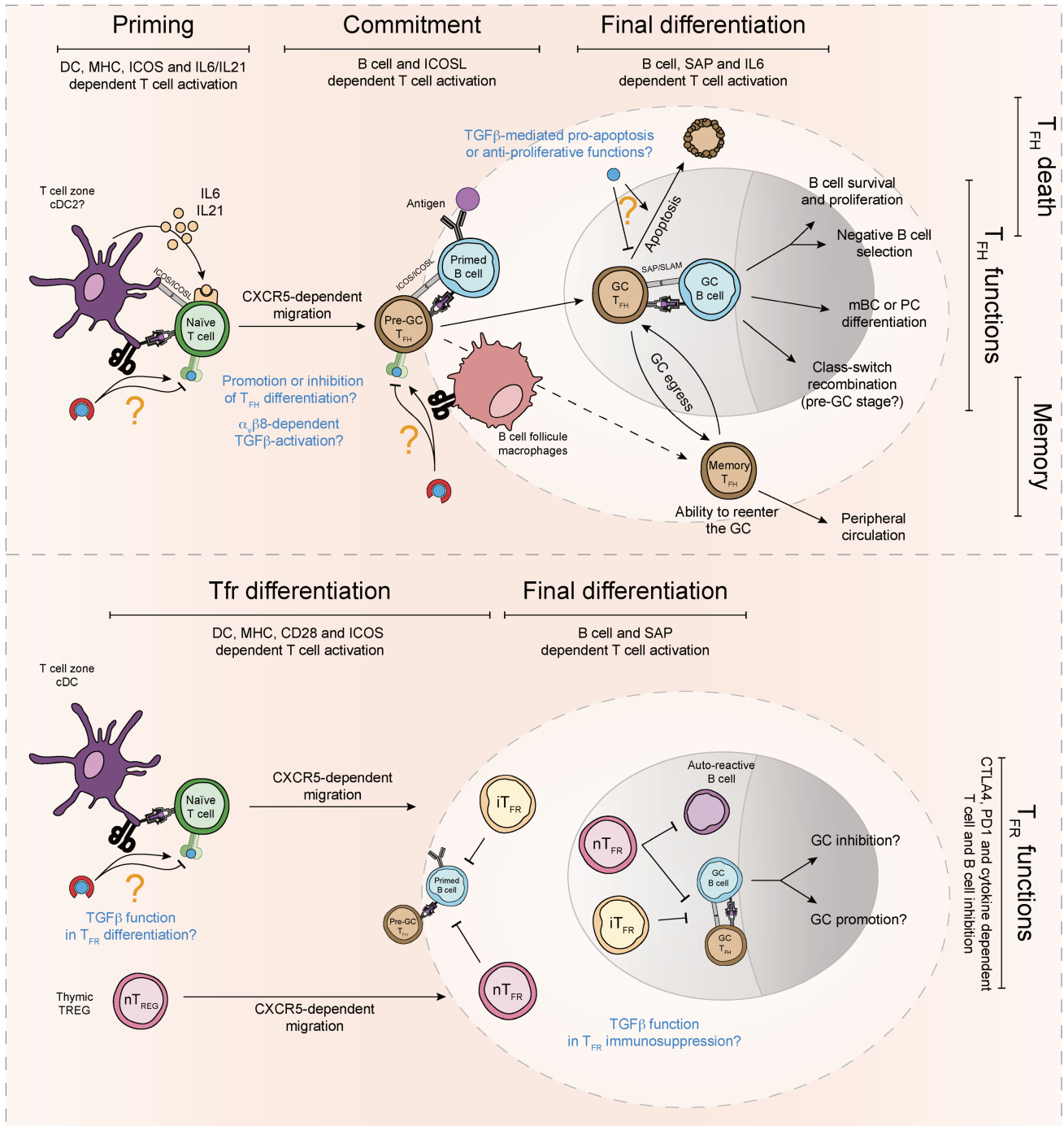
It was initially thought that GC- $T_{FH}$  was the terminal differentiation state of  $T_{FH}$  which could not leave the GC and would then enter into an apoptotic program. However, it has been now well demonstrated that  $T_{FH}$  and GC-

T<sub>FH</sub> can differentiate into memory T<sub>FH</sub> (mT<sub>FH</sub>). These mT<sub>FH</sub> are able to exit the GC compartment, reduce *Bcl6* expression, acquire a less polarized phenotype and can enter the circulation to seed other non-draining SLO. Circulating T<sub>FH</sub> express chemokine receptors with different combinations similar to T<sub>H1</sub> (CXCR3+CCR6-), T<sub>H2</sub> (CXCR3-CCR6-) and T<sub>H17</sub> (CXCR3-CCR6+). These subsets of T<sub>FH</sub> display different effector functions and can differently polarize B cell response (Morita et al. 2011). Upon re-stimulation, memory T<sub>FH</sub> preferentially differentiate back into effector T<sub>FH</sub> and GC-T<sub>FH</sub>. Interestingly, the effector functions of local and circulating mT<sub>FH</sub> are different in their ability to induce B cell responses (Asrir et al. 2017). The mechanisms of mT<sub>FH</sub> differentiation and function are extensively reviewed in (Aloulou and Fazilleau 2019; Hale and Ahmed 2015; Crotty 2014).

It is important to note that presentation of cytokines to naïve T cells and later to pre-GC or GC T<sub>FH</sub> is critical for their commitment and function. If IL21 or IL6 have been shown to be critically and redundantly involved in the commitment of T cells to T<sub>FH</sub>, some evidence for a role of TGFβ in the regulation of T<sub>FH</sub> biology have been reported. To this day, the effects of TGFβ signaling on T<sub>FH</sub> are still controversial.

On one hand, McCarron and Marie describe that the absence of TGFβ signaling on T cells (*Cd4<sup>Cre</sup>.Tgfb2<sup>fllox/fllox</sup>* mouse model) led to the aberrant accumulation of T<sub>FH</sub> and the development of B cell autoreactivity (McCarron and Marie 2014). On the other hand, other studies demonstrated that the development of human follicular T cells and mouse mucosal T<sub>FH</sub> was critically dependent on TGFβ (Marshall et al. 2015; Schmitt et al. 2014). These studies highlight the importance of TGFβ in controlling T<sub>FH</sub> survival and differentiation. These seemingly opposite functions of TGFβ on T<sub>FH</sub> cells have not been reconciled so far. Considering the production of TGFβ in a latent form, it is enticing to consider that the context (tissue localization, time, etc.) in which TGFβ is activated and presented to T cells might be key to resolve these different roles of TGFβ on T<sub>FH</sub> biology.

Very few studies have investigated the requirement for TGFβ activation for T<sub>FH</sub> generation. In 2019, Durand and colleagues have demonstrated that human tonsil T cell zone cDC2 and follicular macrophages were potent inducer of T<sub>FH</sub> cells *in vitro*. They further demonstrated that these cells preferentially express OX40L, secreted TGFβ and expressed *Itgb8* *ex vivo* and after TLR stimulation (Durand et al. 2019). These data suggest that TGFβ activation by cDC2 and macrophages could potentially regulate T<sub>FH</sub> differentiation or activation. The precise *in vivo* biological function of α<sub>v</sub>β<sub>8</sub> and TGFβ in this context was unfortunately not investigated.



**Figure 15 - Models for T<sub>FH</sub> and T<sub>FR</sub> differentiation and function**

(Top) - Overview of the three stages differentiation mechanisms for T<sub>FH</sub> differentiation from naive T cell. The priming by DC, reinforcement by B cell and final commitment in the GC are the 3 proposed steps in T<sub>FH</sub> commitment. Given the described role of TGF $\beta$  in T<sub>FH</sub> differentiation, both in promoting and inhibiting the T<sub>FH</sub> lineage, we propose that the step at which TGF $\beta$  is presented might decide the outcome on T cell differentiation

(Bottom) - Overview of the different T<sub>FR</sub> population. T<sub>FR</sub> can arise from naive T cells (induced T<sub>FR</sub> - iT<sub>FR</sub>) and thymic natural T<sub>REG</sub> (nT<sub>REG</sub>). Upon CXCR5-dependent migration to the B cell follicle, T<sub>FR</sub> can localize to the T:B border or in the GC. The exact function of each T<sub>FR</sub> subset in the regulation of the GC are still not very well characterized. It has been proposed that the origin (Naive T cell or nT<sub>FR</sub>) and the localization of T<sub>FR</sub> in the B cell follicle might decide their function. In the biology of T<sub>FR</sub> the function of TGF $\beta$  is poorly described. Given its importance for the differentiation of T<sub>REG</sub> in the periphery and for the immunosuppressive functions of T<sub>FR</sub>, we've hypothesized that TGF $\beta$  might also critically regulate T<sub>FR</sub> differentiation and function.

### *b. $T_{FH}$ are critical to promote the GCR.*

The canonical function of  $T_{FH}$  is to provide B cell help and promote the GCR, and in particular:

- During the GCR, the selection of B cells depends on the competition of B cells for  $T_{FH}$  help. B cells with a higher affinity BCR can interact preferentially with  $T_{FH}$  which promotes their survival, re-entry into the DZ and proliferation (extensively reviewed in (Shlomchik and Weisel 2012; Mesin, Ersching, and Victora 2016). In the absence of T cell interaction, GC B cells are destined to die through Fas/FasL mediated mechanisms.
- Interaction with  $T_{FH}$  in the LZ also drives the differentiation process of B cells into mBC and PC. Presentation of different co-stimulatory signals and cytokines by  $T_{FH}$  during prolonged T:B interaction drives the differentiation of GCB cells into plasma cells. On the contrary, moderate T cell help signaling might be responsible for mBC commitment. Besides many co-factors, the affinity of TCR:BCR interaction and the length of T:B cell interaction seem to be important determinants of B cell fate.
- Additionally, determination of CSR is also dependent on signals delivered from T cells. Recent work by Roco et al indicates that the presentation of signals required for CSR occurs very early on, during the interaction of pre-GC  $T_{FH}$  with antigen-presenting B cells (Roco et al. 2019), and might not be considered part of the GC.

The molecular determinant of  $T_{FH}$  help to B cells are extensively reviewed in (Crotty 2011; 2015; Vinuesa et al. 2016; Aloulou and Fazilleau 2019; Crotty 2019). In the process of T cell help to B cells, the requirement for  $TGF\beta$  has never been shown to this day.

A schematic representation of the model of  $T_{FH}$  differentiation,  $T_{FH}$  and the potential involvement of  $TGF\beta$  in this process is presented in Fig. 15

### 3.3.3. Follicular regulatory T ( $T_{FR}$ ) cells participate in the regulation of the GCR.

FoxP3-deficiency, either in mice (FoxP3<sup>KO</sup>, Scurfy mice) or in human (IPEX syndrome) could lead to elevated levels of IgG antibodies and the development of auto-reactive antibodies. These observations imply a role for FoxP3<sup>+</sup> T cells in the control of B cell responses. In 2011, three independent studies described for the first time the existence of a CXCR5<sup>+</sup>PD1<sup>+</sup>FoxP3<sup>+</sup> T cell population that was named Follicular Regulatory T cells ( $T_{FR}$ ). (Wollenberg et al. 2011; Chung et al. 2011; Linterman et al. 2011).

In this paragraph we review the recent advances in the field of  $T_{FR}$  cell biology emphasizing on the role of  $TGF\beta$  in their differentiation and functions. The field of  $T_{FR}$  is recent and many aspects of their biology, differentiation and functions remain to be investigated. This paragraph will give a brief overview of these cells, summarized in Fig. 15 but much more details can be found in (Sage and Sharpe 2016; 2015; Fazilleau and Aloulou 2018; Miles and Connick 2018; Stebbeg et al. 2018)

#### *a. Differentiation of $T_{FR}$ .*

Much like  $T_{FH}$ , differentiation of  $T_{FR}$  is critically reliant on the Bcl6 but also Blimp1 transcription factor and other  $T_{FH}$  differentiating factors such as SAP, ICOS or CD28. Similarly, it appears that  $T_{FR}$  development is also



dependent on the interaction of T cells first with cDC for priming and then with B cells for terminal differentiation (Sage and Sharpe 2016).

The three initial studies presented above demonstrated that  $T_{FR}$  arose from thymic FoxP3<sup>+</sup> natural, self-reactive  $T_{REG}$  ( $nT_{REG}$ ) which acquire follicular helper T cell phenotype with the expression of Bcl6, CXCR5, ICOS and PD1 (Wollenberg et al. 2011; Chung et al. 2011; Linterman et al. 2011). However a recent study has demonstrated the ability of  $T_{FR}$  to develop from naïve T cell in condition favoring peripheral  $iT_{REG}$  induction, giving rise to immunizing antigen-specific  $T_{FR}$  in condition of low immunogenicity (IFA) (Aloulou et al. 2016). It is however likely that this population of induced  $T_{FR}$  ( $iT_{FR}$ ) represents a minority of the total  $T_{FR}$  population in the GC. The antigen specificity of these two subsets of  $T_{FR}$  is therefore quite different.  $T_{FR}$  generated from naïve T cells are specific for the peripheral antigen (including the immunizing antigen but also self or self-associated antigens) while  $T_{FR}$  arising from  $nT_{REG}$  ( $nT_{FR}$ ) are predominantly self-reactive. (Fazilleau and Aloulou 2018). Upon differentiation,  $T_{FR}$  will migrate to the B follicle in a CXCR5-dependent manner.  $T_{FR}$  will then seed different compartment of the B cell follicle depending on their expression level of the CXCR5 chemokine receptor (Sayin et al. 2018).

The requirement for cytokine in the differentiation of  $T_{FR}$  has been poorly studied to this day. Apart for the inhibitory effect of IL2 signaling on  $T_{FR}$  differentiation, the importance of other cytokines has not been demonstrated. In particular, TGF $\beta$  is a critical cytokine for the differentiation of  $iT_{REG}$  and the maintenance of FoxP3 expression by T cells. Its implication in the differentiation and maintenance of  $T_{FR}$  would be quite interesting. Notably, McCarron and Marie have found that TGF $\beta$ R KO on T cells, did not result in a modified  $T_{FR}$  population following NP-KLH in CFA immunization (McCarron and Marie 2014). Because CFA immunization does not favor  $iT_{FR}$  generation (Aloulou et al. 2016), we can assume that this experiment shows the absence of requirement of TGF $\beta$  for the generation of  $nT_{FR}$ . Given the role of TGF $\beta$  for  $T_{REG}$  induction in the periphery ( $iT_{REG}$ ), the role of TGF $\beta$  signaling for  $iT_{FR}$  differentiation would be highly interesting but has not been investigated so far.

Additionally, in an *ex vivo* model of HIV infection, Miles and colleagues demonstrate that HIV infection of tonsil cells induces  $T_{FR}$  expansion which is partly mediated by TGF $\beta$  signaling. The author however failed to demonstrate whether this was due to direct TGF $\beta$  signaling to T cell or other cells supporting  $T_{FR}$  differentiation (DC, B cells, ...) and whether this was due to *de novo*  $T_{FR}$  differentiation of pre-existing  $T_{FR}$  proliferation (Miles et al. 2015).

### *b. $T_{FR}$ function.*

The function of  $T_{FR}$  is the most controversial aspect of their biology. Because the methods for probing  $T_{FR}$  function and the context in which  $T_{FR}$  function was assessed can be extremely different between all the published studies, a very wide array of functions have been described. The description of the various models and their principal findings can be found in (Fazilleau and Aloulou 2018).

$T_{FR}$  are characterized by their high expression of inhibitory molecules such as CTLA4, ICOS, Granzyme A, PD1 or GITR. The expression of these markers, and in particular of CTLA4 and PD1 has been shown to be critical for their inhibitory function of the GC response, limiting GC B cell activation and antibody production. This

negative control of the GC is mediated by the inhibition of both B cells, T<sub>FH</sub> responses and the interaction between these two cells. The inhibitory functions of T<sub>FR</sub> can be thought to be mediated by 1/ Direct signaling of CTLA4 and PD1 on their ligand on B and T<sub>FH</sub> cells, 2/ Secretion of suppressive cytokines and 3/ Competition for cytokine or receptor ligand. If the molecular factors involved in this inhibition are not detailed here, they are extensively reviewed in (Sage and Sharpe 2016; 2015; Fazilleau and Aloulou 2018; Miles and Connick 2018; Stebegg et al. 2018). Interestingly, this inhibitory effect of T<sub>FR</sub> has been shown to be important to prevent the development of autoimmunity. The paper by Fu and colleagues, among many others has shown that T<sub>FR</sub> deficient mice exhibited late-onset spontaneous autoimmune diseases (Fu et al. 2018).

However, and surprisingly, the specific deletion of T<sub>FR</sub> using the *Foxp3<sup>Cre</sup>.Bcl6<sup>flox/flox</sup>* mice has also shown a role for T<sub>FR</sub> in promoting the GC. Wu et al, showed that these mice had no significant modification of the T<sub>FH</sub> and GC B cell populations following immunization but rather had a decreased IgG response to foreign antigen. Additionally, T<sub>FH</sub> in these mice had increased IFN $\gamma$ , IL10 and IL21 expression (Wu et al. 2016). Additionally Xi et al. described that T<sub>FR</sub> could promote the GC response by inhibiting the development of Granzyme B-expressing T<sub>FH</sub> (Xie et al. 2019).

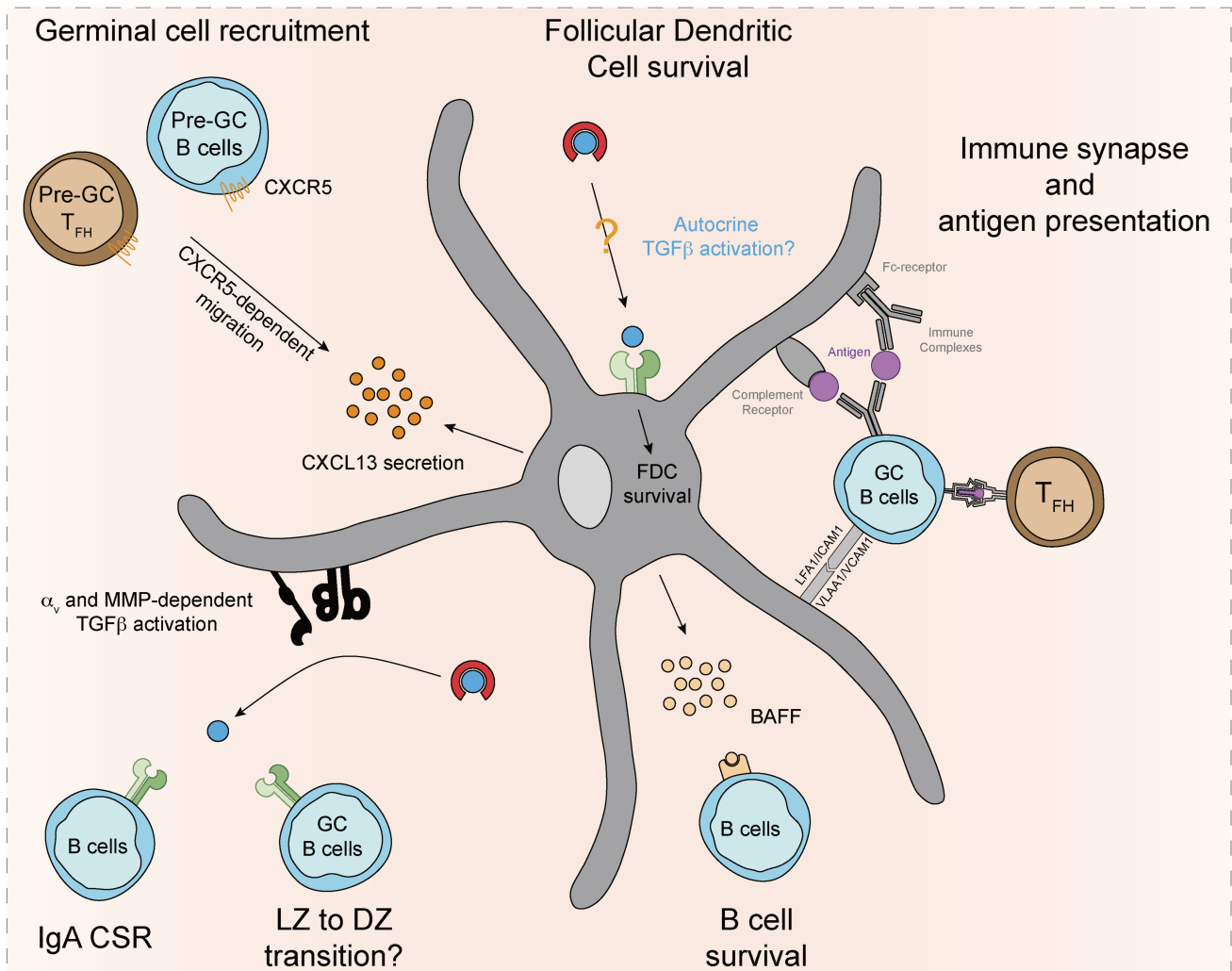
Altogether these results highlight the fact that the function of T<sub>FR</sub> is still not well understood. In their recent review, Fazilleau and Aloulou propose that the heterogeneity in the population of T<sub>FR</sub> that can be generated depending on the condition and the model used (antigen specificity, cytokine secretion, precise follicular localization, ...) can explain the difference in the measured function of these cells.

Once again comparing T<sub>FR</sub> to conventional Bcl6<sup>-</sup> T<sub>REG</sub>, it is interesting to consider their ability to secrete and activate TGF $\beta$ . As described earlier, TGF $\beta$  and its activation by  $\alpha_v\beta_8$  integrins have been shown to be central to the immunosuppressive function of T<sub>REG</sub> and their ability to maintain intestinal homeostasis. The immunosuppressive ability of T<sub>FR</sub> using TGF $\beta$  has been suggested but, to my knowledge, has not been formally proven. A few evidences pointing toward the ability of T<sub>FR</sub> to present and activate TGF $\beta$  can however be found in the literature.

First, Miles and colleagues, demonstrated that HIV infected tonsil cells displayed a higher frequency of LAP-TGF $\beta$  expressing T<sub>FR</sub> (Miles et al. 2015). These T<sub>FR</sub> inhibited the expression of ICOS and the proliferation of T<sub>FH</sub> suggesting an inhibitory role for TGF $\beta$  in T<sub>FR</sub> biology.

Furthermore, in 2017, Wing *et al.* investigated the transcriptional activity of different T cell populations of regulatory T cells, namely CD25<sup>+</sup> and CD25<sup>-</sup> T<sub>FR</sub> and effector and naïve T<sub>REG</sub>. Transcriptional analysis of these two cell populations reveals that *Itgb8* is expressed on effector T<sub>REG</sub> but not naïve T<sub>REG</sub> as previously demonstrated, but also on both CD25<sup>+</sup> and CD25<sup>-</sup> T<sub>FR</sub>. Additionally, Sayin *et al.* demonstrated that T<sub>FR</sub> express elevated levels of the TGF $\beta$ -binding protein GARP, at higher levels than conventional T<sub>REG</sub> (Wing et al. 2017; Sayin et al. 2018).

Altogether, these studies suggest that T<sub>FR</sub> might be able to produce, bind and activate TGF $\beta$  in an  $\alpha_v\beta_8$ -dependent manner although formal demonstration is still required. These studies however did not suggest what the biological function for TGF $\beta$  secretion and activation would be on the GC. The immunomodulatory function of TGF $\beta$  would however suggest that this can inhibit the proliferation of T<sub>FH</sub> and GC B cells.



**Figure 16 - Overview of Follicular Dendritic Cell biology.**

Follicular Dendritic cells (FDC) are critical for the maintenance of the GCR and B cell survival. Its ability to bind the antigen in the GC through Fc and complement receptors and present B cell survival factor (BAFF) is critical in ensuring proper B cell selection in the Light Zone. Furthermore, its ability to secrete CXCL13 is critical in ensuring the proper structure and recruitment of immune cells to GC

TGFβ has been shown to promote FDC survival. Furthermore, FDC derived TGFβ has been shown to promote IgA CSR and suggested to promote LZ to DZ transition of GC B cells. Furthermore, its expression of TGFβ activating molecules in the intestinal compartment suggest that FDC can also activate TGFβ to be presented to B cells.

### 3.3.4. Follicular dendritic cells in the regulation of the GCR.

Follicular Dendritic Cells (FDC) are long reticulated stromal cells which reside in the light zone of the GC. These cells are critical for the maintenance of the GC structure and provide critical help to GC B cells. It is important to highlight their role in GC B cell promotion, through multiple actions.

- FDC are the main producer of the CXCL13 chemokine which is critical to guide GC B cell and T<sub>FH</sub> migration to the GC.
- FDC express high levels of the Complement Receptor and Fc-receptor. Together, they participate in the presentation of naïve antigens to B cells through the binding of immune complexes and antigens carried by the lymph into the lymphoid organs. They are able to form immune synapses with B cells by presenting ICAM1/VCAM1 which binds LFA1/VLA1 on B cells.
- FDC also participate in the generation of a micro-environment that favors B cell survival in the GC. Their secretion of BAFF is critical in the maintenance of GC following immunization.

The precise function of FDC and the molecular determinants involved are review in (Aguzzi, Kranich, and Krautler 2014) and summarized in Fig. 16.

The ability of FDC to secrete TGF $\beta$  has also been involved in the promotion of B cell responses. As previously stated, intestinal FDC can promote the generation of IgA response by secretion of RA and TGF $\beta$  and the expression of TGF $\beta$  activating molecules (Suzuki et al. 2010). Furthermore, Albright and colleagues have suggested that FDC are required for Smad2 signaling in GC B cells (Albright et al. 2019). Whether this is mediated by TGF $\beta$  secretion of activation still requires to be fully investigated.

Additionally, Park et al. describe that TGF $\beta$ -induced Smad2 signaling on FDC themselves decreases their expression of Fas and Caspase 8. This is important for preventing the TNF-induced apoptosis of FDC and thus maintaining a functional FDC network throughout the GCR (Park et al. 2005).

# Results overview

## Objectives

The aim of my PhD was to investigate the importance of  $\alpha_v\beta_8$ -dependent TGF $\beta$ -activation by dendritic cells in the regulation of adaptive immune responses. This study was initially focused on two TGF $\beta$ -dependent immune responses, namely 1/ induced T<sub>REG</sub> generation in the periphery and 2/ intestinal IgA humoral responses. With our unexpected discovery that  $\alpha_v\beta_8$  is expressed by some T<sub>FH</sub> and all T<sub>FR</sub>, my work has, more recently, been extended to the analysis of the biology of TGF $\beta$ -activation by follicular T cells in the control of the germinal center reaction.

This work greatly relies on the generation of a few novel mouse models with which we investigated the biology of  $\alpha_v\beta_8$ -mediated TGF $\beta$  activation. The new *Itgb8*-tdTomato reporter mouse has enabled us to investigate more easily and with much greater detail the expression pattern of this integrin both on CD11c<sup>+</sup> phagocytes and on T cells. The use of several new (*Xcr1*- $\beta_8^{\text{KO}}$ , *huLang*- $\beta_8^{\text{KO}}$ ) and already established (*CD11c*- $\beta_8^{\text{KO}}$  & *CD4*- $\beta_8^{\text{KO}}$ ) conditional KO of the  $\beta_8$  integrin subunit has been particularly useful for investigating *in vivo* the function of  $\alpha_v\beta_8$  expressed by cDC1, cDC2 or T cells in controlling TGF $\beta$ -dependent immune responses. Several *in vitro* approaches have also been developed to study the molecular mechanisms of  $\alpha_v\beta_8$  function on B cell responses. Overall, this work can be subdivided into three main projects which progressively arose along the PhD and with the generation of novel mouse model by the team.

1. *In vivo* requirement of  $\alpha_v\beta_8$  expression on cDC1 and cDC2 to generate TGF $\beta$ -dependent immune responses to the intestinal commensal flora. So far, the requirement for  $\alpha_v\beta_8$  expression on CD11c-expressing phagocytes has been proved *in vivo* and multiple *in vitro* studies had shown the ability of MLN cDC1 and not cDC2 for the generation of iT<sub>REG</sub>. The goal of this study was to investigate further the *in vivo* phenotype of two newly generated mouse models (*Xcr1*<sup>Cre</sup>.*Itgb8*<sup>flox</sup> and *huLangherin*<sup>Cre</sup>.*Itgb8*<sup>flox</sup>), which allow for the specific deletion of  $\alpha_v\beta_8$  on cDC1 and mucosal cDC2 respectively. Our interest in TGF $\beta$ -dependent immune responses led us to investigate the peripheral induction of T<sub>REG</sub> and the generation of IgA immune responses of these mice.
2.  $\alpha_v\beta_8$  integrin-expression by BATF3-dependent dendritic cells facilitates early IgA responses to Rotavirus. MLN cDC1 are the main subset of mucosal DC that express the  $\alpha_v\beta_8$  integrin. The lack of requirement for  $\alpha_v\beta_8$  on cDC1 for the generation of anti-microflora IgA responses (as exposed in the previous chapter), was therefore quite disappointing to us. We therefore sought to investigate the requirement for  $\alpha_v\beta_8$  in the generation of other type of IgA responses. cDC1 are critical in the orchestration of antiviral CD8<sup>+</sup> T cell responses and CD4<sup>+</sup> T<sub>H</sub>1 responses. Furthermore, it has been shown that IgA is the major correlate of protection for certain enteric viral infections. We thus progressively made our way toward the study of  $\alpha_v\beta_8$  expression by cDC1 for the orchestration of anti-rotavirus (RV) IgA responses. This led to an international collaboration with the team of Dr. Katharina Lahl of Lund University in Sweden, specialized in RV infection and cDC1 biology. This study integrates the results from both our teams. This project has led to the writing and submission of a paper, just recently accepted for publication in *Mucosal Immunology*. My specific contribution is detailed below.

3. Investigating the relevance of  $\alpha_V\beta_8$  integrin expression by follicular T cell for the TGF $\beta$ -mediated control of B cell responses. The surprising discovery, about midway through my second year of PhD, first by RT-qPCR then by analysis the *Itgb8*-tdTomato reporter mice, that follicular T cells ( $T_{FH}$  and  $T_{FR}$ ) can express the  $\alpha_V\beta_8$  integrin quickly sparked vivid interest in the lab. My PhD project was therefore re-oriented towards understanding the importance of  $\alpha_V\beta_8$  expression by follicular T cells in the control of the GCR. This led to the development of a few different new mouse models and experimental paradigm through which we questioned this surprising discovery. Although still preliminary, this works gives some insight into the importance of TGF $\beta$ -activation in the control of the GCR.

## Formatting of the Results section

The first part of this section has also been formatted in the format of a paper. We are planning to submit this work to a peer-reviewed journal as soon as possible, after confirming and adding a few experiments to this study. The second part of this Results section presents a paper, written in collaboration with the team of Dr. Katharina Lahl, and has been accepted for publication in Mucosal Immunology. Finally, the last part of my PhD work, has also been formatted for peer-reviewed publication. However, the results being so preliminary, more critical thinking and discussion has been added to the main text to put these results into context. An additional section, highlighting the future considerations and experiments for this study has also been added.

## Personal involvement

### Part 1

Apart for 1/ the DSS experiments which have been carried out by Dr. Helena Païdassi, Principal Investigator of this study and Elise Raoul-Rea during her internship in the lab (Fig. 4), and 2/ the validation of the *Itgb8*-tdTomato reporter mice carried out by Mathilde Boucard-Jourdin (Fig. 1A-B), all the data presented in this study has been generated by myself. Technical help from Mathilde Boucard-Jourdin, Véronique Barateau, Lucie Daniel and other member of the lab has been most helpful in the generation of these data.

### Part 2

The data in this paper has been generated by me and our collaborators in Sweden. All the data relating to RV-infection have been obtained in Sweden, mainly by Joy Nakawesi. I generated the rest of the data with the help of Helena Païdassi and Véronique Barateau.

### Part 3

All the data presented here have been produced by me with the technical help of Véronique Barateau and Loïc Peyrot.

# Results

## Part 1 – In vivo requirement of $\alpha\text{v}\beta\text{8}$ expression on cDC1 and cDC2 to generate TGF $\beta$ -dependent immune responses

### Introduction

The gastrointestinal tract is home to a diverse population of commensal microorganisms, many of which are beneficial to the host as they provide important metabolic help and protection from pathogenic infections. However, it is also an important site of entry of pathogens. The mucosal immune system must therefore walk a fine line to protect against such infections while preventing destructive inflammatory responses to the host's commensal microbiota. A complex and active network of specialized immune cells together participates in intestinal immune homeostasis and the maintenance of barrier integrity, with Foxp3<sup>+</sup> regulatory T cells (T<sub>REG</sub>) and IL17-producing helper T cells (T<sub>H</sub>17) being key players. Foxp3<sup>+</sup> T<sub>REG</sub> are critical in the maintenance of immune tolerance to the microbiota and dietary antigens, in promoting tissue repair and to prevent excessive activation of the pro-inflammatory responses. In particular, induced T<sub>REG</sub> (iT<sub>REG</sub>) generated from naïve T cells in Gut-Associated Lymphoid Tissues (GALT) are critical to prevent the development of mucosal inflammation (Whibley, Tucci, and Powrie 2019). T<sub>H</sub>17, generated in response to bacteria and fungi, have been implicated in autoimmune and inflammatory diseases, however, they are also present in non-inflamed intestine, where they play a key role in anti-microbial immunity and the enhancement of epithelial barrier integrity. (O'Connor, Zenewicz, and Flavell 2010).

Conventional dendritic cells (cDC) directly participate in the regulation of T cell responses. cDC are specialized antigen-presenting cells (APC) that sense environmental, inflammatory and danger signals and instruct adaptive immune responses accordingly. In mice, CD11c<sup>+</sup>MHC<sup>+</sup> cDC are divided into two main subsets, namely type 1 (cDC1) and type 2 cDC (cDC2), both playing key roles in T cell activation and differentiation (Sichien et al. 2017; Chang, Ko, and Kweon 2014; Durai and Murphy 2016). cDC1 and cDC2 development is driven by *Batt3* and *Irf8*, or *Notch2* and *Ifr4* respectively. In the gut, the majority of cDC express  $\alpha\text{E}(\text{CD}103)\beta\text{7}$  integrin and can be distinguished by the expression of CD11b: CD103<sup>+</sup>CD11b<sup>-</sup> cDC1 and CD103<sup>+</sup>CD11b<sup>-</sup> cDC2. The organ localization, microenvironment and inflammatory conditions perceived by these cells determines their precise function in antigen capture and induction of T cell responses (Merad et al. 2013). This process relies, upon activation in the intestinal LP, on the ability of migratory DC (mig. DC) to transport intestinal antigens to Mesenteric draining Lymph Nodes (MLN) for presentation to naïve T cells.

A longstanding subject in the field has been to determine the relative functions of these two subsets. Mouse models allowing to specifically delete one subset or the other have demonstrated that cDC1 are specialized for the induction of Type 1 immune responses to viral and intracellular pathogens, and in particular CD8<sup>+</sup> T cell priming and T<sub>H</sub>1 induction. On the contrary, depletion of cDC2 results in decreased T<sub>H</sub>2 responses to helminth infection as well as reduced T<sub>H</sub>17 generation in the intestinal LP (reviewed in (Durai and Murphy 2016)).

However, while the preferential ability of CD103<sup>+</sup> DC (which comprise both cDC1 and cDC2) to generate iT<sub>REG</sub> in the gut was established 13 years (Coombes et al. 2007; C.-M. Sun et al. 2007), the relative contribution of

cDC1 and cDC2 for the induction of intestinal iT<sub>REG</sub> remains ambiguous. Further studies using conditional deletion of either mucosal cDC1 or cDC2 have failed to show a large modification of steady state intestinal T<sub>REG</sub> frequency (Persson et al. 2013; Esterházy et al. 2016; Arnold et al. 2019a; Luda et al. 2016b). Because depletion of both CD103<sup>+</sup> cDC1 and cDC2 induces a loss of intestinal T<sub>REG</sub>, it is assumed that either a functional cooperation exists between the two cDC subsets and/or that one subset can compensate for the lack of the other.

Importantly, the generation of both iT<sub>REG</sub> and T<sub>H</sub>17 is under the control of Transforming Growth Factor  $\beta$  (TGF $\beta$ ) (C.-M. Sun et al. 2007; Marie et al. 2005; Li, Sanjabi, and Flavell 2006; Coombes et al. 2007; Veldhoen et al. 2006). Unlike most cytokines, TGF $\beta$  is secreted in a latent form. This inactive form of TGF $\beta$  is encapsulated in the Latent Associated Peptide (LAP) that prevents its binding to TGF $\beta$  receptor. Therefore, TGF $\beta$  must be dissociated from the LAP, or “activated” in order to induce TGF $\beta$  signaling in T cells. In the immune system, alpha-v ( $\alpha_v$ ) integrins are key players of TGF $\beta$  activation due to their ability to bind to an Arg-Gly-Asp (RGD) tripeptide in the LAP (Worthington, Klementowicz, and Travis 2011). In fact, a single point mutation in the RGD sequence in the LAP which disrupts  $\alpha_v$  integrins binding site (RGD  $\rightarrow$  RGE), is sufficient to recapitulate the phenotype of *Tgfb1*<sup>-/-</sup> mice, showing the critical importance of integrin-mediated TGF $\beta$ -activation in the regulation of immune responses (Yang et al. 2007).

Previously, we and other have shown that expression of  $\alpha_v\beta_8$  integrin by CD11c-expressing phagocytes (including cDC) is critical for the regulation of TGF $\beta$ -dependent T cell responses (reviewed in (McEntee, Gunaltay, and Travis 2019; Nolte and Margadant 2020)). In particular, the targeted deletion of  $\beta_8$  integrin on CD11c<sup>+</sup> phagocytes (*Cd11c*<sup>Cre</sup>.*Itgb8*<sup>fllox/fllox</sup>, named CD11c- $\beta_8$ <sup>KO</sup> thereafter) leads to the development of severe spontaneous colitis, which is associated with a specific loss of T<sub>REG</sub> and T<sub>H</sub>17 in the intestinal LP (Travis et al. 2007; Lacy-Hulbert et al. 2007; Melton et al. 2010; Acharya et al. 2010; Worthington et al. 2011; Païdassi et al. 2011). Numerous *in vitro* and *ex vivo* experiments have determined that MLN migratory cDC1 predominantly express the  $\beta_8$  integrin subunit, which specializes them for activation and presentation of active TGF $\beta$  to T cells for the generation of T<sub>REG</sub> (Païdassi et al. 2011; Worthington et al. 2011; Boucard-Jourdin et al. 2016; Shiokawa et al. 2017). In addition, previous studies have established the importance of  $\alpha_v\beta_8$ -mediated TGF $\beta$  activation by CD11c<sup>+</sup> phagocytes for the generation of T<sub>H</sub>17 in EAE models of inflammation and parasitic infection (Acharya et al. 2010; Melton et al. 2010; Steel et al. 2019). However, the relative contribution of mucosal DC subsets for the activation of TGF $\beta$  and the orchestration of TGF $\beta$ -dependent T<sub>REG</sub> and T<sub>H</sub>17 differentiation *in vivo* remains an open question. In particular, the formal *in vivo* demonstration that  $\alpha_v\beta_8$  expression by cDC1 is necessary and sufficient for their ability to induce T<sub>REG</sub> is still missing. Furthermore, cDC2 have been shown to induce both TGF $\beta$ -dependent T<sub>H</sub>17 and T<sub>REG</sub> responses, despite a seemingly low expression of  $\alpha_v\beta_8$  (Boucard-Jourdin et al. 2016). Here, we question whether the expression of the TGF $\beta$ -activating  $\alpha_v\beta_8$  integrin by cDC1 or cDC2 is required for their ability to induce T<sub>REG</sub> and T<sub>H</sub>17 at immune homeostasis or in inflammatory conditions.

In this study, we took advantage of a new *Itgb8* reporter mouse model to establish the precise expression pattern of  $\beta_8$  integrin at a single cell level in DC in the intestine and GALT. We confirm that  $\beta_8$  is preferentially



expressed by MLN mig. cDC1. In addition, we show that a small subset of cDC2 also express  $\beta 8$  integrin. We further use several models of conditional KO of  $\alpha_v\beta 8$  on cDC1 or cDC2 to question the requirement of both these DC subsets to induce TGF $\beta$ -dependent intestinal immune responses i.e. T<sub>REG</sub> and T<sub>H</sub>17 generation and control intestinal inflammation. Unexpectedly, while cDC1 is the major *Itgb8*-expressing cDC subset, induction of intestinal iT<sub>REG</sub> is only minimally impaired when  $\beta 8$  is restricted to cDC1. Our preliminary results also indicate that deletion of  $\beta 8$  on cDC1 does not impact the induction and recovery from DSS-induced colitis. By contrast, cDC2-restricted deletion of  $\beta 8$  has a more profound effect on T<sub>REG</sub> generation despite the lower expression of  $\beta 8$  on this cDC subset. By contrast, either cDC1- or cDC2-restricted deletion of  $\beta 8$  only minimally impairs T<sub>H</sub>17 generation in MLN. Altogether these results point towards a cooperation between cDC1 and cDC2 in the activation of TGF $\beta$  by  $\alpha_v\beta 8$  and the control of TGF $\beta$ -mediated immune responses.

## Methods

### Mice

All animals were housed under specific pathogen-free conditions at Plateau de Biologie Expérimentale de la Souris (Lyon, France). All mice were on C57Bl/6 background. Age- and sex-matched littermate controls were used for all experiments. Male and female mice were used.

Transgenic *Cd11c*<sup>Cre</sup> (B6.Cg-Tg(*Itgax-cre*)1-1Reiz/J) were obtained from Nathalie Bendriss-Vermare (CRCL, France), *Xcr1*<sup>Cre</sup> (*Xcr1-IRES-iCre-2A-mTFP1*) from Bernard Malissen (CIML, France) and *HuLang*<sup>Cre</sup> (B6.Cg-CD207<sup>tm2.1(cre)Bjcc</sup>) mice from Katharina Lahl (Lund University, Sweden). They were crossed to *Itgb8*<sup>+/-</sup> or *Itgb8*<sup>fllox/-</sup> obtained from Julien Marie (CRCL, France).

$\beta 8$  integrin expression reporter mice were generated on a C57Bl/6 background by expression of an internal ribosomal entry site-fluorescent tandem dimer (td)-Tomato cassette (IRES-Td-Tomato) immediately following the stop codon in exon 14 of *Itgb8* (Fig. 1A) (GenOway, Lyon, France).

Female C57Bl/6 mice were purchased from Charles River Laboratories (L'Arbresle, France).

Animal experiments were performed under appropriate licenses within local and national guidelines for animal care.

### DSS

Colitis was induced by administration of 1.5% Dextran Sodium Sulfate (DSS) [w/v] [molecular weight (MW), 36000–50000; MP Biomedicals, Santa Anna, USA] in drinking water for 6 days, while control mice received water only. Mice were examined daily with respect to their general condition, body weight and consistency of stools. When indicated, mice were sacrificed on day 9-10 for FACS analysis.

### Cell isolation and Flow Cytometry

For the analysis of lymphocyte population, single-cell suspensions from spleen, LN, and Peyer's Patches were obtained by mechanical disruption through 70  $\mu$ m cell strainer in Complete RPMI buffer (10% FBS, 1% Pen/Strep (Gibco), 1% HEPES (Gibco) and 0.1%  $\beta$ -mercaptoethanol (Gibco)). Mononuclear phagocytes from spleen, LN and PP were isolated as previously described (Boucard-Jourdin et al. 2016). Briefly, spleen, LN and

PP were digested in RPMI containing 1mg/mL Collagenase IA (Sigma) and 100µg/mL DNase 1 (Roche). LP mononuclear cells were isolated as previously described (Moro-Sibilot et al. 2015). Briefly, mucus and epithelial cells were removed with 1.5µg/mL DTE (Sigma) and 10mM EDTA solution before digestion with 12.5µg/mL Liberase TM (Roche) and 100µg/mL DNase1 (Roche). Before FACS sorting, filtered cell suspensions were enriched for DC using an OptiPrep gradient (Sigma).

Data acquisition was performed using LSR-Fortessa cytometers while DC subsets were sorted using an BD FACSAriaII cytometer (BD Biosciences). Data analysis was performed using FlowJo software v9 (Treestar Inc).

### Histological staining

A short piece of colon in the proximal section was harvested and fixed in PFA 2% overnight before dehydration and paraffin inclusion. Slices were obtained on a microtome before staining with Hematoxylin and Eosin (Sigma Aldrich). White light microscopy of the histological sections was performed on an AxioImagerZ1 (Zeiss).

### Genomic PCR, RT-qPCR and Western Blot analysis of $\beta 8$

MLN dendritic cells were sorted as gated in Figure 1C and DNA, RNA or protein was extracted using Proteinase K, Trizol/Chloroform or RIPA extraction respectively, as previously described (Boucard-Jourdin et al. 2016).

*Itgb8* gene expression analysis by quantitative RT-PCR was performed as previously described (Boucard-Jourdin et al. 2016) and normalized to *Gapdh* expression. Cre-mediated deletion in *Itgb8* locus was analysed by PCR on genomic DNA using the following set of primers: 5'-CCC ACT AAG ATA ACT GGC CGT ATC-3', 5'-GAG GGG TGG GGA AAT TTT TGT ATC-3', 5'-GTG GAT TCT ACA GGC AAG C-3'.

$\beta 8$  protein expression was measured by Western Blot, as previously described (Boucard-Jourdin et al. 2016) using an anti- $\beta 8$  antiserum generated in rabbit against the C-terminal tail of the human  $\beta 8$  cytoplasmic region (provided by Joseph McCarty) (McCarty et al. 2005).

### Statistics

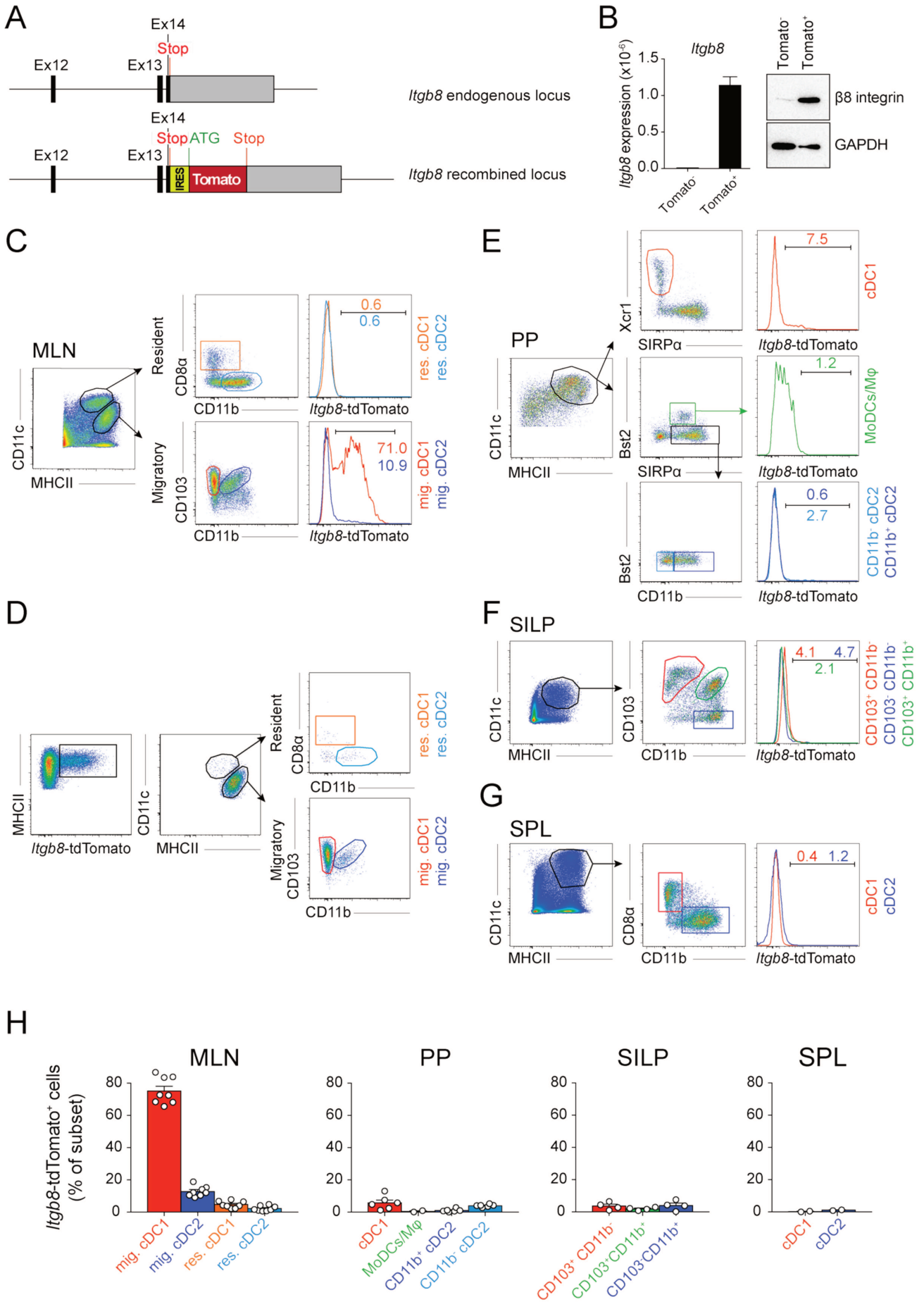
Statistical significance was evaluated as indicated in each figure legend using GraphPad Prism software (GraphPad).

## Results

### $\alpha_v\beta 8$ is preferentially expressed by a subpopulation of migratory MLN cDC1

Determining the precise pattern of  $\alpha_v\beta 8$  integrin expression in mice has been limited by the lack of appropriate tools to detect  $\beta 8$  integrin expression by flow cytometry. So far, studies of  $\beta 8$  integrin expression only relied on the analysis of sorted cell populations for total protein and gene expression by Western Blot and RT-qPCR. Thus, the study of  $\beta 8$  expression on DC population at the single cell level has not been reported so far. To address this question, we generated a reporter mouse model for  $\beta 8$  gene expression in which the tdTomato fluorescent protein is expressed under the control of  $\beta 8$  integrin gene (*Itgb8*) promoter (Fig. 1.1A) (Nakawesi et al. 2020). As shown in Fig. 1.1B, expression of both  $\beta 8$  transcript (as assessed by RT-qPCR) and protein (as

assessed by Western blot) correlates with tdTomato fluorescence thereby validating our  $\beta$ 8 reporter mouse model.



### **Figure 1.1 - $\alpha\beta 8$ is preferentially expressed by migratory cDC1 from the MLN**

(A-B) – *Itgb8*-tdTomato mice allow the detection of  $\beta 8$  integrin expression by flow cytometry. Genetic construction (A) and model validation (B) of the new mouse reporter *Itgb8*-tdTomato for *Itgb8* expression. (A) Black rectangles represent *Itgb8* coding sequences, grey rectangle indicates non-coding exon portions, solid line represents introns. Start (ATG) and Stop codons are indicated, and yellow and red rectangles represent the IRES and the reporter (tdTomato) sequences, respectively. (B) Tomato<sup>+</sup> and Tomato<sup>-</sup>CD3<sup>+</sup> T cells were sorted from the spleen of naive *Itgb8*-tdTomato mice.  $\beta 8$  integrin gene (*Itgb8*) and protein expression were assessed by qRT-PCR and Western Blot. Histogram shows *Itgb8* expression expressed relative to *Gapdh*.

(C-G) – Mononuclear phagocyte subsets from MLN (C-D), PP (E), SI LP (F) and spleen (G) of *Itgb8*-tdTomato mice were analyzed for *Itgb8* expression. Cells are pre-gated on Live CD45<sup>+</sup>CD19<sup>-</sup>CD3<sup>-</sup> cells. Histograms show the % *Itgb8*-tdTomato<sup>+</sup> cells for each subset. Note that the CD11b<sup>+</sup> cDC2 gate contains both PP and DAV (Dome-associated villus) cDC2. (n=2-7).

(H) Percentage of *Itgb8*-tdTomato<sup>+</sup> cells for each subset.

Data were collected from 2-4 independent experiments.

To investigate the pattern of  $\beta 8$  expression by the MLN cDC populations, *Itgb8*-tdTomato fluorescence was examined in 4 subsets: 1/ migratory (CD11c<sup>low</sup>MHC<sup>hi</sup>) cDC1 (CD103<sup>+</sup>CD11b<sup>-</sup>), 2/ migratory cDC2 (CD103<sup>+</sup>CD11b<sup>+</sup>), 3/ resident (CD11c<sup>hi</sup>MHC<sup>low</sup>) cDC1 (CD8 $\alpha$ <sup>+</sup>CD11b<sup>-</sup>) and 4/ resident cDC2 (CD8 $\alpha$ <sup>+</sup>CD11b<sup>+</sup>).

As illustrated by Fig. 1.1C, we first confirm that  $\beta 8$  is not expressed by resident DC populations as we were unable to detect more tdTomato fluorescence in these cells than in non-transgenic animals. Second, we show that  $\beta 8$  integrin is preferentially expressed by migratory cDC1, with tdTomato being detected on 60 to 80% of these cells, confirming our previously published observations (Boucard-Jourdin et al. 2016). The analysis of tdTomato fluorescence at the single cell level also reveals for the first time that a small proportion of migratory cDC2 (10-20%) also expresses the  $\beta 8$  integrin. The latter conclusion is further substantiated by deconvolution of the  $\beta 8$ -expressing population (gated as Tomato<sup>+</sup>MHCII<sup>+</sup>) that clearly identifies a minor cDC2 subset within the migratory DC compartment (Fig. 1.1D).

Expression of  $\beta 8$  by PP and LP DC has been controversial (Worthington et al. 2011; Païdassi et al. 2011; Reboldi et al. 2016; Nakawesi et al. 2020). Except for a few percent (7-8%) of PP Xcr1<sup>+</sup> cDC1, we show here that none of the PP or intestinal LP DC subsets express the  $\beta 8$  integrin (Fig. 1.1E-F). Lastly, we show that spleen (SPL) DC do not have any detectable *Itgb8*-tdTomato fluorescence, hence confirming the restricted expression of  $\beta 8$  to the MALT compartment (Fig. 1.1G). The frequency of  $\beta 8$ -expressing DC in each DC subset and across the different investigated organs is represented in Fig. 1.1H.

Altogether these results show for the first time that  $\beta 8$  expression is largely confined to the migratory DC compartment of MLN but is absent from other gut-associated (SILP and PP) or peripheral (SPL) lymphoid tissues. They also demonstrate that  $\beta 8$  is not homogeneously expressed by migratory MLN cDC1 and cDC2 but rather is expressed in a bimodal pattern, the functional relevance of which remains to be investigated.

### **Mice lacking $\beta 8$ integrin on cDC1 or on cDC2 do not develop colitis**

Conditional deletion in of  $\beta 8$  in CD11c<sup>+</sup> phagocytes (CD11c- $\beta 8$ <sup>KO</sup>) leads to severe spontaneous colitis associated with the specific loss of T<sub>REG</sub> and T<sub>H</sub>17 in the LP. (Travis et al. 2007). In this model,  $\beta 8$  deletion is targeted in both cDC1 and cDC2, thus precluding analysis of the contribution of each subset to the observed phenotype. Because we found expression of  $\beta 8$  on most migratory cDC1 and on some cDC2, we next investigated how targeted deletion of  $\beta 8$  on either one or the other of these DC subtypes affects sensitivity to spontaneous colitis induction. To address this question, *Itgb8*<sup>fllox</sup> mice were crossed with either Xcr1<sup>Cre</sup> mice

(Xcr1- $\beta 8^{KO}$ ) or human-Langerin<sup>Cre</sup> mice (huLang- $\beta 8^{KO}$ ) leading to the targeted deletion of  $\beta 8$  in cDC1 or some cDC2 respectively (Mattiuz et al. 2018; Ohta et al. 2016; Welty et al. 2013). Importantly, huLang<sup>Cre</sup> mice allows to specifically target the LP CD103<sup>+</sup>CD11b<sup>+</sup> cDC2, which upon migration to the MLN form the migratory cDC2 population (Welty et al. 2013).

The specific invalidation of  $\beta 8$  integrin in the expected MLN subsets for each mouse model was confirmed by genomic PCR of the *Itgb8* locus (Fig. 1.2A). While Xcr1- $\beta 8^{KO}$  mice displayed a specific  $\beta 8$  deletion of mig. and res. cDC1, the penetrance of deletion in cDC2 in the huLang- $\beta 8^{KO}$  mice appears to be incomplete. The targeting specificity of the huLang<sup>Cre</sup> promoter to cDC2 has however been well demonstrated (Welty et al. 2013). We are thus confident that, although incomplete, the deletion of  $\beta 8$  in huLang- $\beta 8^{KO}$  mice is restricted to cDC2. Both Xcr1- $\beta 8^{KO}$  and huLang- $\beta 8^{KO}$  mice were born at expected frequency and developed normally with no evidence of defects in the development of hematopoietic cells.

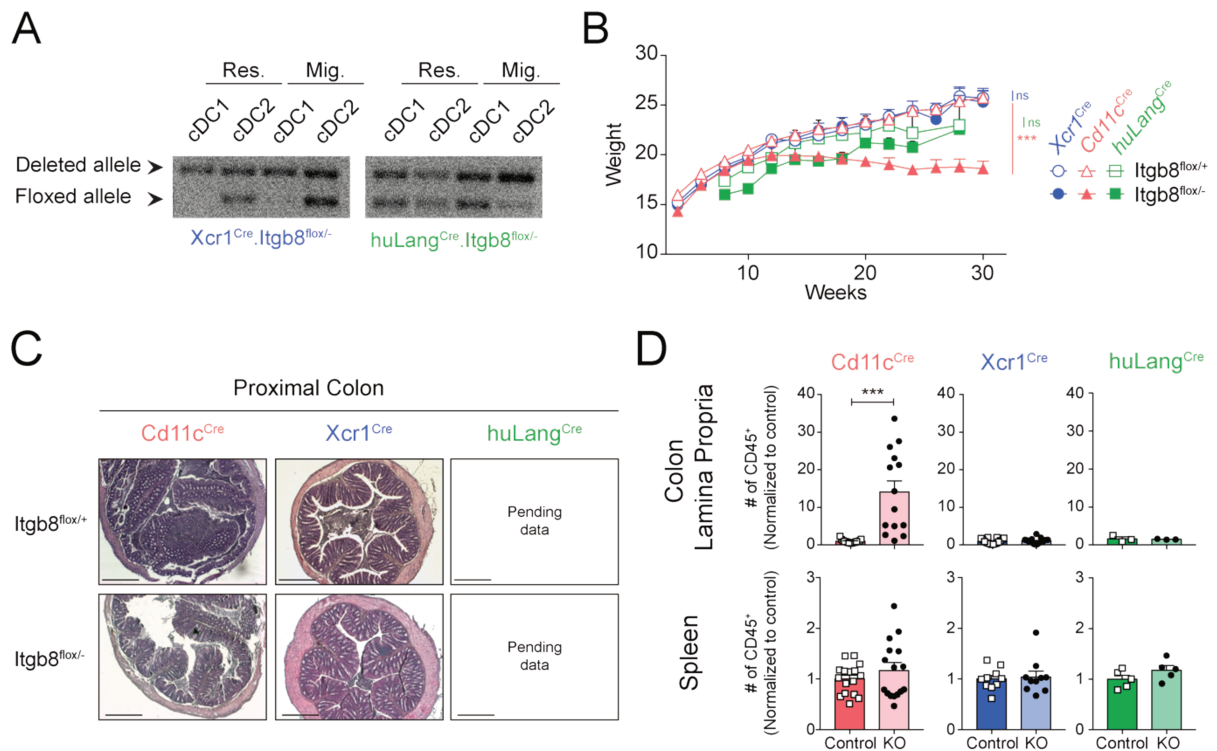
We first investigated the clinical, subclinical and inflammatory phenotype of these two new mouse models. Contrary to CD11c- $\beta 8^{KO}$  which develop a wasting syndrome and severe spontaneous inflammation, (Fig. 1.2B-C), with the recruitment of inflammatory cells to the colon (Fig. 1.2D) (Travis et al. 2007), Xcr1- $\beta 8^{KO}$  and huLang- $\beta 8^{KO}$  mice gain weight normally and do not present clinical sign of colitis (Fig. 1.2B). Furthermore, no histological inflammation (Fig. 1.C) or recruitment of immune cells (Fig. 1.2D) to the MLN or SPL could be observed. Altogether these data indicate that targeting  $\beta 8$  invalidation on either cDC1 or cDC2 does not phenocopy conditional invalidation of  $\beta 8$  in CD11c<sup>+</sup> phagocytes.

### *$\alpha_V\beta 8$ expression by cDC1 and cDC2 is only partially required for generation of iT<sub>REG</sub> in the GALT.*

In the particular micro-environment of the intestine, expression of the transcription factors FoxP3 and ROR $\gamma$ t allows to distinguish 3 CD4<sup>+</sup> T cell populations: FoxP3<sup>+</sup>ROR $\gamma$ t<sup>+</sup> T<sub>REG</sub> and FoxP3<sup>+</sup>ROR $\gamma$ t<sup>-</sup> T<sub>REG</sub>, and FoxP3<sup>-</sup>ROR $\gamma$ t<sup>+</sup> T<sub>H</sub>17 cells (Fig. 1.3A). It has been shown that most iT<sub>REG</sub> induced in the gut co-express FoxP3 and ROR $\gamma$ t and contribute in controlling intestinal inflammation (Sefik et al. 2015). Accordingly, FoxP3<sup>+</sup>ROR $\gamma$ t<sup>+</sup> iT<sub>REG</sub> do not express Nrp1, which has been shown to be expressed by thymic derived nT<sub>REG</sub> (Fig. 1.3B) (J. M. Weiss et al. 2012).

Initial analysis of CD11c- $\beta 8^{KO}$  mice showed a reduced frequency of FoxP3<sup>+</sup> T<sub>REG</sub> (Travis et al. 2007; Lacy-Hulbert et al. 2007). Here, we show that this reduction is restricted to iT<sub>REG</sub> with 35-fold reduction of FoxP3<sup>+</sup>ROR $\gamma$ t<sup>+</sup> iT<sub>REG</sub> in the colon LP (Fig. 1.3C). On the contrary, the proportion of FoxP3<sup>+</sup>ROR $\gamma$ t<sup>-</sup> T<sub>REG</sub> (containing both nT<sub>REG</sub> and some FoxP3<sup>+</sup>ROR $\gamma$ t<sup>-</sup> iT<sub>REG</sub> (Whibley, Tucci, and Powrie 2019)) is slightly reduced in CLP but is rather increased in MLN. We therefore show for the first time that that ROR $\gamma$ t<sup>+</sup> iT<sub>REG</sub> is the regulatory T cell population which is the most profoundly reduced upon  $\beta 8$  invalidation in CD11c<sup>+</sup> phagocytes.

As shown above,  $\alpha_V\beta 8$  is preferentially expressed by MLN migratory cDC1. But to our surprise, specific deletion of  $\beta 8$  in cDC1 (Xcr1- $\beta 8^{KO}$  mice) only led to a slight reduction of the proportion of FoxP3<sup>+</sup>ROR $\gamma$ t<sup>+</sup> iT<sub>REG</sub> in the CLP (1.5-fold) and MLN (1.4-fold). On the contrary, conditional deletion of  $\beta 8$  in cDC2 (huLang- $\beta 8^{KO}$ ) unexpectedly resulted in a 2.2-fold reduction of ROR $\gamma$ t<sup>+</sup> iT<sub>REG</sub> in the MLN.



**Figure 1.2 - Mice lacking  $\beta 8$  integrin on cDC1 or on cDC2 do not develop colitis**

XCR1- $\beta 8^{\text{KO}}$  and huLang- $\beta 8^{\text{KO}}$  mice were generated to specifically delete  $\alpha \beta 8$  in cDC1 or mucosal cDC2 DCs.

(A) - Genomic PCR for the *Itgb8* locus in sorted MLN DC subpopulation as gated in Figure 1C.

(B-D) - XCR1- $\beta 8^{\text{KO}}$  and huLang- $\beta 8^{\text{KO}}$  mice do not develop colitis. Weight (B) and representative histology of colon (C) of XCR1- $\beta 8^{\text{KO}}$ , CD11c- $\beta 8^{\text{KO}}$ , huLang- $\beta 8^{\text{KO}}$  and control littermates. Statistics in (B) consistently relate to every time point from 12 weeks onward and were assessed using a two-way ANOVA between genotypes followed by Sidak correction for multiple comparison test for each individual timepoint. Scale bar in (C) represent 500 $\mu\text{m}$ . (D) Number of total CD45<sup>+</sup> cells were quantified in the CLP and SPL of CD11c- $\beta 8^{\text{KO}}$ , XCR1- $\beta 8^{\text{KO}}$  and huLang- $\beta 8^{\text{KO}}$  mice by flow cytometry.

Statistical analysis: Mann-Whitney U test,  $\emptyset p > 0.05$ , \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

In parallel, we assessed the role of  $\beta 8$  integrin on cDC1 or cDC2 in the generation of intestinal  $T_H17$  responses. While in  $CD11c\text{-}\beta 8^{KO}$  mice, the frequency of  $FoxP3^+ROR\gamma t^+$   $T_H17$  are reduced by 6- and 3.5-fold in the LP and MLN respectively, targeted  $\beta 8$  deletion in cDC2 in  $huLang\text{-}\beta 8^{KO}$  led only to a small 1.4-fold decrease of MLN  $T_H17$  (Fig. 1.3C). In  $Xcr1\text{-}\beta 8^{KO}$  mice, CLP and MLN  $T_H17$  were however not significantly affected.

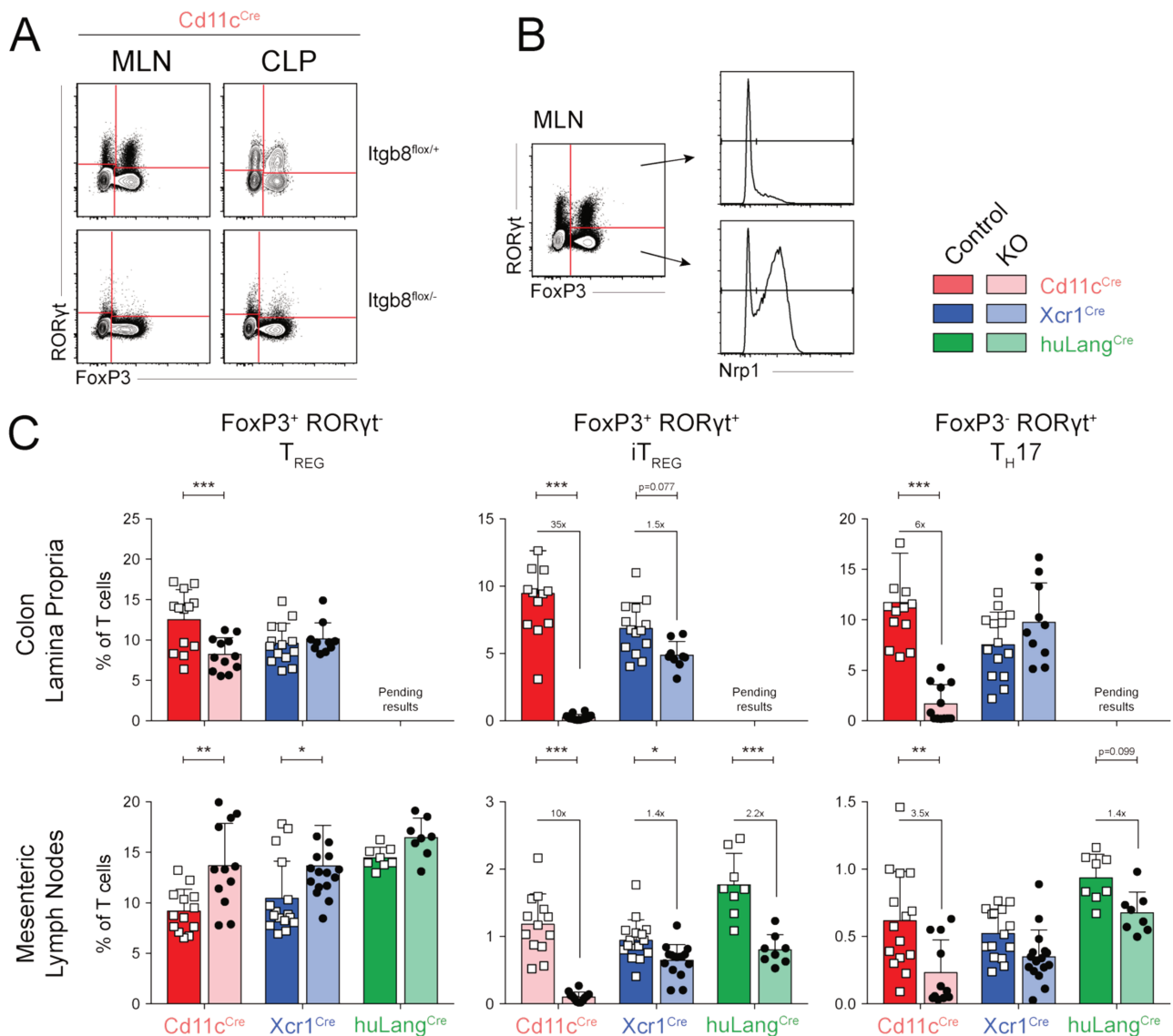
These results suggest that  $\alpha_v\beta 8$  expression on both cDC1 and cDC2 is only partially required for their ability to induce  $iT_{REG}$  at steady state. Furthermore, the absence of defects in  $T_H17$  frequency in the  $Xcr1\text{-}\beta 8^{KO}$  model suggests that  $\alpha_v\beta 8$  expression by cDC1 is dispensable for steady state  $T_H17$  generation. Unfortunately, the low number of mice and absence of data for the colon LP of  $huLang\text{-}\beta 8^{KO}$  mice prevents us from concluding now on the role of  $\alpha_v\beta 8$  expression by intestinal  $CD103^+CD11b^+$  cDC2 in the generation of  $T_H17$  responses.

### *cDC1-specific deletion of $\beta 8$ integrin does not modify the susceptibility to DSS-induced colitis.*

To investigate the functional relevance of this decreased  $iT_{REG}$  in  $Xcr1\text{-}\beta 8^{KO}$  and  $huLang\text{-}\beta 8^{KO}$ , we next sought to investigate the response to intestinal inflammatory challenge using the standard DSS-induced colitis model. Using this model, we monitored the impact of cDC1-targeted  $\beta 8$  invalidation on 1/ their initial susceptibility to DSS-induced colitis and 2/ their recovery from DSS-induced colitis, known to be critically dependent on  $T_{REG}$  generation and function. DSS was administered in the drinking water of  $Xcr1\text{-}\beta 8^{KO}$  mice and control littermates for 6 days and their weight was monitored (Fig. 1.4A). As expected, DSS-treated groups lost significant amount of weight, however,  $Xcr1\text{-}\beta 8^{KO}$  mice exhibited the same response kinetics than their control littermates (Fig. 1.4B).

Altogether we show that targeted deletion of  $\beta 8$  on cDC1 or cDC2 results in a partial loss in the frequency of intestinal  $T_{REG}$ . However, this is not sufficient to result in the induction of spontaneous colitis or increase the susceptibility to DSS-induced inflammatory response. In agreement with the modest impact of cDC1-targeted  $\beta 8$  invalidation on  $iT_{REG}$  frequencies, these data concur to show that despite the large distribution of  $\beta 8$  on cDC1, this DC subset is either marginally involved or redundant mechanisms can compensate for the loss of  $\beta 8$  on cDC1 to control  $TGF\beta$ -dependent T cell responses. The targeted deletion of intestinal cDC2 results in a small but non-significant reduction of MLN  $T_H17$  suggesting that  $\alpha_v\beta 8$  expression by these cells could be one of the mechanisms by which they are able to generate  $T_H17$ .





**Figure 1.3 -  $\beta 8$  deletion in cDC1 or cDC2 leads to reduced intestinal iT<sub>REG</sub> responses**

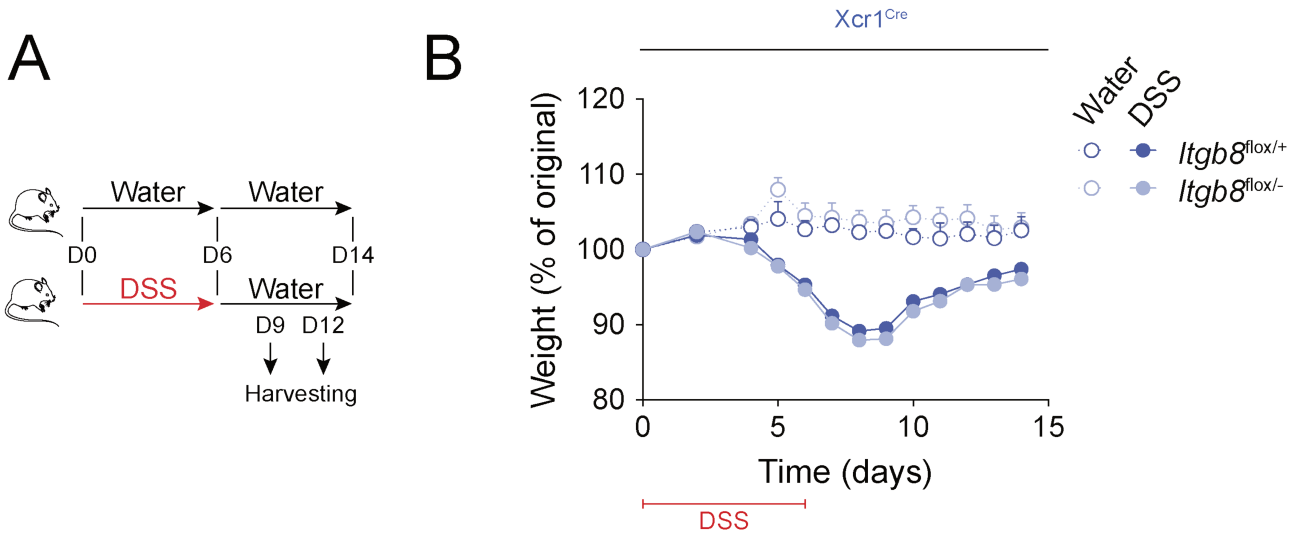
(A) - Representative flow cytometry plots for the analysis of CD4<sup>+</sup> T<sub>REG</sub> and T<sub>H</sub>17 populations in the MLN and CLP of CD11c- $\beta 8^{\text{KO}}$  mice.

(B) - Representative gating strategy and flow cytometry histograms for the analysis of Nrp1 expression of FoxP3<sup>+</sup>ROR $\gamma$ t<sup>+</sup> (top) and FoxP3<sup>+</sup>ROR $\gamma$ t<sup>+</sup>CD4<sup>+</sup> T cell population in the MLN and CLP of control littermates.

(C) - Percentage of indicated CLP and MLN CD4<sup>+</sup> T cell populations from CD11c- $\beta 8^{\text{KO}}$  (red), XCR1- $\beta 8^{\text{KO}}$  (blue) and huLang- $\beta 8^{\text{KO}}$  (green) mice, analyzed according to the gating strategy in (A) for FoxP3 and ROR $\gamma$ t expression. Histogram show the percentage of each T cell subsets as a percentage of all CD4<sup>+</sup> T cells.

Control mice are always control littermates. Data were collected from 1-4 independent experiments, (n = 5-15)

Statistical analysis was performed using a 2-way ANOVA test for genotype and mice model with Sidak multiple comparison correction test.  $\emptyset$ p>0.05, \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001



**Figure 1.4 - cDC1-specific deletion of  $\beta 8$  integrin does not modify the susceptibility to DSS-induced colitis**

A model of T-cell mediated colitis using 1.5% DSS in drinking water was induced on C57/B6, *Itgb8*-tdTomato and XCR1- $\beta 8^{\text{KO}}$  or huLang- $\beta 8^{\text{KO}}$  mice as indicated in (A).  
 (B) – Weight of individual mice was followed throughout the DSS experiment to monitor weight loss and recovery. Plotted here is a percent change compared to day 0.

## Discussion

Intestinal cDC are crucial for initiating cellular and humoral responses in the GALT (Durai and Murphy 2016). cDC are key in maintaining the fine balance between immune tolerance and effective immunity, through their ability to sense their environment, sample and present local antigens to naïve T cells thereby orchestrating T cell responses according to context and tissue microenvironment.

Conventional DC comprise two main populations, namely cDC1 and cDC2, with LN containing both migratory and resident counterparts of each population. All these DC subpopulations have been credited with diverse functional properties. Even though the progeny of these cells is now well established (Merad et al. 2013), and while the functional specialization of DC subsets has been extensively studied, how DC acquire these functions and how specific functions are compartmentalized remain elusive. Here we focus on orchestration of intestinal adaptive immune responses under the prism of TGF $\beta$ , which plays a critical role in the orchestration of cellular and humoral immune responses in the gut.

While the ability to induce T<sub>H</sub>17 is generally attributed to cDC2, the relative contribution of cDC1 and cDC2 to T<sub>REG</sub> generation remains ambiguous. The generation of both T<sub>H</sub>17 and T<sub>REG</sub> from naïve T cells critically relies on TGF $\beta$  signaling. TGF $\beta$  is secreted under a latent form and requires activation in order to bind to TGF $\beta$ R and induce downstream signaling. We and others have previously shown that expression of the TGF $\beta$ -activating  $\alpha_v\beta_8$  integrin by CD11c-expressing phagocytes is critical for maintenance of intestinal homeostasis and the generation of intestinal T<sub>REG</sub> and T<sub>H</sub>17 (Lacy-Hulbert et al. 2007; Travis et al. 2007; Païdassi et al. 2011; Worthington et al. 2011; Acharya et al. 2010; Melton et al. 2010; Boucard-Jourdin et al. 2016). However, the relative contribution of cDC1 and cDC2 in this process is not properly described. Using models of conditional *Itgb8* deletion and a reporter mouse model for *Itgb8* expression, this study aimed at deciphering the contribution of  $\alpha_v\beta_8$  on cDC1 and cDC2 in the generation of peripheral T<sub>REG</sub> and T<sub>H</sub>17 responses. We show that: 1/ in MLN,  $\alpha_v\beta_8$  is expressed on the large majority (60 to 80%) of migratory cDC1 and on a small population of migratory cDC2 (10 %). 2/ In other lymphoid tissues,  $\alpha_v\beta_8$  expression is restricted to a small population of PP cDC1. 3/ Deletion of  $\beta_8$  integrin either on cDC1 or on cDC2 alone is not sufficient to induce spontaneous colitis and  $\beta_8$  deletion in cDC1 does not modify susceptibility to DSS-induced colitis. 4/ Deletion of  $\beta_8$  expression by induces a 2-fold decrease in iT<sub>REG</sub> frequency, suggesting a preferential involvement of the minor  $\beta_8$ -expressing cDC2 subset in generation of iT<sub>REG</sub>. 5/  $\alpha_v\beta_8$  expression by cDC2 could be one of the mechanisms by which they are able to generate T<sub>H</sub>17 although studies are currently on-going to formerly prove this point.

The *Itgb8*-tdTomato reporter mouse model has allowed us for the first time to explore by flow cytometry the expression pattern of  $\beta_8$  integrin at the single cell level on different DC subsets. Our results raise interesting questions regarding cDC biology that we address below.

- First, the bimodal expression pattern of  $\beta_8$  in MLN migratory cDC1 reveals heterogeneity within this population regarding the capacity to activate TGF $\beta$ . Further studies are ongoing to investigate the functional differences between  $\beta_8^+$  and  $\beta_8^-$  MLN mig cDC1 subsets in particular, in terms of induction of TGF $\beta$ -dependent T cell response (T<sub>REG</sub> and T<sub>H</sub>17 induction) and of their state of differentiation and/or activation.

- Second, the lack of *Itgb8*-tdTomato fluorescence in PP cDC2 is in contrast with previously published data suggesting that this subset can induce IgA responses through  $\alpha_v\beta8$ -mediated TGF $\beta$  activation (Reboldi et al. 2016). We are so far unable to explain this difference. However, given the dynamic expression pattern of *Itgb8* expression through time and its regulation by various signaling pathway (Boucard-Jourdin et al. 2016), it is reasonable to assume that the difference in intestinal microflora, environment and housing conditions might induce different expression pattern of the integrin.
- In human lamina propria,  $\alpha_v\beta8$  is expressed by CD1c<sup>+</sup> cDC2 (Fenton et al. 2016) while it is not detected in LP DC in naïve mice. This is either due to 1/ different mechanisms of regulation of *Itgb8* expression in mice and human or 2/ the difference in the intestinal environment and microbiota between SPF raised inbred laboratory mice and humans with a much more diverse microbiota, susceptible to being challenged by pathogenic species and diverse dietary antigens. This highlights the necessity to improve the currently available mouse models to better mimic the wild life intestinal microenvironment and better model complex pathological mucosal disease such as IBD. Species-related differences in regulation of the *Itgb8* locus between human and mice also deserve further investigations.

Given that  $\beta8$  is preferentially expressed by cDC1 at steady state, which specializes this subset for generation of iT<sub>REG</sub> *in vitro* through  $\alpha_v\beta8$ -mediated activation of TGF $\beta$  (Païdassi et al. 2011; Boucard-Jourdin et al. 2016), the minimal reduction in intestinal iT<sub>REG</sub> and the absence of spontaneous colitis in *Xcr1- $\beta8$ <sup>KO</sup>* mice were unexpected.

In parallel, we have described here for the first time that a small population of MLN mig. cDC2 (10%) expresses  $\beta8$  integrin. Until now, the bulk analysis of  $\beta8$  expression cDC subsets had likely prevented this observation. Besides, even though limited to a small proportion of cells,  $\beta8$  expression by cDC2 is likely to play a role in the regulation of TGF $\beta$ -dependent T cell responses, as *huLang- $\beta8$ <sup>KO</sup>* mice display a weak but significant reduction of intestinal iT<sub>REG</sub> frequency.

However, even though deletion of  $\beta8$  in cDC1 or cDC2 leads to a small reduction of iT<sub>REG</sub>, *Xcr1- $\beta8$ <sup>KO</sup>* and *huLang- $\beta8$ <sup>KO</sup>* present a mild phenotype and do not develop colitis as observed in *CD11c- $\beta8$ <sup>KO</sup>* mice. Multiple hypothesis can be put forward for this observation:

- Given that both cDC1 and cDC2 can express  $\beta8$  integrin, it is possible that the loss of  $\beta8$  expression by a particular subset is compensated by the ability of the other subset to induce iT<sub>REG</sub>,
- Other mechanisms of TGF $\beta$  activation could be used to compensate for the loss of  $\beta8$ .
- Other CD11c-expressing APCs such as macrophages or monocyte-derived DC could also play a redundant role with cDC in the induction of iT<sub>REG</sub> through  $\alpha_v\beta8$ -mediated activation of TGF $\beta$

Furthermore, our data suggest that  $\alpha_v\beta8$  deletion on cDC2 but not cDC1 results in a partial decrease of intestinal T<sub>H</sub>17 frequency. This suggests that  $\alpha_v\beta8$ -mediated TGF $\beta$  activation might be one of the mechanisms by which cDC2 induce T<sub>H</sub>17 in MLN. We hypothesize that the environmental cues perceived by each DC subset might determine in which context  $\alpha_v\beta8$  expression by each subset might be most relevant. However, the precise

molecular mechanisms underlying this functional specialization of cDC1 and cDC2 in inducing T<sub>REG</sub> and T<sub>H17</sub> still needs further investigation.

Experiment are currently being setup to investigate these different hypotheses and are detailed later in the manuscript. Furthermore, we have so far only analyzed the frequencies of iT<sub>REG</sub> and T<sub>H17</sub> in the GALT. It would be interesting to investigate if the generated T<sub>REG</sub> and T<sub>H17</sub> have similar functional properties in the absence of  $\alpha_v\beta_8$  on cDC.

Given the importance of cDC1 in generating CD8<sup>+</sup> and T<sub>H1</sub> T cell responses to viral infection and the restricted ability of cDC2 to induce T<sub>H17</sub> responses, we propose that the importance of  $\alpha_v\beta_8$  expression by cDC1 or cDC2 in mediating T<sub>REG</sub> generation will likely be dependent on the immunological and pathological context. At steady state, it seems that  $\alpha_v\beta_8$  is minimally required on both cDC1 and cDC2 to induce T<sub>REG</sub> and T<sub>H17</sub> responses. In a Th1 driven viral pathology, the requirement for  $\alpha_v\beta_8$  expression by cDC1 to also induce T<sub>REG</sub> might be increased. In a Th2 or T<sub>H17</sub> driven inflammatory setting,  $\alpha_v\beta_8$  expression by cDC2 might be of greater importance.

Our investigation of the importance of  $\alpha_v\beta_8$  expression in the context of DSS-induced colitis has shown that cDC1-specific deletion of  $\beta_8$  integrin does not modify the susceptibility to DSS-induced colitis. However, the clinical outcome estimated by mice weight loss might not be the best readout. We are currently setting up experiments to investigate specifically the T<sub>REG</sub> and T<sub>H17</sub> populations in these mice following DSS colitis.

Alternatively, the investigation of a T cell-dependent colitis model huLang- $\beta_8^{\text{KO}}$  mice might bring some interesting insight. Indeed a recent study has shown the requirement of cDC2 for the priming of T cells in T<sub>H17</sub> induction in such a model (Pool, Rivollier, and Agace 2020). Investigating whether  $\alpha_v\beta_8$  is involved in this process and  $\beta_8$ -deficient animals could be protected from the colitis would be extremely interesting.

Overall, these data suggest that in the case of regulation of TGF $\beta$ -dependent immune responses in the intestinal mucosa, a complex interplay between cDC1, cDC2 and other immune and non-immune cells is at play to maintain immune homeostasis by generation of T<sub>REG</sub> and T<sub>H17</sub>. Furthermore, this study also highlights the fact that no single cDC subset is able to generate T<sub>REG</sub> responses through TGF $\beta$  activation, but this is more likely a common feature triggered under different antigen and DAMP stimulation in the GALT. All in all, many compensatory and redundant mechanisms are likely at play to generate these protective immune responses in the gut.

## Part 2 – $\alpha_V\beta_8$ integrin-expression by BATF3-dependent dendritic cells facilitates early IgA responses to Rotavirus

### **Authors:**

J. Nakawesi<sup>1,†</sup>, S. This<sup>2,†</sup>, J. Hütter<sup>3,#</sup>, M. Boucard-Jourdin<sup>2</sup>, V. Barateau<sup>2</sup>, K. Getachew Muleta<sup>1</sup>, L. J. Gooday<sup>1</sup>, K. Fog Thomsen<sup>3</sup>, A. Garcias López<sup>3</sup>, I. Ulmert<sup>3</sup>, D. Poncet<sup>4</sup>, B. Malissen<sup>5</sup>, H. Greenberg<sup>6</sup>, O. Thauinat<sup>2</sup>, T. Defrance<sup>2</sup>, H. Paidassi<sup>2,‡,\*</sup>, and K. Lahl<sup>1,3,‡,\*</sup>

### **Affiliations:**

<sup>1</sup>Immunology Section, Lund University, Lund, Sweden,

<sup>2</sup>CIRI, Inserm, U1111, Université Claude Bernard Lyon 1, CNRS, UMR5308, École Normale Supérieure de Lyon, Univ Lyon, Lyon, France,

<sup>3</sup>Division of Biopharma, Institute for Health Technology, Technical University of Denmark (DTU), Kongens Lyngby, Denmark,

<sup>4</sup>Institute for Integrative Biology of the Cell (I2BC), CEA, CNRS, Université Paris-Sud, INRA, Université Paris-Saclay, Gif-sur-Yvette Cedex, France,

<sup>5</sup>Centre d'Immunologie de Marseille-Luminy, Aix Marseille Université, INSERM, CNRS UMR, Marseille 13288, France; Centre d'Immunophénomique, Aix Marseille Université, INSERM, CNRS, Marseille 13288, France,

<sup>6</sup>Departments of Medicine and Immunology and Microbiology, Stanford University School of Medicine, Stanford, CA 94305, USA; VA Palo Alto Health Care System, Palo Alto, CA 94304, USA,

**<sup>†</sup>Equal contribution**

**<sup>‡</sup>Helena Paidassi and Katharina Lahl are joint senior authors.**



## ARTICLE

# $\alpha\text{v}\beta\text{8}$ integrin-expression by BATF3-dependent dendritic cells facilitates early IgA responses to Rotavirus

J. Nakawesi<sup>1</sup>, S. This<sup>2</sup>, J. Hütter<sup>3,8</sup>, M. Boucard-Jourdin<sup>2</sup>, V. Barateau<sup>2</sup>, K. Getachew Muleta<sup>1</sup>, L. J. Gooday<sup>1</sup>, K. Fog Thomsen<sup>3</sup>, A. Garcias López<sup>3</sup>, I. Ulmert<sup>3</sup>, D. Poncet<sup>4</sup>, B. Malissen<sup>5</sup>, H. Greenberg<sup>6,7</sup>, O. Thauinat<sup>2</sup>, T. Defrance<sup>2</sup>, H. Paidassi<sup>1,2</sup> and K. Lahl<sup>1,3</sup>

**Q1** Secretory intestinal IgA can protect from re-infection with rotavirus (RV), but very little is known about the mechanisms that induce IgA production during intestinal virus infections. Classical dendritic cells (cDCs) in the intestine can facilitate both T cell-dependent and -independent secretory IgA. Here, we show that BATF3-dependent cDC1, but not cDC2, are critical for the optimal induction of RV-specific IgA responses in the mesenteric lymph nodes. This depends on the selective expression of the TGF $\beta$ -activating integrin  $\alpha\text{v}\beta\text{8}$  by cDC1. In contrast,  $\alpha\text{v}\beta\text{8}$  on cDC1 is dispensable for steady state immune homeostasis. Given that cDC2 are crucial in driving IgA during steady state but are dispensable for RV-specific IgA responses, we propose that the capacity of DC subsets to induce intestinal IgA responses reflects the context, as opposed to an intrinsic property of individual DC subsets.

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## INTRODUCTION

Rotavirus (RV) is a double-stranded RNA virus that replicates in mature enterocytes at the tips of the small intestinal villi<sup>1,2</sup> and was first identified by electron microscopy in intestinal biopsies of children diagnosed with acute gastroenteritis.<sup>3</sup> It continues to be the leading cause of life-threatening diarrheal diseases among young children in the developing world.<sup>4</sup> Intestinal RV-specific IgA is the major correlate of protection against natural infection,<sup>5,6</sup> but the mechanisms inducing protective RV-specific IgA are not well understood. RV-specific secretory IgA arises as early as three days after primary infection, directly preceding RV-clearance.<sup>6</sup> This rapid response suggests at least some T-cell independent B cell activation, an idea supported by the fact that  $\alpha/\beta$  TCR deficient or nude mice lacking all T cells shed much less RV than RAG-deficient mice lacking both T and B cells.<sup>7</sup> Nevertheless, most of the anti-RV response in intact animals requires T cell help.<sup>8</sup>

**Q2–Q5** Classical dendritic cells (cDCs) are the major antigen-presenting cells in the body and integral to the differentiation of helper T cells. Several populations of cDCs are located throughout the intestinal lamina propria (LP), as well as within gut-associated lymphoid tissues including Peyer's patches and isolated lymphoid follicles. Most cDCs in the LP express the integrin  $\alpha\text{E}(\text{CD}103)\beta\text{7}$  and can be divided into two distinct populations by the expression of CD11b: Interferon regulator factor 8 (IRF8)- and Basic leucine zipper transcription factor ATF-like 3 (BATF3)-dependent CD103<sup>+</sup>CD11b<sup>-</sup> DCs (cDC1) and IRF4-dependent CD103<sup>+</sup>CD11b<sup>+</sup> DCs (cDC2).<sup>9–12</sup> Although both subsets of

intestinal DCs are implicated in intestinal immune homeostasis via the induction and maintenance of regulatory T cells (Tregs),<sup>13</sup> they engulf different types of antigens and differ in their ability to induce specific types of T cell responses. Thus, cDC2 are implied in supporting Th2 and Th17 responses against parasites and extracellular bacteria,<sup>14–16</sup> while cDC1 excel at priming CD8<sup>+</sup> T cell responses and Th1 responses<sup>11,17</sup> to viral and intracellular bacteria. Importantly, cDC1 play the dominant role in cross-presenting intestinal epithelium-derived cell-associated antigen to CD8<sup>+</sup> T cells both in steady state<sup>18</sup> and during rotavirus infection.<sup>19</sup>

cDCs also participate in the initiation of B cell responses and several groups have documented a role for DCs in facilitating IgA production in the intestine (reviewed in<sup>20,21</sup>). However, much less is known on the division of labour between the different cDC subsets for the induction of B cell responses. IgA class-switch recombination in mice can occur via both T cell-dependent and T cell-independent pathways, with T cell-dependent IgA class-switching usually occurring within germinal centers of the mLN and Peyer's Patches.<sup>22,23</sup> In contrast, T cell-independent IgA class switching depends on myeloid cells and plasmacytoid DCs (pDC) expressing BAFF and APRIL,<sup>24</sup> potentially located in the LP itself.<sup>25,26</sup> In any case, TGF $\beta$  is critical for both TI and TD IgA responses in vivo, as mice with defective TGF $\beta$  signalling in B cells show complete IgA deficiency.<sup>27</sup> In addition, TGF $\beta$  and retinoic acid provided by mucosal cDCs synergize in the induction of IgA.<sup>28</sup>

<sup>1</sup>Immunology Section, Lund University, Lund, Sweden; <sup>2</sup>CIRI, Inserm, U1111, Université Claude Bernard Lyon 1, CNRS, UMR5308, École Normale Supérieure de Lyon, Univ Lyon, Lyon, France; <sup>3</sup>Division of Biopharma, Institute for Health Technology, Technical University of Denmark (DTU), Kongens Lyngby, Denmark; <sup>4</sup>Institute for Integrative Biology of the Cell (I2BC), CEA, CNRS, Université Paris-Sud, INRA, Université Paris-Saclay, Gif-sur-Yvette Cedex, France; <sup>5</sup>Centre d'Immunologie de Marseille-Luminy, Aix Marseille Université, INSERM, CNRS UMR, Marseille 13288, France; Centre d'Immunophénomique, Aix Marseille Université, INSERM, CNRS, Marseille 13288, France; <sup>6</sup>Departments of Medicine and Immunology and Microbiology, Stanford University School of Medicine, Stanford, CA 94305, USA and <sup>7</sup>VA Palo Alto Health Care System, Palo Alto, CA 94304, USA

Correspondence: H Paidassi (helena.paidassi@inserm.fr) or K Lahl (Katharina.lahl@med.lu.se)

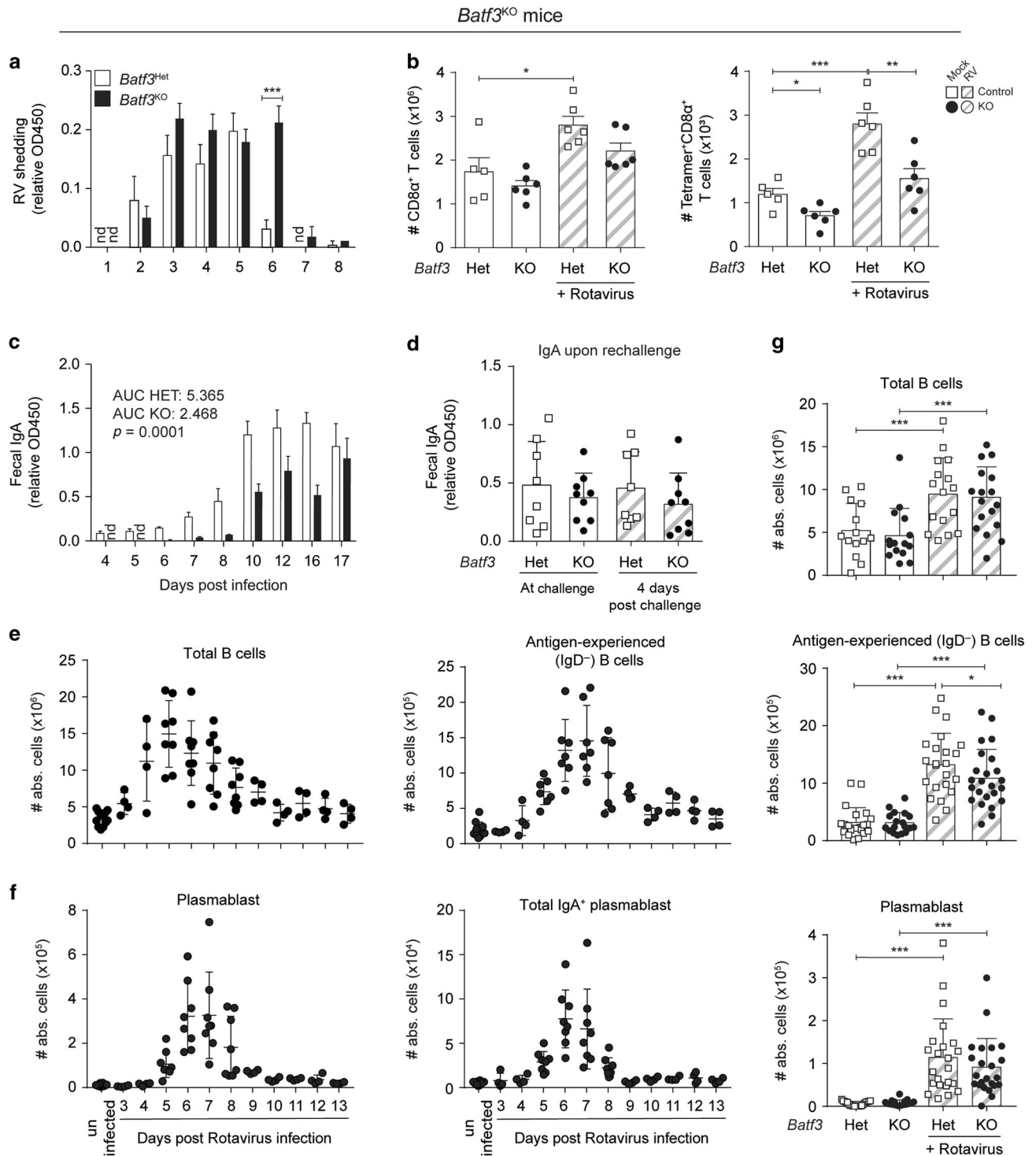
<sup>8</sup>Present address: Bayer Pharma AG, Pharmaceuticals Division, Berlin, Germany

These authors contributed equally: J. Nakawesi, S. This

These authors jointly supervised this work: Helena Paidassi, Katharina Lahl

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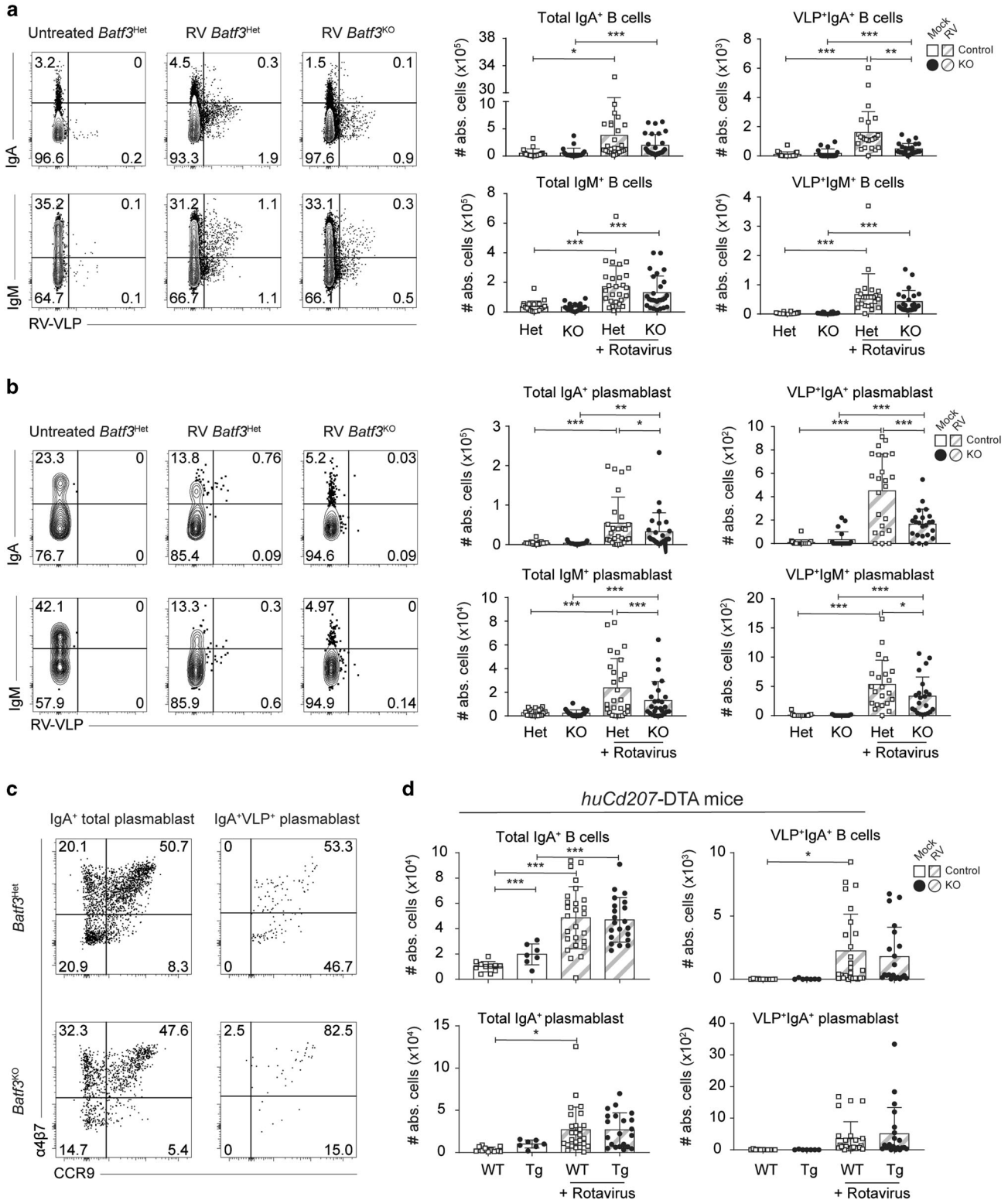
**Fig. 1** *Batf3*-dependent dendritic cells are required for the optimal induction of adaptive immunity toward Rotavirus. **a–c**, *Batf3*<sup>WT/Het</sup> and *Batf3*<sup>KO</sup> mice were orally infected with RV. **a** Levels of RV antigen shedding in feces was measured by ELISA. **b** The total number of total CD8<sup>+</sup> T cells (left) and RV-specific CD8<sup>+</sup> T cells (right) was assessed at 7 days post infection. **c** The levels of anti-RV IgA secreted into the feces after primary infection at indicated time-points was measured by ELISA. AUC area under the curve, *p* value determined using an unpaired *T* test. **d** The levels of anti-RV IgA secreted into the feces before re-challenge at 8 weeks after primary infection (d0) and 4 days after rechallenge were measured by ELISA. **e, f** show kinetics for the total number of different B cells subsets accumulating in the MLNs after infection in C57Bl/6 mice (total B cells: live CD19<sup>+</sup>B220<sup>+</sup>, antigen-experienced: live CD19<sup>+</sup>B220<sup>+</sup>IgD<sup>-</sup>, plasmablasts: live CD19<sup>+</sup>IgD<sup>-</sup>CD138<sup>+</sup>) in *BATF3* mice at 7 days post infection. Data pooled from **a, b, d–f** two (*n* = 4), or **g** six (*n* = 3–5) independent experiments, and **c** one representative experiment out of three (*n* = 4 mice each). **a, c**: unpaired *T* test, **b, d–g**: two-way ANOVA, Tukey post hoc. \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001. White symbols: HET; black symbols: KO; shaded bars: RV-infected. AUC Area under the curve.



TGFβ is produced in a latent form before it can bind to its receptor. By binding to an Arg-Gly-Asp (RGD) tripeptide in latent TGFβ, avβ8 integrin is an important pathway for TGFβ activation in vivo, with mutation of the av integrin-binding site in the RGD sequence of latent TGFβ recapitulating many of the phenotypes of TGFβ knockout mice.<sup>29</sup>

avβ8 integrin on CD11c<sup>+</sup> mononuclear phagocytes (MNP), including DCs, is important for intestinal immune homeostasis, as CD11c-targeted avβ8 deficiency leads to spontaneous colitis in mice.<sup>30</sup> Previously, we and others have shown that expression of avβ8 on CD11c<sup>+</sup> MNPs activates TGFβ, facilitating the generation of intestinal regulatory T cells (Treg)<sup>31,32</sup> and Th17 cells.<sup>33,34</sup> A

Batf3<sup>KO</sup> mice



**Fig. 2** **BATF3-dependent cDC1 dendritic cells are required for the optimal induction of anti-RV-specific IgA responses in the mLN.** **a–c** *Batf3*<sup>Het</sup> and *Batf3*<sup>KO</sup> mice were orally infected with RV. **a** Representative flow cytometry plots for RV-VLP staining in the B220<sup>+</sup>CD138<sup>−</sup> B cells (pre-gated on CD19<sup>+</sup>IgD<sup>−</sup>) and the total number of IgA<sup>+</sup> and IgM<sup>+</sup> and RV-specific B cells in the mLN. **b** Representative flow cytometry plots for RV-VLP staining in plasmablasts (B220<sup>−</sup>CD138<sup>+</sup>; pre-gated on CD19<sup>+</sup>IgD<sup>−</sup>) and the total number of IgA<sup>+</sup> and IgM<sup>+</sup> and RV-specific plasmablasts at 7 days post infection. **c** Representative flow cytometry plots for CCR9 and α<sub>4</sub>β<sub>7</sub> expression on the different IgA<sup>+</sup> plasmablasts (concatenated data from three mice, representative of two independent experiments). **d** *huCD207*-DTA and wildtype mice were orally infected with RV. The total number of IgA<sup>+</sup> and RV-specific B cells (top) or plasmablasts at 7 days post infection. Cells were pre-gated as mentioned in **a**, **b** above. Dots represent data from individual mice. Data were collected from **a**, **b** six independent experiments, or **d** four independent experiments with 3–6 infected mice per experiment. Two-way ANOVA with Tukey post hoc test was performed for statistical analysis. \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001. White symbols: HET; black symbols: KO; shaded bars: RV-infected.

recent study suggests that αvβ8 on cDC2 in Peyer's Patches is important for driving IgA responses to commensals in steady state mice,<sup>35</sup> but it is not known whether αvβ8-mediated TGFβ activation by cDCs is required for the induction of IgA responses during infections such as RV.

In this context, protective IgA responses in the lung induced by polyinosinic:polycytidylic acid (poly(I:C)) adjuvanted vaccination against influenza virus require cDC1 in the nasal tissues.<sup>36</sup> In contrast, cDC2 induce IgA responses to commensal bacteria in the steady state<sup>35</sup> or after immunization with flagellin derived from *Salmonella typhimurium*.<sup>37</sup> Here, we show that BATF3-dependent cDC1, but not cDC2, are required for optimal RV-specific IgA responses in the mLN of adult mice infected orally with RV. This requires TGFβ-activating αvβ8 integrin on cDC1 even though αvβ8 on cDC1 is dispensable for immune homeostasis in the absence of infection.

## RESULTS

BATF3-dependent cDC1 are required for the optimal induction of anti-RV-specific IgA responses in MLN

Because antibodies play a major role in protection of infants from RV-reinfection,<sup>38</sup> we sought to explore the cellular requirements for B cell activation during primary RV infection. cDC1 efficiently sense and coordinate the immune response towards viral infections.<sup>17,18,39</sup> The transcription factor BATF3 is essential for the development of cDC1, and BATF3-deficient mice lack both CD8α<sup>+</sup> and CD103<sup>+</sup> cDC1 in mLN<sup>12</sup> and small intestinal lamina propria cDC1 in steady-state and upon RV infection (Supplementary Fig. S1A). In order to assess the role of cDC1 in inducing humoral anti-RV responses in adult mice, we infected *Batf3*<sup>Het</sup> and *Batf3*<sup>KO</sup> littermates orally with RV. As expected from a previous study,<sup>19</sup> and in line with the prominent role of cDC1 in driving immunity towards viruses, *Batf3*<sup>KO</sup> mice cleared the infection with a delay of one day (Fig. 1a). This correlated with fewer total and RV-specific CD8<sup>+</sup> T cells in the mLN 7 days post infection (Fig. 1b) together with significantly delayed secretion of fecal RV-specific IgA (Fig. 1c). Fecal RV-specific IgA in *BATF3*<sup>KO</sup> mice reached wild-type levels around day 17 after infection and plateaued for at least 8 weeks (Fig. 1d).

To explore the basis of the defective IgA levels in the absence of cDC1 early after infection, we first examined the kinetics of B cell proliferation and differentiation in wild type C57Bl/6 mice. As reported previously,<sup>40</sup> RV infection triggered a massive temporary increase in total B cell numbers in the mLN (Fig. 1e), with the number of naïve IgD<sup>+</sup> B cells peaking at day 5 post infection, followed by a peak in Ag-experienced (IgD<sup>−</sup>) B cells on days 6–7. This was accompanied by significantly increased numbers of total and IgA<sup>+</sup> CD138<sup>+</sup> B cells at these times (Fig. 1f). Further characterization of CD138<sup>+</sup> B cells showed that they expressed the proliferation marker Ki67 and CXCR4, indicating that they were plasmablasts (Supplementary Fig. S1B). All subsets of B cells in the mLN returned to baseline levels by day 9 after infection. cDC1-deficient *Batf3*<sup>KO</sup> mice showed the same increases in the numbers of total B cells and a minor decrease in Ag-experienced (IgD<sup>−</sup>) B cells 7 days post infection (Fig. 1g), showing that cDC1 might

influence B cell activation, but are dispensable for the general B cell expansion occurring in the context of RV infection.

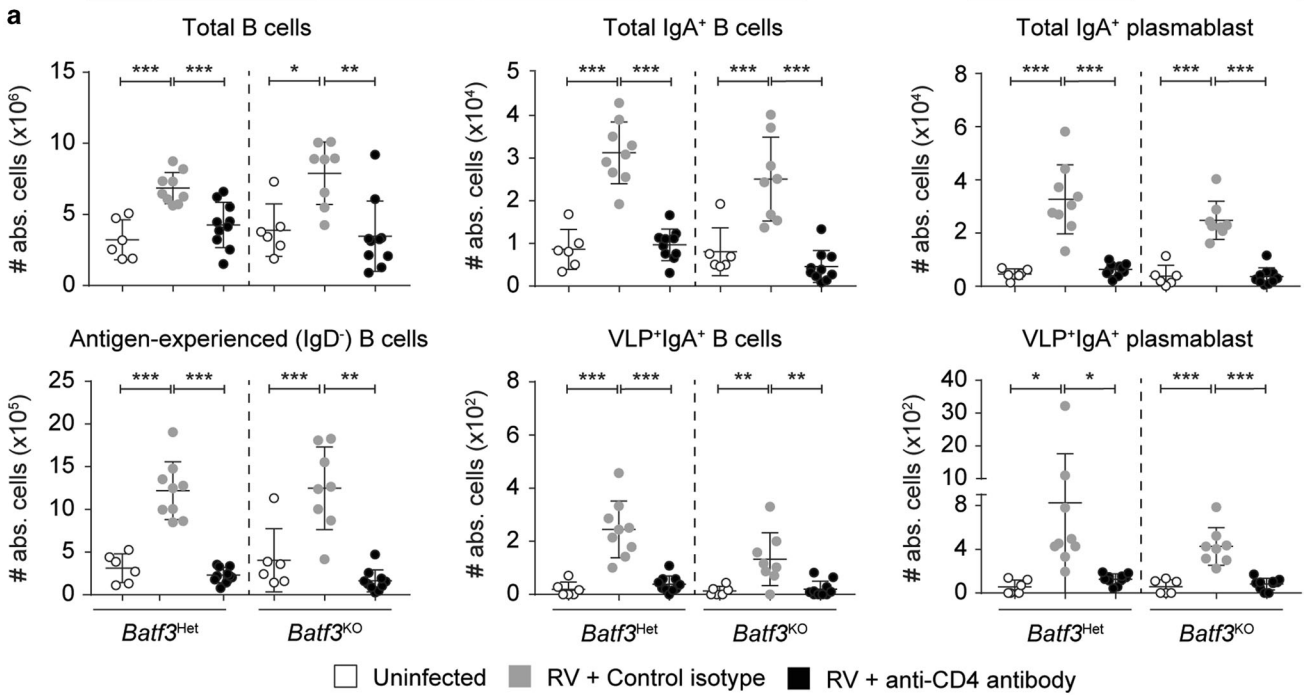
In order to better understand the reduced levels of RV-specific IgA in the intestine of *Batf3*<sup>KO</sup> mice, we identified specific B cells using RV-like particles (VLPs) constructed from the viral proteins 2 and 6 bound to GFP.<sup>41</sup> Rotavirus triggered a mild increase in the generally low abundance of IgA<sup>+</sup> B cells in the mLN. Among those, we detected significantly fewer RV-specific VLP<sup>+</sup>IgA<sup>+</sup> B cells in *Batf3*<sup>KO</sup> mice when compared with their *Batf3*<sup>Het</sup> littermates (Fig. 2a and Supplementary Fig. S1C), despite not significantly decreased total IgA<sup>+</sup> B cells. There was also a non-significant trend towards fewer VLP<sup>+</sup>IgM<sup>+</sup> B cells (Fig. 2a), which also showed significance when assessing cumulative percentage (Supplementary Fig. S1C). In parallel, *Batf3*<sup>KO</sup> mice had reduced numbers of CD138<sup>+</sup> VLP<sup>+</sup>IgA<sup>+</sup> and to a minor extent VLP<sup>+</sup>IgM<sup>+</sup> plasmablasts in mLN, while total IgA<sup>+</sup> and IgM<sup>+</sup> plasmablast were also reduced (Fig. 2b). Lower numbers of specific IgA producing B cells was specific to the mLN, as there were no differences in any B cell subsets in the Peyer's Patches (Supplementary Fig. S1D–F). We were unable to enumerate RV-specific B cells in the small intestinal lamina propria, due to loss of staining caused by the enzymatic digestion process involved in isolating these cells. All B cell subsets in *Batf3*<sup>Het</sup> and *Batf3*<sup>KO</sup> mice expressed identical levels of the gut homing receptors CCR9 and α<sub>4</sub>β<sub>7</sub> integrin, suggesting that differential homing to the small intestine is unlikely to explain lower RV-specific IgA secretion in the intestinal secretion of cDC1-deficient mice (Fig. 2c).

Anti-RV-specific IgA responses in the mLN do not require cDC2  
cDC2 coordinate IgA class switching in the Peyer's Patches at steady state<sup>35</sup> and are required for IgA induction in the mLN in response to flagellin.<sup>37</sup> We therefore tested whether cDC2 were also required for RV-specific IgA responses. *huCD207*.DTA mice specifically lack small intestinal CD103<sup>+</sup> cDC2 and Langerhans cells<sup>42</sup> and when infected orally with RV, these mice generated total and VLP<sup>+</sup> IgA<sup>+</sup> B cell responses in mLN that were comparable to controls (Fig. 2d). *huCD207*.DTA mice also, as previously published,<sup>19</sup> did not differ from wild type mice in shedding of RV or in the levels of fecal RV-specific IgA (data not shown). Together, these results indicate that *Batf3*-dependent cDC1 are required for the optimal induction of anti-RV-specific IgA<sup>+</sup> plasmablasts in the mLN, while cDC2 are dispensable.

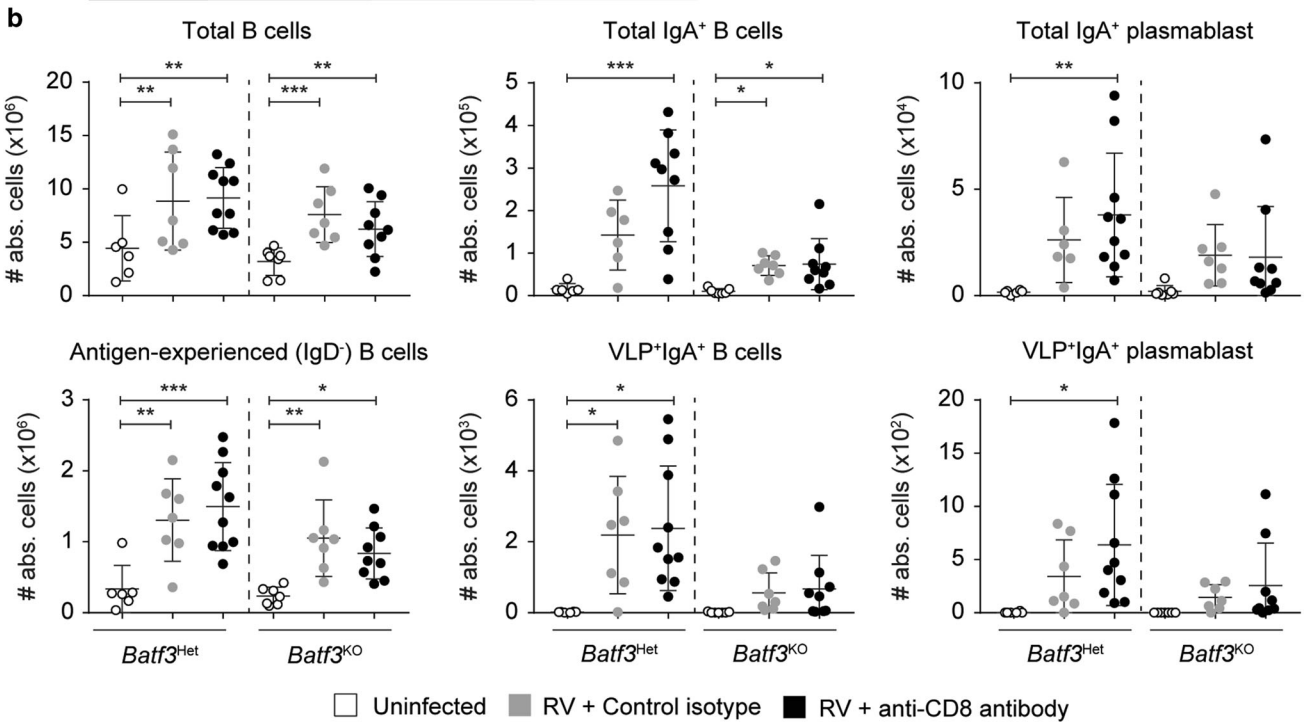
Optimal IgA<sup>+</sup> B cell generation in the mLN requires CD4<sup>+</sup> T Cells, but not CD8<sup>+</sup> T Cells

To examine whether the cDC1-dependent, RV-specific B cell responses in the mLN were T cell dependent, we first depleted CD4<sup>+</sup> T cells in infected *Batf3*<sup>Het</sup> and *Batf3*<sup>KO</sup> mice. Administration of anti-CD4 antibody depleted >90% of CD4<sup>+</sup> mLN T cells (Supplementary Fig. S2A) and ablated the expansion of total IgA<sup>+</sup> and VLP<sup>+</sup>IgA<sup>+</sup> B cells and plasmablasts that normally occurred during RV infection (Fig. 3a). In contrast, administration of anti-CD8 antibody to *Batf3*<sup>Het</sup> and *Batf3*<sup>KO</sup> mice did not reduce the numbers of VLP<sup>+</sup> or total IgA<sup>+</sup> B cells in the mLN of infected mice (Fig. 3b), despite causing >90% reduction in conventional CD8αβ<sup>+</sup> T cells within the intraepithelial lymphocyte compartment, mLN or

*Batf3*<sup>KO</sup> mice & CD4<sup>+</sup> T cell depletion



*Batf3*<sup>KO</sup> mice & CD8<sup>+</sup> T cell depletion



**Fig. 3** The bulk of the antibody response to RV depends on CD4<sup>+</sup> T cell help but does not require CD8<sup>+</sup> T cells. **a** *Batf3*<sup>Het</sup> and *Batf3*<sup>KO</sup> mice treated with either anti-mouse CD4 (GK 1.5) Mab or Rat IgG2b,κ isotype control and oral RV infection. **b** *Batf3*<sup>Het</sup> and *Batf3*<sup>KO</sup> mice treated with either anti-mouse CD8β Mab or IgG1,κ isotype control and oral RV infection. The graphs depict the total number of B cells (live CD19<sup>+</sup>B220<sup>+</sup>) and antigen-experienced (live CD19<sup>+</sup>B220<sup>+</sup>IgD<sup>-</sup>) cells (left panels), the total number of IgA<sup>+</sup> and RV-specific B cells (live CD19<sup>+</sup>B220<sup>+</sup>IgD<sup>-</sup>IgA<sup>+</sup>VLP<sup>+/+</sup>) (middle panels) and the total number of IgA<sup>+</sup> and RV-specific plasmablasts (live CD19<sup>+</sup>IgD<sup>-</sup>CD138<sup>+</sup>IgA<sup>+</sup>VLP<sup>+/+</sup>) (right panels) at 7 days post infection. Dots represent data from individual mice. Data was collected from two independent experiments. Two-way ANOVA was performed for statistical analysis with Tukey post hoc testing. Statistics are depicted for within genotype comparisons only. \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001.

PPs (Supplementary Fig. S2B). Indeed, there was a consistent trend towards more IgA<sup>+</sup> B cells in CD8<sup>+</sup> T cell-depleted *Batf3*<sup>Het</sup> mice compared with control *Batf3*<sup>Het</sup> mice, probably reflecting the higher virus burden in CD8<sup>+</sup> T cell-deficient mice.

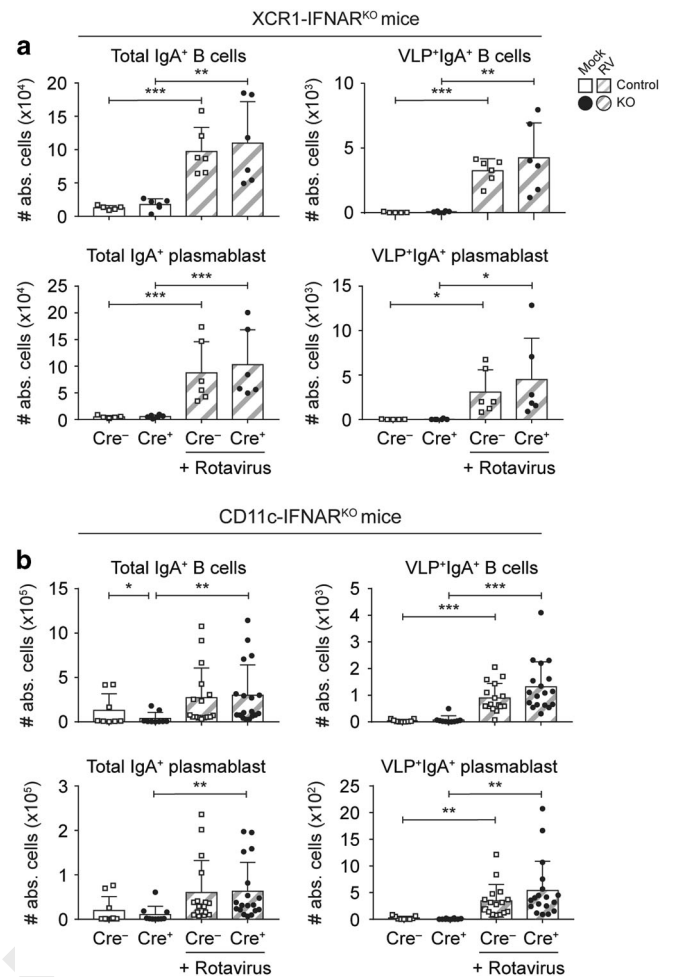
Thus CD4<sup>+</sup> T cells are required to generate RV-specific IgA responses, whereas CD8<sup>+</sup> T cells do not contribute to this process. Furthermore our results argue that the differences in IgA induction observed in cDC1-deficient mice do not reflect a secondary effect of altered CD8 T cell induction in these mice.

Type 1 IFN receptor signalling on cDC1 is dispensable for the induction of antibody responses during Rotavirus infection. Type I interferons (IFNs) are produced rapidly during viral infections<sup>43,44</sup> and type I IFN signalling in DCs has been implicated in their ability to drive the generation of T<sub>FH</sub> cell in response to systemic vaccination in a model using OVA and LPS.<sup>45</sup> Furthermore, we have shown previously that pDCs and type I interferon (IFN) signalling play an important role in early B cell activation, RV-specific IgA secretion and RV clearance.<sup>46</sup> Therefore, to examine whether type I IFN sensing in cDC1 was needed for their role in RV-specific IgA responses, we generated a novel mouse model expressing cre recombinase under the control of the cDC1-specific XCR1 promoter crossed to *ifnar*<sup>flx/flx</sup> mice.<sup>47</sup> The resulting XCR1-IFNAR<sup>KO</sup> mice that lack IFNAR specifically on cDC1s generated comparable numbers of VLP<sup>+</sup> and total IgA<sup>+</sup> B cell and plasmablasts in mLN to those seen in *ifnar*<sup>flx/flx</sup> mice used as controls (Fig. 4a). Similar results were obtained in CD11c-IFNAR<sup>KO</sup> mice that lack the IFNAR on all DC subsets, with unaltered VLP<sup>+</sup> and total IgA<sup>+</sup> B cell and plasmablast induction in the mLNs in response to RV infection, together with normal viral clearance (Fig. 4b and data not shown). Together, these experiments show that IFNAR signalling on DCs is dispensable for the induction of IgA antibody responses during RV infection.

avβ8 integrin expression by cDC1 is not required for steady-state mucosal immune responses. As discussed above, TGFβ is one of the key factors for the induction of IgA responses *in vivo*<sup>27</sup> and the TGFβ activating integrin avβ8 expressed on CD11b<sup>+</sup> PP DCs has been implicated in the generation of IgA responses towards commensal bacteria.<sup>35</sup> However avβ8 is expressed preferentially by migratory cDC1 in steady state mLN, where it is required for their ability to induce Tregs via TGFβ activation.<sup>48</sup> Therefore, we examined whether avβ8-mediated TGFβ activation by cDC1 could also account for their ability to drive IgA class-switching during viral infection.

We generated a novel mouse model in which a tdTomato fluorescent reporter is expressed under the control of the *Itgb8* promoter (*Itgb8*-tdTomato mice) (Fig. 5a,b). Analysis of DC subsets in these mice confirmed that *Itgb8*-tdTomato expression was confined mostly to migratory cDC1 in the mLN (Fig. 5c-e), with 65–70% of XCR1<sup>+</sup> migratory cDC1 expressing β8 integrin (Fig. 5c, d). Thus, while migratory cDC1 represent 23.7 % of total mLN DCs, they represent 80% of the *Itgb8*-tdTomato<sup>+</sup> mLN DCs (Fig. 5e). In contrast, <10% of XCR1<sup>+</sup>SIRPα<sup>-</sup> cDC1 in the PPs were tdTomato<sup>+</sup> and expression of the transgene was absent from other myeloid populations in PP (Fig. 5c,d), which recapitulates Immgen-derived RNA-seq data on *Itgb8* expression at the cell population level (Fig. 5f). Thus, avβ8 is expressed preferentially by migratory cDC1 in the mLNs in steady state and not in the PPs.

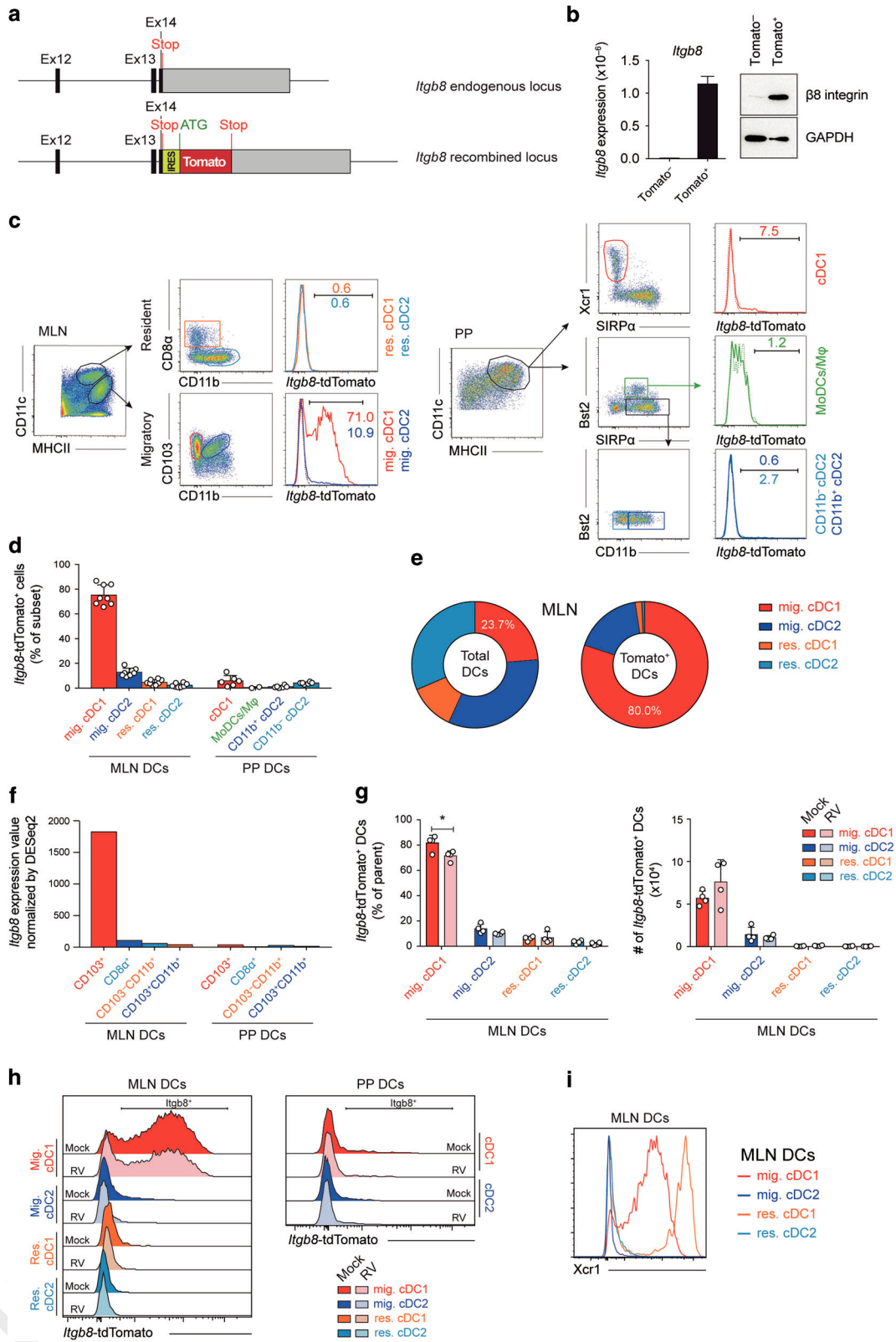
We have previously shown that avβ8 expression by DCs is tightly regulated and depends on a combination of lineage and tissue-microenvironment factors, as well as activation status.<sup>48</sup> Interestingly, upon *in vitro* TLR3 engagement by the synthetic analog of dsRNA, polyinosinic-polycytidylic acid (poly(I:C)), splenic cDC1 not expressing β8 in the steady state<sup>48</sup> get activated, up-regulate CD86 expression and induce expression of β8 integrin (Supplementary Fig. S3B,C). This is not the case for cDC2. In order to determine whether β8 expression levels change during



**Fig. 4 Type 1 IFN receptor (IFNAR) signalling on DCs is dispensable for the induction of antibody responses during Rotavirus infection.** **a** XCR1-IFNAR<sup>KO</sup> and **b** CD11c-IFNAR<sup>KO</sup> mice were orally challenged with RV. The total number of total or RV-specific IgA<sup>+</sup> B220<sup>+</sup>CD138<sup>-</sup> B cells (top; pre-gated on CD19<sup>+</sup>IgD<sup>-</sup>) and total or RV-specific IgA<sup>+</sup> plasmablasts (bottom; B220<sup>-</sup>CD138<sup>+</sup>; pre-gated on CD19<sup>+</sup>IgD<sup>-</sup>) at 7 days post infection. Dots represent data from individual mice. Data were collected from **a** two and **b** four independent experiments with three mice per group. Two-way ANOVA was performed for statistical analysis with Tukey post hoc testing. \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001. White symbols: HET; black symbols: KO; shaded bars: RV-infected.

infection, *Itgb8*-tdTomato mice were orally infected with RV. Importantly, three days post infection the percentage but not the number of β8<sup>+</sup> migratory cDC1 was slightly decreased in the mLN (Fig. 5g), while total percentage and number of DCs remained stable (Supplementary Fig. 3D). We observed no induction of β8 in other mLN or any PP DC subsets (Fig. 5h). Therefore, the β8 expression pattern is conserved during RV infection with the maintenance of preferential expression in migratory cDC1 mLN DCs.

In order to study the role of avβ8 on cDC1 in regulating intestinal IgA responses, we generated mice lacking avβ8 integrin expression specifically on cDC1 (*Xcr1*<sup>Cre</sup>.*Itgb8*<sup>flx/-</sup>, called XCR1-β8<sup>KO</sup> thereafter). As expected, only cDC1 (migratory CD103<sup>+</sup>CD11b<sup>-</sup> and resident CD8α<sup>+</sup> mLN DCs expressing XCR1 (Fig. 5i)), but not cDC2, deleted the floxed *Itgb8* allele in these mice (Fig. 6a). XCR1-β8<sup>KO</sup> mice developed normally, did not develop wasting disease or colitis (Fig. 6b, c) and had normal cellularity of total lymphocytes (Fig. 6d) and populations of Tregs and T<sub>H</sub>17 cells in the colonic lamina propria (Fig. 6e). Numbers of



total IgA producing B cells and plasma cells in spleens, PPs, MLNs and colonic lamina propria were also unaltered (Fig. 6f), translating to normal total IgA levels in the serum and intestinal washes from XCR1- $\beta 8^{\text{KO}}$  mice (Fig. 6g) and in line with normal T<sub>FH</sub> and T<sub>FR</sub> fractions in the MLNs and PPs (Fig. 6h). This was in sharp contrast

to mice in which  $\alpha\beta 8$  is deleted in all DCs (CD11c- $\beta 8^{\text{KO}}$ ), which developed spontaneous colitis and dysregulated intestinal B and T cell responses (Fig. 6b-e and Supplementary Fig. S4). Thus, expression of  $\alpha\beta 8$  integrin by cDC1 is dispensable for immune

**Fig. 5 avβ8 integrin expression is restricted to migratory CD103<sup>+</sup> XCR1<sup>+</sup> cDC1 mLN DCs.** **a, b** Genetic construction (**a**) and model validation (**b**) of the new mouse reporter *Itgb8*-tdTomato for *Itgb8* expression. **a** Black rectangles represent *Itgb8* coding sequences, grey rectangle indicates non-coding exon portions, solid line represents introns. Start (ATG) and Stop codons are indicated, and yellow and red rectangles represent the IRES and the reporter (tdTomato) sequences, respectively. **b** Tomato<sup>+</sup> and Tomato<sup>-</sup> CD3<sup>+</sup> T cells were sorted from the spleen of naive *Itgb8*-tdTomato mice. β8 integrin gene (*Itgb8*) and protein expression was assessed by qRT-PCR and Western Blot. The bar graph shows *Itgb8* expression expressed relative to *Gapdh*. **c** Mononuclear phagocyte subsets from mLN or PP of *Itgb8*-tdTomato mice were analyzed for *Itgb8* expression as indicated. Cells are pre-gated on live CD45<sup>+</sup> CD19<sup>-</sup> CD3<sup>-</sup> CD11c<sup>+</sup> (mLN) or live CD45<sup>+</sup> CD19<sup>-</sup> CD3<sup>-</sup> (PP). **d** Percentage *Itgb8*-tdTomato<sup>+</sup> cells for each subset. Note that the CD11b<sup>+</sup> cDC2 gate contains both PP and DAV (Dome-associated villus) cDC2. (data pooled from three experiments with at least two mice each). **e** Percentage of each subset among total mLN DCs (left) or among *Itgb8*-tdTomato<sup>+</sup> DCs (right). **f** *Itgb8* expression value normalized by DESeq2 from ImmGen Open Source Mononuclear Phagocytes RNA-seq data. **g, h** *Itgb8*-tdTomato mice were infected orally with RV. *Itgb8* expression in mLN and PP was monitored by flow cytometry 3 days post infection (data pooled from 2 experiments with 2 mice each). **g** Percentages of parent (DC subset, left) and number (right) of *Itgb8*-tdTomato<sup>+</sup> DCs were plotted for the different mLN DC subsets. **h** Histograms of *Itgb8*-tdTomato expression in mLN and PP DC subsets. **i** Histogram shows the analysis of XCR1 expression in mLN DCs subsets from C57/Bl6 mice gated as in **c**. Two-way ANOVA was performed for statistical analysis with a Tukey post hoc test. \*\**p* < 0.01.

homeostasis in the intestine, including the generation of steady state IgA responses.

The TGFβ-Activating avβ8 integrin on cDC1 is essential for optimal RV-specific IgA responses

As XCR1-β8<sup>KO</sup> mice did not develop colitis and had normal total IgA production, we were able to use them to assess the impact of cDC1-restricted deletion of avβ8 deletion on RV-specific IgA responses without the confounding effects of inflammation. As we had found in *Batf3*<sup>KO</sup> mice, mice lacking avβ8 specifically on cDC1 had normal numbers of total and IgA<sup>+</sup> B cell numbers 7 days after infection with RV (Fig. 7a), but had decreased numbers of IgA<sup>+</sup> (both total and VLP<sup>+</sup>) plasmablasts in mLN compared with XCR1-β8<sup>het</sup> littermate controls (Fig. 7a). This did not affect the number of IgM<sup>+</sup> B cells in mLN (Supplementary Fig S5A). Thus, the TGFβ-activating avβ8 integrin on cDC1 is required for optimal induction of RV-specific IgA in the mLN at 7 days post infection.

avβ8-mediated TGFβ activation is required for cDC1 to induce optimal IgA class-switching in vitro

To confirm directly that avβ8 on cDC1 plays a role in IgA class-switching via TGFβ activation, we switched to an in vitro model. Splenic NP-specific B cells from QM mice were cultured in vitro in serum-free medium to minimize TGFβ contamination and stimulated with anti-CD40 Ab and NP-Ficoll. Total mLN DCs from WT mice stimulated IgA class-switch in vitro and this was significantly enhanced by addition of either active or latent TGFβ (Fig. 7b). Binding and activation of latent TGFβ by avβ8 occurs via an Arg-Gly-Asp (RGD) tripeptide in the latency-associated peptide and a cyclic form of the RGD peptide inhibits the binding and activation of latent TGFβ by avβ8 integrins in the context of T cell-dependent responses.<sup>33,48</sup> This RGD mimetic blocked the IgA class switching enhancing effect of added latent TGFβ in DC-B cell co-cultures in these cultures as well as in cultures containing keyhole limpet hemocyanin-NP instead of NP-Ficoll (Fig. 7b and Suppl. Fig. 5B,C). Both cDC1 and cDC2 subsets were able to induce IgA class switching in this in vitro model efficiently but blocking avβ8 with RGD only affected the IgA induction efficiency by cDC1s, while cDC2s were unaltered (Fig. 7c). Likewise, only migratory mLN cDC1s but not cDC2s sorted from XCR1-β8<sup>KO</sup> mice were significantly impaired in their capacity of inducing IgA class switching (Fig. 7d and Suppl. Fig. S5C). Thus, mLN cDC1s appear to activate latent TGFβ to promote IgA class switching via avβ8, a process that could be replicated in vitro using FACS-sorted migratory CD103<sup>+</sup>CD11b<sup>-</sup> mLN cDC1. Taken together these in vitro experiments support the idea that intestine-derived cDC1 can induce IgA switching in B cells via avβ8 integrin-mediated activation of latent TGFβ (for a graphical summary see Fig. 8).

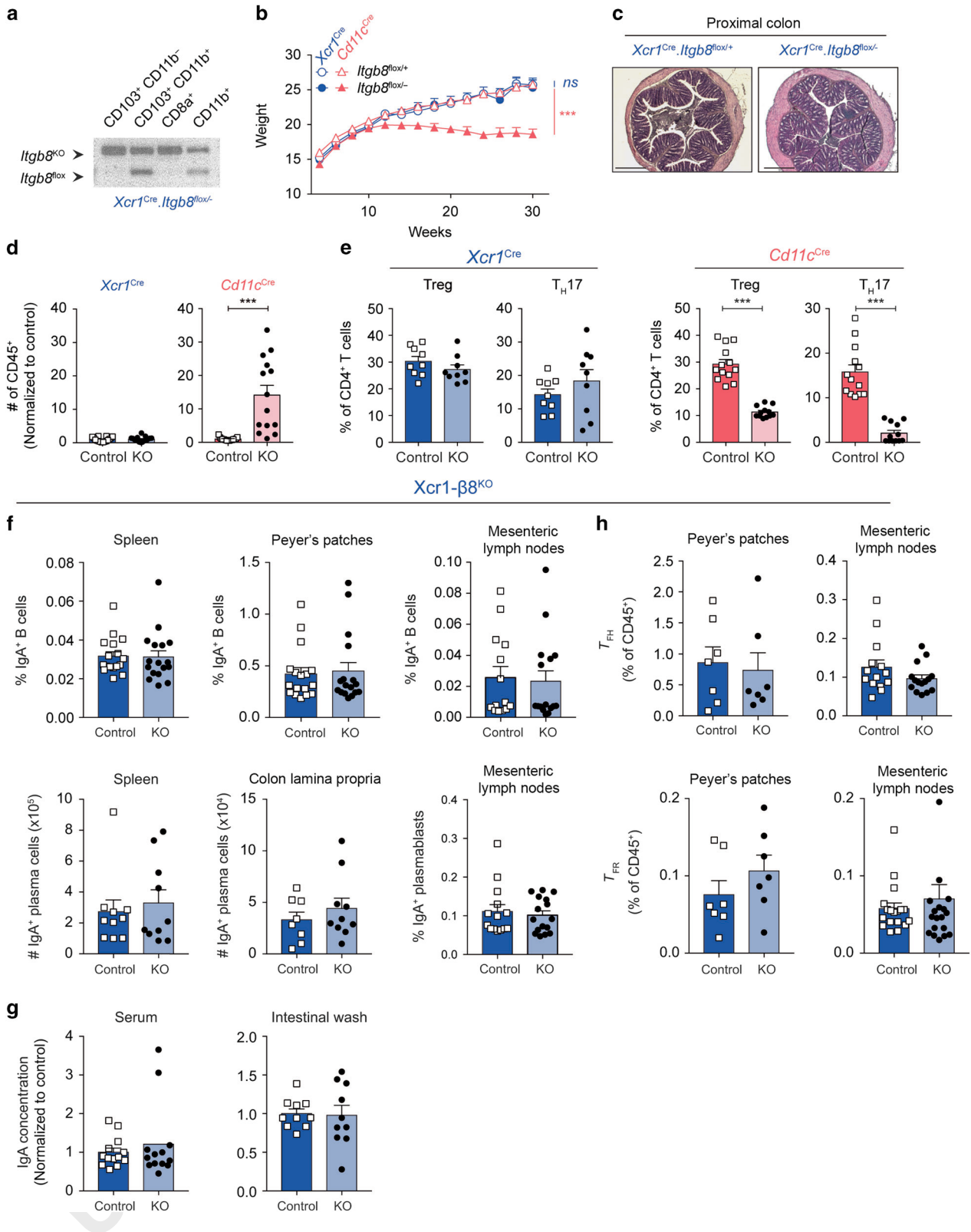
## DISCUSSION

Specific IgA production is the major correlate of protection from reinfection with rotavirus,<sup>5,6</sup> but very little is known about the mechanisms that induce IgA responses against intestinal viruses. Here we show that the cDC1, but not cDC2 subset of DCs, is critical for generating optimal T-cell dependent RV-specific IgA responses in the mLNs early during primary infection. Type I IFN signalling in DCs is dispensible for their role in B cell priming upon infection. Instead, induction of optimal IgA responses depends, at least in part, on avβ8 expression by cDC1, driving activation of TGFβ.

As we confirm here, intestinal cDC1 are crucial for the optimal immune response towards RV, with RV-specific CD8<sup>+</sup> T cells depending on this subset for their priming.<sup>19</sup> This finding is consistent with the ability of RV to infect the intestinal epithelium and cause epithelial cell death, as well as the fact that cDC1 are the primary DC subset that presents epithelial-derived antigens to CD8<sup>+</sup> T cells in the mLN.<sup>18</sup> Importantly, we and others<sup>19</sup> found that some RV-specific CTLs remained present in the absence of cDC1, suggesting that other cells can also prime CD8<sup>+</sup> T cell responses to RV. It is not known whether RV can infect DCs directly in vivo, but if this occurs, the residual CTL response to RV may also reflect direct presentation by infected non-cDC1 DCs. Alternatively, the few remaining cDC1 that are known to be present in *Batf3*-deficient mice on the C57Bl/6 genetic background<sup>49</sup> could suffice to prime RV-specific CTLs. This is thought to reflect the ability of other BATF transcription factors to take over the role of *Batf3* in an IL-12 dependent manner in the presence of inflammation,<sup>49</sup> although it is notable that we did not find remaining cDC1 in *Batf3*<sup>KO</sup> mice during RV infection (Supplementary Fig. S1).

Given these findings, we hypothesized that cDC1 might also influence B cell priming and IgA class-switching in response to RV. Indeed, we found delayed secretion of fecal RV-specific IgA in infected *Batf3*<sup>KO</sup> mice, together with a marked defect in the expansion of antigen-specific IgA<sup>+</sup> plasmablasts in the mLNs. The numbers of total IgA<sup>+</sup> and IgM<sup>+</sup> plasmablasts, but not those of total IgA<sup>+</sup> or IgM<sup>+</sup> non-plasmablasts were also slightly affected by the absence of cDC1, indicating that the initial massive increase in B cells that occurs in response to RV is independent of cDC1 and that the defect is biased towards RV-specific IgA. Strikingly, the absence of intestinal cDC2 had no effect on RV-specific IgA<sup>+</sup> B cells numbers in the mLN, contrasting with the known role of this DC subset in intestinal IgA production in the steady-state and in response to extracellular bacteria.<sup>35,37</sup> cDC1s excel at transporting apoptotic intestinal epithelial cells to the draining mLN,<sup>18,50</sup> and RV infection induces ample cell death.<sup>51,52</sup> Together, this suggests that the induction of IgA class-switching is not restricted to intestinal cDC2, but rather depends on the context and nature of the antigen.

The defective generation of RV-specific IgA B cell responses in *Batf3*<sup>KO</sup> mice also appears to be an anatomically restricted



phenomenon, as we found no effect in the PP. This might be due to a redundancy in DC subsets at sites such as PPs that are likely heavily loaded with antigen or could reflect the relative absence of  $\alpha\beta 8$  integrin expression by cDC1 in PP (see below). It might also reflect the specific ability of cDC1 to deliver epithelial-derived antigen to the mLN.<sup>18</sup>

To our knowledge this is the first demonstration that cDC1 play a role in the generation of antigen-specific IgA responses in the intestine. However, previous studies have shown a similar requirement for cDC1 in IgA responses in the lungs after administration of an influenza hemagglutinin subunit with poly(I:C), a synthetic mimic of double-stranded RNA.<sup>36</sup> This effect

**Fig. 6 Mice with a cDC1-specific αvβ8 deletion display normal steady-state mucosal responses.** XCR1-β8<sup>KO</sup> mice were generated to specifically delete αvβ8 in cDC1. **a** Genomic PCR for the *Itgb8* locus in sorted mLN DC subpopulation as gated in Fig. 5a. **b, c** XCR1-β8<sup>KO</sup> mice do not develop colitis. Weight (at least seven individual mice per timepoint and per genotype) (**b**) and representative histology of the colon (**c**) of XCR1-β8<sup>KO</sup>, CD11c-β8<sup>KO</sup> and littermate controls. Statistics in **b** consistently relate to every time point from 12 weeks onward and were assessed using a two-way ANOVA between genotypes followed by Sidak correction for multiple comparison test for each individual timepoint. Scale bar in **c**: 500 μm. **d, e** Number of total CD45<sup>+</sup> cells (**d**) and fraction of Treg and T<sub>H</sub>17 (**e**) were quantified in the Colon Lamina Propria (CLP) of XCR1-β8<sup>KO</sup> and CD11c-β8<sup>KO</sup> mice by flow cytometry. **f** Percentage of IgA<sup>+</sup> B cells (CD45<sup>+</sup>CD3<sup>-</sup>B220<sup>+</sup>CD19<sup>+</sup>CD138<sup>-</sup>IgA<sup>+</sup>) and plasma cells (CD45<sup>+</sup>CD3<sup>-</sup>B220<sup>-</sup>CD138<sup>+</sup>IgA<sup>+</sup>) from indicated organs from XCR1-β8<sup>KO</sup> mice measured by flow cytometry. **g** Serum and intestinal wash IgA levels in XCR1-β8<sup>KO</sup> versus control littermates measured by ELISA. **h** T<sub>FH</sub> and T<sub>FR</sub> percentages of total CD45<sup>+</sup> live cells from PPs and mLNs analyzed by flow cytometry. Cell percentage are plotted as a percent of CD45<sup>+</sup> cells. Data were collected from 2-4 independent experiments (total *n* = 7-15). Statistical analysis for **d-h**: two-way ANOVA was performed for statistical analysis with a Tukey post hoc test, \*\*\**p* < 0.001.

depended on a functional TLR3-TRIF pathway and correlated with the presence of T follicular helper cells. As we found in the intestines, there were no differences in total B cell numbers in the lung in the absence of cDC1, suggesting a specific defect in IgA class switching.

Having established a role for cDC1 in the induction of RV-specific IgA in the mLN, we then explored the mechanisms involved. cDC1 have been reported to drive germinal center-derived IgA responses in the respiratory tract<sup>36</sup> and consistent with previous observations,<sup>8</sup> we found that the RV-specific B cell recruitment and IgA responses in the mLN were dependent on CD4<sup>+</sup> α/β T cells. As published earlier by Sun et al., IFNγ production by virus-specific CD4<sup>+</sup> T cells was, however, not altered (ref. 19 and own observations). While total numbers of mLN T<sub>FH</sub> cells were also not altered in the absence of cDC1 (data not shown), the requirement for germinal center reactions for early IgA plasmablast induction upon RV infection requires further investigation. As *Batf3*<sup>KO</sup> mice lacking cDC1 have markedly reduced CD8<sup>+</sup> T cell responses during RV infection (our data and ref. 19) and IFNγ produced by CD8<sup>+</sup> T cells can induce class switching in B cells,<sup>53</sup> we assessed whether CD8<sup>+</sup> T cells contributed to RV-specific IgA production and whether that could explain the decrease in RV-specific IgA in BATF3-deficient mice. However, depletion of CD8<sup>+</sup> T cells did not reduce IgA<sup>+</sup> B cell responses in RV-infected mice, indicating that CD8<sup>+</sup> T cells do not contribute to IgA class switching in this model.

Rotavirus elicits type I IFN responses<sup>54</sup> and stimulation of DCs with type I IFN enhances humoral immunity, promotes isotype switching<sup>55</sup> and increases the development of lymph node-resident T<sub>FH</sub> cells.<sup>45</sup> Using a complete knock-out for the type I IFN receptor, we have previously reported a role for type I IFN in the early induction of bulk B cell responses during RV infection.<sup>46</sup> Given the similar viral load in type I IFN deficient and wild type mice infected with homologous RV strains,<sup>56</sup> this is however unlikely to contribute to viral clearance. Here, we show that deletion of the type I IFN receptor specifically on DCs or cDC1 did not alter RV-specific IgA responses. This negative finding is consistent with other work examining cDC1-dependent IgA production in the lung in response to poly(I:C)<sup>36</sup>; adaptive immunity to norovirus infection also does not require type I IFN receptor signalling on DCs.<sup>57</sup> Thus, the requirement for type I IFN signalling in bulk B cell activation early after infection does not appear to involve the DC compartment and is more likely to reflect a direct role for the type I IFN receptor on B cells.<sup>46</sup>

The initiation of class switch recombination towards IgA requires activation of the cytokine TGFβ, with binding of its latent form to cell surface αvβ8 integrin being an important mechanism of TGFβ activation.<sup>29,30</sup> In the gut, αvβ8 integrin on MNP is critical to maintain mucosal immune homeostasis, as mice selectively lacking β8 in all CD11c-expressing cells develop severe spontaneous colitis,<sup>30</sup> associated with loss of intestinal Treg and Th17 cells. Subsequently, we and others have shown that induction of intestinal TGFβ-dependent T cell responses is under the strict control of DC αvβ8, which licenses TGFβ signaling to T cells via local activation of TGFβ.<sup>31-34</sup> As we confirm here, αvβ8 is

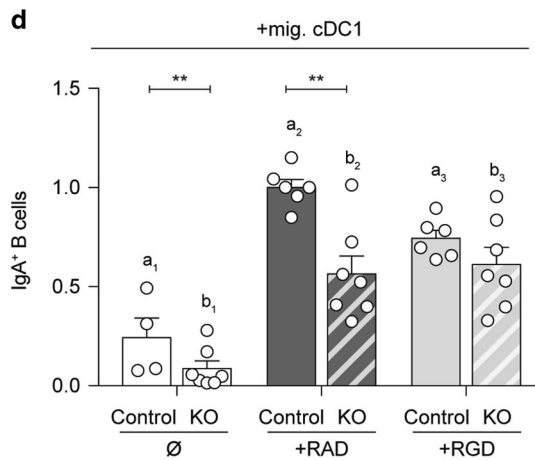
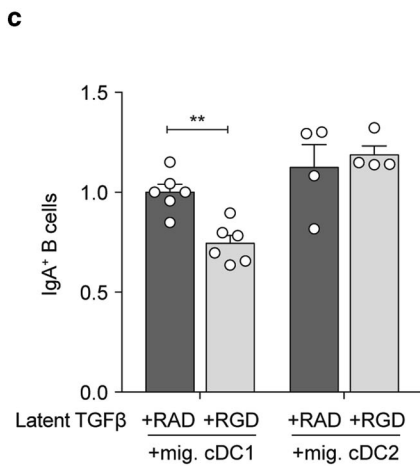
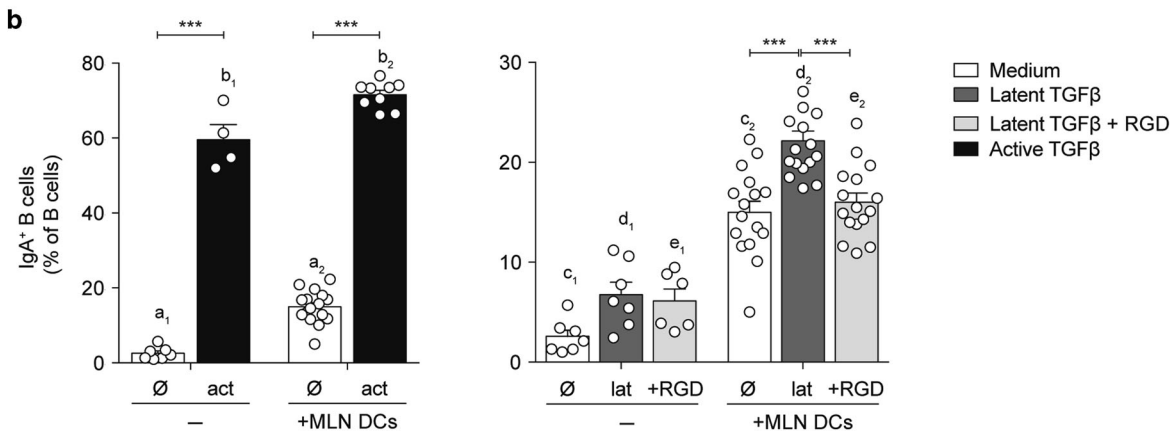
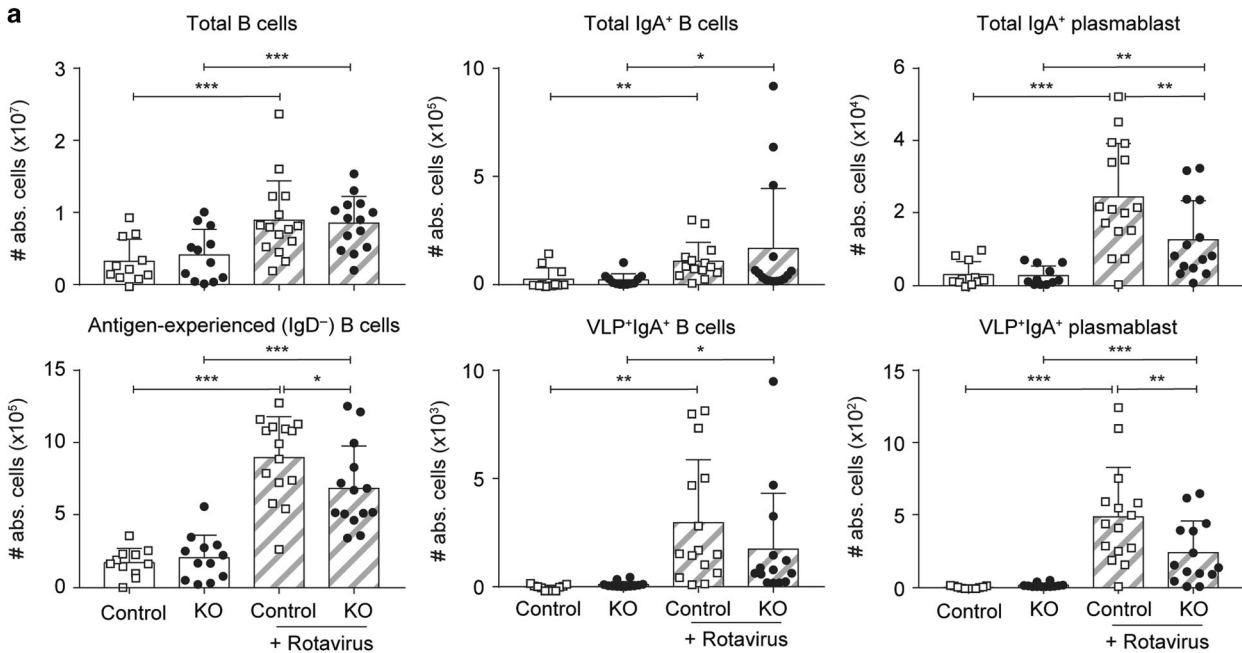
expressed preferentially by migratory cDC1 in the normal mLN,<sup>13,48</sup> but in contrast to its deletion in all CD11c<sup>+</sup> cells, selective β8 deletion in cDC1 under the control of the XCR1 promoter does not disrupt mucosal immune homeostasis. The reasons for the discrepancy between these models are unclear, but may suggest that the low abundance of β8<sup>+</sup> mLN cDC2 (~12%) and PP DCs (1-4%) can compensate for αvβ8-deficiency on cDC1 to maintain steady state conditions. Along those lines, we have previously shown that stimulation *in vitro* can reveal αvβ8 expression on cells other than cDC1.<sup>48</sup> Importantly, together with targeted deletion of β8 integrin in specific myeloid cell populations, our *Itgb8*-tdTomato reporter mice will serve as a valuable tool to dissect the contribution of different cell subsets to αvβ8-dependent immune homeostasis in the future.

Here, we show that β8 expression in any other PP or mLN mononuclear phagocyte population is minor compared to β8 expression in migratory cDC1 in the mLN, both by FACS and by qRT-PCR or RNA-seq (Fig. 5 and data not shown). A recent study reported that both CD11b<sup>+</sup> and CD8a<sup>-</sup>CD11b<sup>-</sup> DCs in PP express αvβ8.<sup>35</sup> In their study, Reboldi et al.<sup>35</sup> examined β8 expression at the population level only in the PPs, while our analysis both at the single-cell level using *Itgb8*-specific reporter mice and in sorted cell populations (ImmGen data, Fig. 5f) revealed much higher proportions of β8<sup>+</sup> cells and higher levels of β8 transcripts on DCs in mLNs compared with PPs. This did not change in the context of RV infection. As discussed above, the low percentage of β8<sup>+</sup> cells in DC subsets other than mLN cDC1 does not preclude them from playing a critical role in mucosal immune homeostasis, which will need to be explored in the future. In addition, as we have shown previously that β8 expression by mLN DC can be influenced by several factors in the intestinal microenvironment such as dietary metabolites, the microbiota and the presence of inflammation,<sup>48,60</sup> we cannot exclude that the apparent discrepancies in β8 expression profiles may reflect differences in these factors. Furthermore, it remains possible that other triggers such as bacterial or fungal infections might induce β8 expression by cDC2 in mLN and/or PP, allowing them to participate in TGFβ activation for the induction of IgA responses. For example, ATP promotes αvβ8 expression in colonic LP CD103<sup>-</sup>CX3CR1<sup>+</sup>CD11b<sup>+</sup> MNP.<sup>61</sup> An alternative possibility is that cDC2 may be able to activate TGFβ via other, αvβ8-independent, mechanisms such as proteases, ROS or thrombospondin-1.<sup>62,63</sup> Finally, several studies have suggested that DC may promote IgA class switching *in vivo* by non-TGFβ-dependent mechanisms, such as retinoic acid, TNF/iNOS or APRIL.<sup>25,64,65</sup> Therefore, the molecular mechanisms driving IgA responses may be complex and context dependent.

A novel finding of the current study was the significant reduction in IgA<sup>+</sup> plasmablasts in the mLN of mice lacking αvβ8 specifically in cDC1, providing a mechanism for the dependency of RV-specific IgA on the presence of this subset of DC consistent with the ability of αvβ8 to activate this cytokine.<sup>66</sup> Thus, αvβ8 expressing cDC1 may play a critical and specific role in the generation of IgA responses to viruses and other antigens that require cross-presentation in the intestine. It has to be noted that the defect in IgA plasmablasts in XCR1-β8<sup>KO</sup> mice was not as



Xcr1-β8<sup>KO</sup> mice



dramatic as was seen in cDC1-deficient mice. Intestinal cDC1 excel in carrying epithelium-derived antigens to the mLN,<sup>18</sup> possibly explaining the pronounced phenotype in mice lacking cDC1. The difference in phenotypes could also reflect a role for other

molecules capable of converting TGFβ into its bioactive form, such as other integrins or metalloproteinases (MMPs)<sup>67</sup> and this requires further study. In addition, as noted above, TGFβ is not the only mediator that can facilitate class-switching towards IgA at

**Fig. 7 The TGFβ-activating avβ8 integrin on cDC1 DCs licences IgA induction by this subset in vivo and in vitro.** **a** XCR1-β8<sup>KO</sup> and littermate control (XCR1-β8<sup>Het</sup>) mice were orally challenged with RV. The total number of B cells, antigen-experienced IgD<sup>-</sup> cells (left), total or RV-specific IgA<sup>+</sup>B220<sup>+</sup>CD138<sup>-</sup> B cells (pre-gated on CD19<sup>+</sup>IgD<sup>-</sup>) (middle) and total or RV-specific IgA<sup>+</sup> plasmablasts (B220<sup>-</sup>CD138<sup>+</sup>; pre-gated on CD19<sup>+</sup>IgD<sup>-</sup>) at 7 days post infection is plotted. Dots represent data from individual mice. Data were collected from three independent experiments with 4–5 mice per group each. **b** Splenic QM B cells were isolated by magnetic purification and cultured alone or with total CD11c<sup>+</sup>MHC<sup>+</sup> DCs (+mLN DCs) isolated from mLN of C57/B6 mice in serum-free medium and in the presence of NP-Ficoll, anti-CD40 Ab and RA. Active TGFβ (act), latent TGFβ (lat) and/or a synthetic RGD (or RAD)-containing peptide were added as indicated. After 5 days cells were harvested and analysed for IgA expression by flow cytometry. Additional statistics: a<sub>1</sub>-a<sub>2</sub>, b<sub>1</sub>-b<sub>2</sub>, c<sub>1</sub>-c<sub>2</sub>, d<sub>1</sub>-d<sub>2</sub> and e<sub>1</sub>-e<sub>2</sub>: \*\*\* (total n = 4-17) **(c, d)** CD103<sup>+</sup> CD11b<sup>-</sup> migratory cDC1 (+mig cDC1, **(c, d)**) or CD103<sup>+</sup> CD11b<sup>+</sup> migratory cDC2 (+mig cDC2, **(c)**) were sorted from the mLN of XCR1-β8<sup>WT</sup> (C,D) or XCR1-β8<sup>KO</sup> **(d)** mice as previously described and cultured with FACS-sorted QM B cells (gated as CD19<sup>+</sup> B220<sup>+</sup>). Data is normalized to control cDC1 cultured with latent TGFβ + RAD for each replicate (total n = 4-7). Additional statistics: a<sub>1</sub>-a<sub>2</sub>, b<sub>1</sub>-b<sub>2</sub>, b<sub>1</sub>-b<sub>3</sub>:\*\*\*, a<sub>1</sub>-a<sub>3</sub>: \*\* Dots represent data from independent biological replicates. Data were collected from four **(b)** or three **(c, d)** independent experiments. Two-way ANOVA with Tukey post hoc was performed for statistical analysis. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001.

mucosal sites. These include retinoic acid, which can act synergistically with TGFβ in this role.<sup>65,68</sup> Migrating intestinal DCs including cDC1 possess all the enzymes necessary to convert vitamin A into RA, indicating that lack of RA might help explain the larger defect in IgA production in *Batf3*<sup>KO</sup> mice. However, the induction of the gut homing molecules CCR9 and α4β7 on B cells was normal in *Batf3*<sup>KO</sup> mice, suggesting that overall levels of RA may be relatively unaffected even in the absence of cDC1. In addition, this result is also likely to exclude defective B cells migration from the mLN to the intestinal mucosa as a mechanism of the lower levels of secreted IgA we observed in these animals.

In summary, we show here that BATF3-dependent cDC1, but not cDC2, are required for optimal T-cell dependent RV-specific IgA responses in the mLNs during primary RV infection in adult mice. This strongly suggests that both major DC subsets in the intestines can induce IgA production but vary in their contribution according to the nature of the trigger. While type I IFN receptor signalling in DCs is dispensable for their role in IgA induction, their expression of the TGFβ activating avβ8 integrin is in part accountable for their capacity of inducing IgA during RV infection. Given the prominent role of RV-specific IgA in protection against re-infection, we hypothesize that strong RV-specific IgA induction in the mLNs may account for functionally relevant immune memory. Of note, BATF3-deficient mice are fully protected from re-infection eight weeks after primary infection (data not shown). This is in line with our data showing that RV-specific IgA titers in the feces eventually reach wildtype levels in the absence of cDC1 (Fig. 1c, d), which are maintained for at least eight weeks and probably accounting for the lack of recall following this timeline (data not shown). Further analysis of the RV-specific affinity and longevity of the B cell memory pool remains to be performed in order to define whether targeted engagement of cDC1 is a superior strategy for future vaccine design against rotavirus and other mucosal viral pathogens.

## METHODS

### Mice

All animals were housed under specific pathogen-free conditions at Lund/Malmö (Sweden) or Lyon (France). All mice were on the C57Bl/6 background. Littermate or age-matched mice were used for all experiments. Male and female mice were used between 8 and 16 weeks of age. *Xcr1*-IRES-iCre-2A-mTfP1 gene-targeted mice (also known B6-*Xcr1*tm2Ciphe mice and called XCR1.cre mice here) mice permit to specifically delete floxed gene in cDC1.<sup>69</sup> *Batf3*<sup>KO</sup> and *Het* (B6.129S(C)-*Batf3*<sup>tm1Kmm/J</sup>), CD11c-IFNAR<sup>KO</sup> ((B6.Cg-Tg(Itgax-cre)1-1Reiz/J<sup>70</sup> crossed to IFNAR floxed mice (obtained from U. Kalinke<sup>47</sup>)), XCR1-IFNAR<sup>KO</sup> (XCR1.cre crossed to IFNAR floxed mice) and *huCD207-DTA*<sup>42</sup> mice were bred and maintained in the Clinical Research Center at Lund University. β8 integrin-reporter mice were generated on a C57Bl/6 background by expression of an internal ribosomal entry site-fluorescent tandem dimer (td)-Tomato cassette (IRES-Td-Tomato) immediately

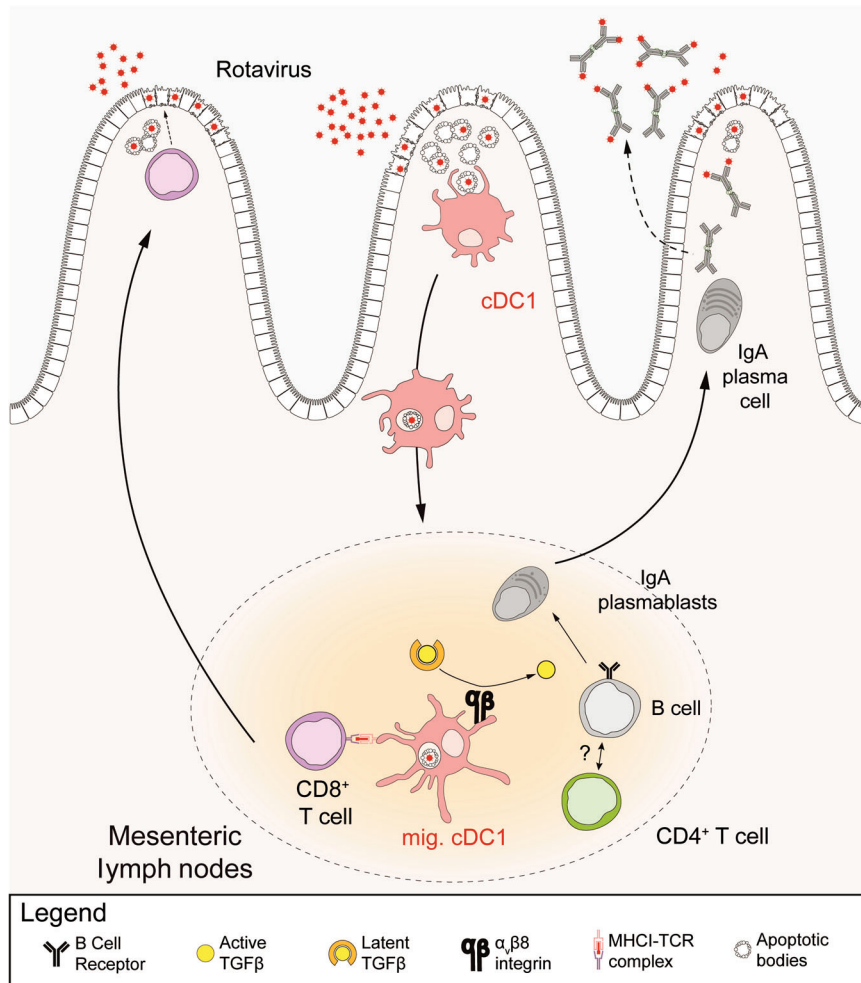
following the stop codon in exon 14 of *Itgb8* (Fig. 5a) (GenOway, Lyon, France). *Itgb8*-IRES-TdTomato mice had no gross abnormalities and were born at the expected female:male ratios. *Itgb8*-IRES-TdTomato, Quasi-monoclonal (QM) mice<sup>71</sup> (kindly provided by Dr. M. Cascalho, University of Michigan, Medical School, Ann Arbor, MI, USA) and XCR1-β8<sup>KO</sup> mice (XCR1.cre crossed to *Itgb8*<sup>fllox/-</sup><sup>30</sup>) were bred at Plateau de Biologie Expérimentale de la Souris (PBES, ENS Lyon, France). Animal experiments were performed under appropriate licenses within local and national guidelines for animal care.

### Rotavirus infections

The virulent wildtype EC<sub>w</sub> strain of RV was obtained from cleared intestinal homogenates of suckling mice orally gavaged with RV at 3 days of age and sacrificed 2 days after infection. For infection of adult mice, RV was inoculated orally at a dose of 3 × 10<sup>3</sup> DD<sub>50</sub>, with Peyer's patches and mLNs being collected and lymphocytes isolated at intervals as described below.

### Cell Isolation and flow cytometry

Single-cell suspensions from spleen, mLNs and Peyer's Patches were generated by mechanical disruption through 70 μm cell strainer in FACS buffer (5% FBS, 500 ml PBS (Gibco)). Non-specific binding was blocked with normal rat serum (Sigma) and rat anti-mouse CD16/CD32 Fc block (2.4G2, BD Biosciences) for 20 min at 4 °C. To stain for RV-specific B cells, cells were incubated with rotavirus-like particles (VLP2/6) containing RV VP2 and VP6 bound to GFP (obtained from Didier Poncet, I2BC CNRS Gif sur Yvette, France)<sup>41</sup> for 45 min at 4 °C. The following antibodies were used: anti-CD19 (1D3), anti-CD138 (281-2), anti-B220 (RA3-6B2), anti-IgM (R6-60.2) (BD Horizon), anti-GL-7 (GL7), anti-IgD (11-26c.2a), anti-CD45.2 (104) (BioLegend), anti-IgA (mA-6E1) (eBioscience), and anti-CD95 (Jo2) (BD Biosciences). The Live/Dead<sup>TM</sup> Fixable Near-IR Dead cell stain kit (L10119, Life Technologies) was used to assess cell viability, while VP7<sub>VGPVFP</sub> tetramers coupled to BV421 obtained from the NIH Tetramer core facility were used at a concentration of 12 μg/ml to stain for RV-specific CD8<sup>+</sup> T cells. B cells were sorted from splenocytes of QM mice<sup>71</sup> either by magnetic separation (R&D) or by flow cytometry as indicated using the following Ab: anti-CD19 (1D3), anti-B220 (RA3-6B2), anti-CD11c (HL3), anti-CD3 (145-2C11) (all from BD Biosciences, Pont de Claix, France). DCs subsets from spleen, mLN and PP were isolated as described previously.<sup>48</sup> Briefly, after tissue digestion, cell suspensions were enriched for DCs using an OptiPrep gradient. Total spleen DCs were then isolated by positive magnetic separation using anti-CD11c beads (Miltenyi). DC subsets from mLN and spleen were FACS-sorted using a BD FACSArial cytometer and the following antibodies: anti-CD11c (HL3), anti-I-A/I-E (2G9), anti-CD8α (53-6.7), anti-CD45RB/B220 (RA3-6B2), anti-CD103 (M290) (all from BD Biosciences, Pont de Claix, France), anti-CD11b (M1/70; eBioscience, Paris, France) and anti-XCR1 (ZET; Biolegend). Data acquisition was performed using LSRII or



**Fig. 8 Proposed model for the regulation of anti-rotavirus IgA responses.** While subepithelial dome cDC2 are required in the PP for induction of IgA responses to extracellular bacteria, we show that mLN cDC1 promote both cellular and humoral immune responses to RV infection. cDC1 in the LP take up viral antigen in apoptotic bodies, activate and migrate to the mLN, where they induce RV-specific cytotoxic T cells. We here show that they also contribute to the induction of RV-cDC1-driven T cell-dependent IgA responses in the mLN.  $\alpha\beta 8$  expression, uniquely prominent on migratory cDC1 in the mLN is required to instruct the class switch towards IgA through provision of bioactive TGF $\beta$ . CD8<sup>+</sup> T cells and plasma cells eventually migrate to the LP to kill infected enterocytes and produce secretory IgA, respectively.

LSR-Fortessa cytometers and DIVA software (BD Bioscience). The analysis was performed using FlowJo software 10 (Treestar Inc).

**Measurement of rotavirus antigen shedding and IgA in feces**  
Fecal samples from infected mice were weighed and soaked in PBS, 1% BSA, 1 mM EDTA, soybean trypsin inhibitor (0.05 mg/ml), 2 mM PMSF (phenylmethylsulfonyl fluoride) (Sigma) and 0.025% azide for 2 h at 100 mg/ml, homogenized and centrifuged at 13000 rpm for 10 min. The supernatant was analysed for the presence of RV antigen and RV-specific IgA as previously described in ref. <sup>38</sup> using guinea pig anti-RV hyperimmune serum, rabbit anti-RV hyperimmune serum (both from the Greenberg laboratory), goat anti-rabbit IgG-HRP (Millipore) and TMB Substrate Reagent Set (RUO, 555214, BD Biosciences).

**Depletion of CD8<sup>+</sup> T cells and CD4<sup>+</sup> T cells in vivo**  
Mice were depleted of CD8<sup>+</sup> T cells by administration of 300  $\mu$ g InVivoMAb anti-mouse CD8 $\beta$  (53-5.8, Nordic BioSite) intraperitoneally on days -2, -1 before RV infection, on the day of infection and days 3 and 5 post RV infection. Non-depleted mice received purified mouse IgG1 $\kappa$  isotype as a control (MOPC-21, Nordic BioSite). For depletion of CD4<sup>+</sup> T cells, mice received 50  $\mu$ g InVivoMAb anti-mouse CD4 (GK 1.5, Nordic Biosite) on day -1

before RV infection and day 3 post RV infection. Non-depleted mice received purified Rat IgG2b,  $\kappa$  isotype as a control.

#### $\beta 8$ integrin expression analysis

*Itgb8* gene expression analysis by quantitative RT-PCR was performed as previously described.<sup>48</sup>  $\beta 8$  integrin protein expression was measured by Western blot using an anti- $\beta 8$  antiserum generated in rabbit against the C-terminal tail of the human  $\beta 8$  cytoplasmic region (provided by Joseph McCarty<sup>72</sup>) as previously described.<sup>48</sup>

#### IgA class switch by B cells in vitro

B cells were isolated from QM mice<sup>71</sup> either by magnetic isolation (MagCelect Mouse B Cell Isolation Kit, R&D) or sorted by FACS as indicated in Figure legends. B cells ( $2 \times 10^4$ ) were co-cultured with cDCs ( $2 \times 10^4$ ) with 4-Hydroxy-3-nitrophenylacetic (NP) conjugated to Ficoll (NP-Ficoll, Biosearch Technologies, 15 ng/mL), anti-CD40 (Enzo Life Sciences, 2  $\mu$ g/mL) and RA (Sigma, 2  $\mu$ M) in the presence or absence of latent TGF $\beta$  (R&D, 4 ng/mL), active TGF $\beta$  (R&D, 1 ng/mL), cRGD (Enzo Life Sciences, 20  $\mu$ g/mL), cRAD (Enzo Life Sciences, 20  $\mu$ g/mL) and anti-TGF $\beta$  Ab (BD, 40  $\mu$ g/mL) for 5 days in X-vivo 15 medium (Lonza) supplemented with sodium pyruvate (1 mM), MEM NEAA, HEPES buffer (10  $\mu$ M),

2-mercaptoethanol (50 μM) and L-glutamine (2 mM). IgA<sup>+</sup> B cells were identified by flow cytometry using the following antibodies: anti-IgA-FITC (11-44-2; SouthernBiotech), anti-CD19-PECF594 (1D3), anti-B220-BV786 (RA3-6B2) (BD Biosciences). Viability was assessed using ThermoFisher Fixable Viability Dye FVD-ef780.

#### DC stimulation in vitro

DCs isolated from the spleen of *Itgb8*-tdTomato were stimulated in vitro as previously described.<sup>48</sup> Briefly, spleen DCs were cultured at 37 °C in complete RPMI medium in the presence of 1 μmg/ml high molecular weight poly(I:C) (InvivoGen) for 8 h. *Itgb8*-tdTomato expression in DC subsets was then assessed by flow cytometry using the following antibodies: anti-CD11c (HL3), anti-I-A/I-E (2G9), anti-CD45RB/B220 (RA3-6B2), anti-CD19 (1D3), anti-CD3 (145-2C11) (all from BD Biosciences, Pont de Claix, France), anti-CD11b (M1/70; eBioscience, Paris, France), and anti-XCR1 (ZET) and anti-CD86 (GL-1; both from Biolegend).

#### Statistical analysis

Statistics were performed using two-way ANOVA (post hoc: Tukey) considering treatment and experiment as factors for the analysis. Kruskal–Wallis test was applied to compare more than two groups (e.g.: different genotypes ( $n = 3$ ) within the same treatment) (post hoc: Dunn<sup>73</sup>) when the experiment was performed only once. Statistical significance was estimated by using R Studio.

R Script:

Two-way ANOVA: `aov(value ~ genotype + day, data = Data)`

Post-Hoc test Tukey: `TukeyHSD(Data_anova2, which = "genotype")`

Kruskal–Wallis test: `kruskal.test(value ~ treatment, data = Data)`

Post-Hoc test Dunn: `dunnTest(value ~ treatment, data = Data, method = "bh")`

Where genotype accounts for analysis of different genotypes within the same treatment. Using treatment instead of genotype allows for analysis of different treatments within a genotype. Data refers to the data to be analysed.

Area under the curve calculation (unpaired *T* test as statistical method) and two-way ANOVA with Sidak correction were performed using Graphpad Prism Software.

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#### AUTHOR CONTRIBUTIONS

J.N., S.T., J.H., H.P., and K.L. conceived and designed the project; J.N., S.T., J.H., I.U., A.G. L., H.P., and K.L. designed experiments; J.N., S.T., J.H., M.B.-J., V.B., K.G.M., L.J.G., K.F.T., H. P., I.U., A.G.L., and K.L. performed experiments and analysed the data; D.P., B.M., and H.G. provided essential tools; O.T. and T.D. provided advice on conceptualization and experimental design, and participated in editing the paper; J.N., S.T., H.P., and K.L. wrote the paper; H.P. and K.L. supervised the study and acquired funding; all authors reviewed the paper.

#### ADDITIONAL INFORMATION

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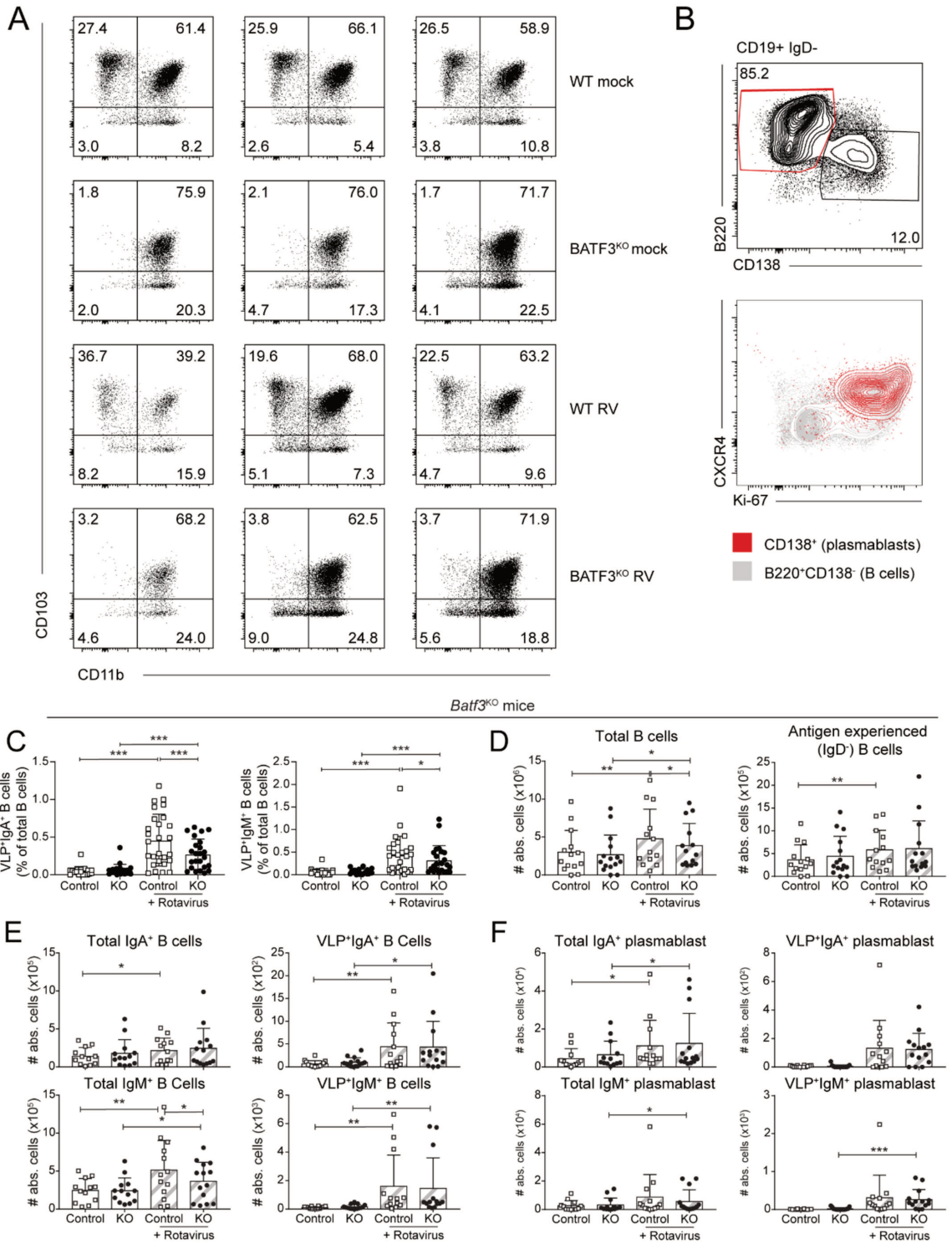
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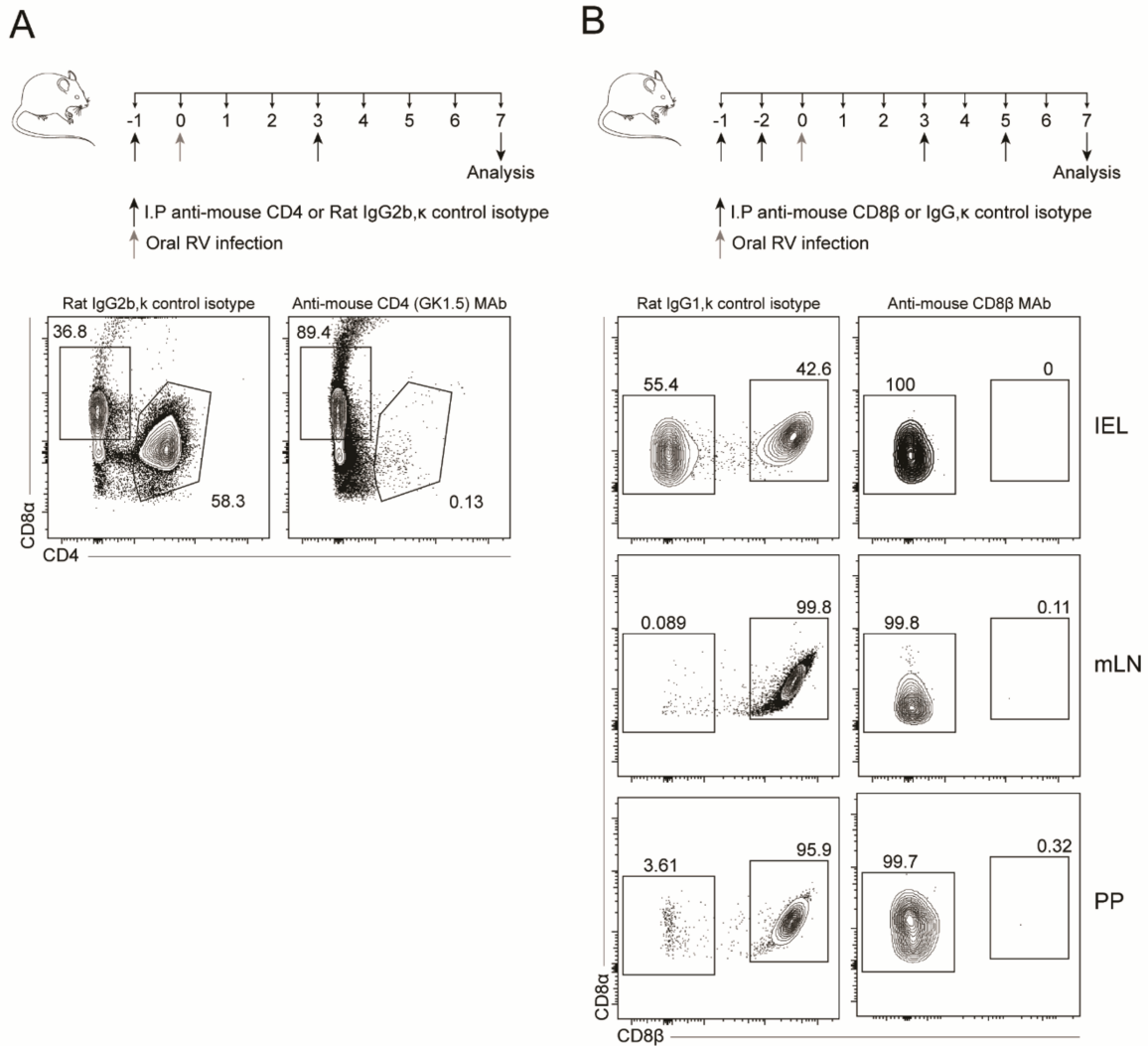


Suppl. Fig. 1



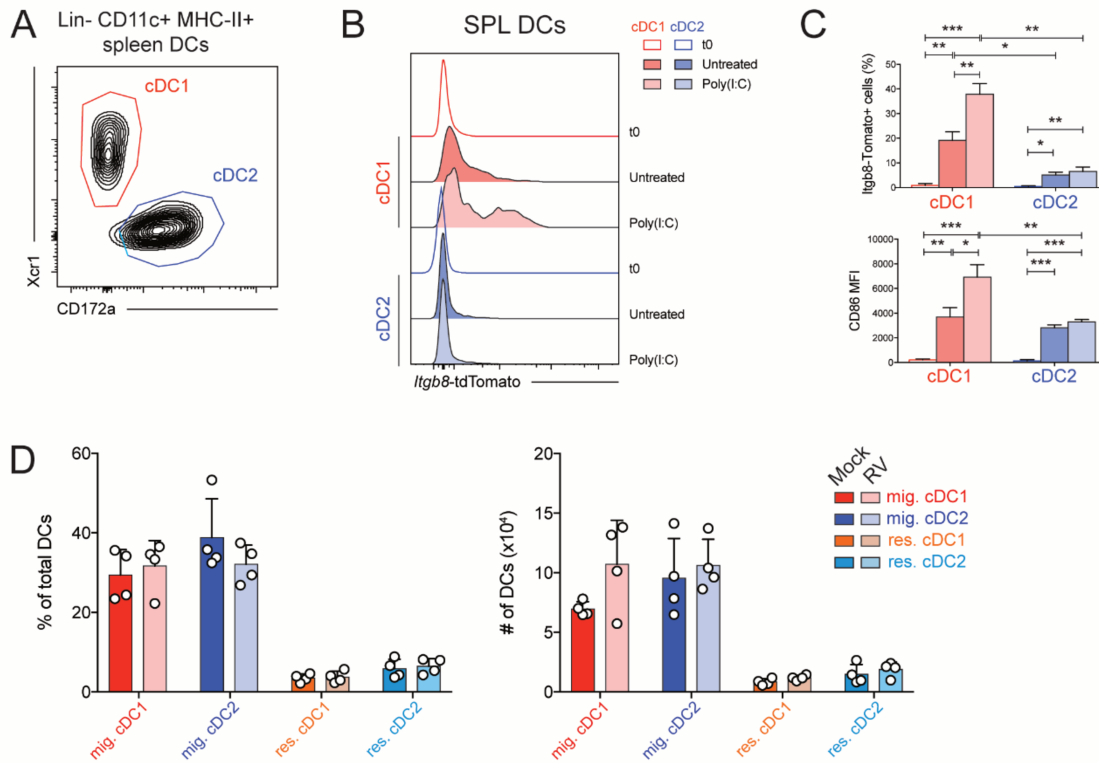
**Fig. S1. The absence of cDC1 is maintained RV in response to infection in *Batf3*<sup>KO</sup> mice and cDC1 are dispensable for the optimal induction of anti-RV specific IgA plasma blasts in the Peyer's Patches.** *Batf3*<sup>Het</sup> and *Batf3*<sup>KO</sup> mice were orally infected with RV. (A) Small intestinal lamina propria cells were isolated from WT and *Batf3*<sup>KO</sup> mice of mock- or d7 orally RV infected mice. FACS plots show live single cells, which were further gated on lineage negative (CD3, CD19, B220, NK1.1, CD64), CD11c<sup>+</sup>, MHCII<sup>+</sup>. Plots from all three mice per group in the experiment are depicted. (B) Representative plot from pre-gated CD19<sup>+</sup>IgD<sup>-</sup> live single cells from day 7 RV-infected *Batf3*<sup>Het</sup> mLN (left). Overlay plot depicting CXCR4 and Ki67 as surrogate markers for plasmablasts (right). (C) Percent of VLP<sup>+</sup>IgA<sup>+</sup> (left) and VLP<sup>+</sup>IgM<sup>+</sup> (right) cells among non-plasma B cells (live, single cell, CD19<sup>+</sup>, B220<sup>+</sup>, CD138<sup>-</sup>) in the mLN of d7 RV- and mock-infected *Batf3*<sup>KO</sup> and *Batf3*<sup>HET</sup> mice (relates to Fig. 2A). (D) Total number of B cells, Naïve B cells and mature (IgD<sup>-</sup>) B cells at 7 days post-infection in the PPs. (E) Total number of IgA<sup>+</sup> and IgM<sup>+</sup> plasmablasts at 7 days post-infection in the PPs. For the gating strategy see supplementary figure S1. Dots represent data from individual mice. Data were collected from 4 independent experiments. Two-way ANOVA with Tukey post hoc was performed for statistical analysis. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001.

Suppl. Fig. 2

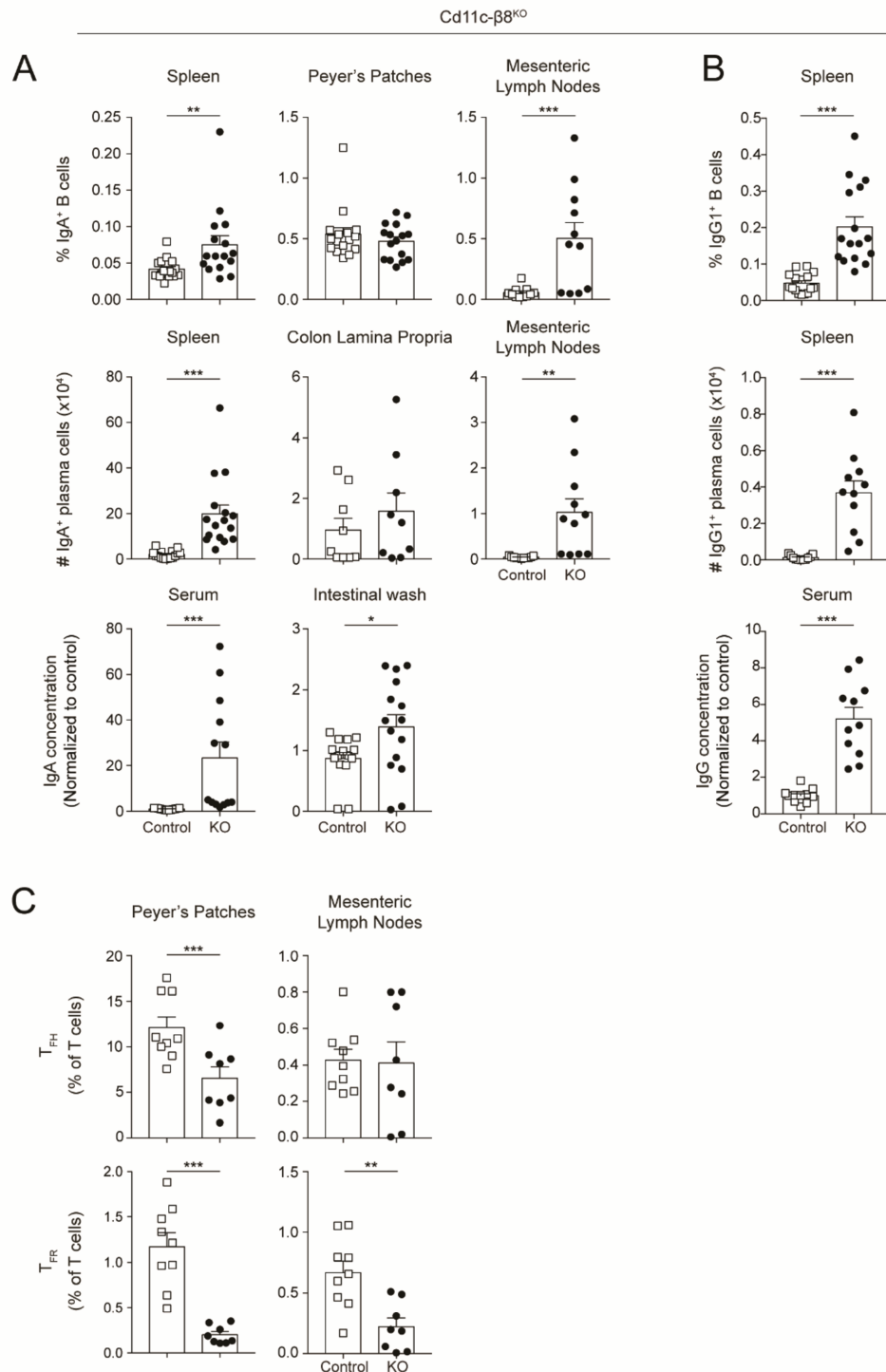


**Fig. S2. Depletion of CD4<sup>+</sup> and CD8<sup>+</sup> T cells in *Batf3*<sup>KO</sup> and *Het* mice.** (A) (Upper panel) Scheme showing *in vivo* depletion of CD4<sup>+</sup> T cells and oral RV infection in both *Batf3*<sup>Het</sup> and *Batf3*<sup>KO</sup> mice. Mice were treated with either anti-mouse CD4 (GK 1.5) Mab or Rat IgG2b,κ isotype Control, I.P and oral RV infection. (Lower panel) Representative flow cytometry plots for depletion of CD4<sup>+</sup> T cells in the mLNs. (B) *Batf3*<sup>Het</sup> and *Batf3*<sup>KO</sup> mice treated with either anti-mouse CD8β Mab or IgG1,κ control isotype and oral RV infection. Representative flow cytometry plots for depletion of CD8<sup>+</sup> T cells in the IELs, mLNs and PP.



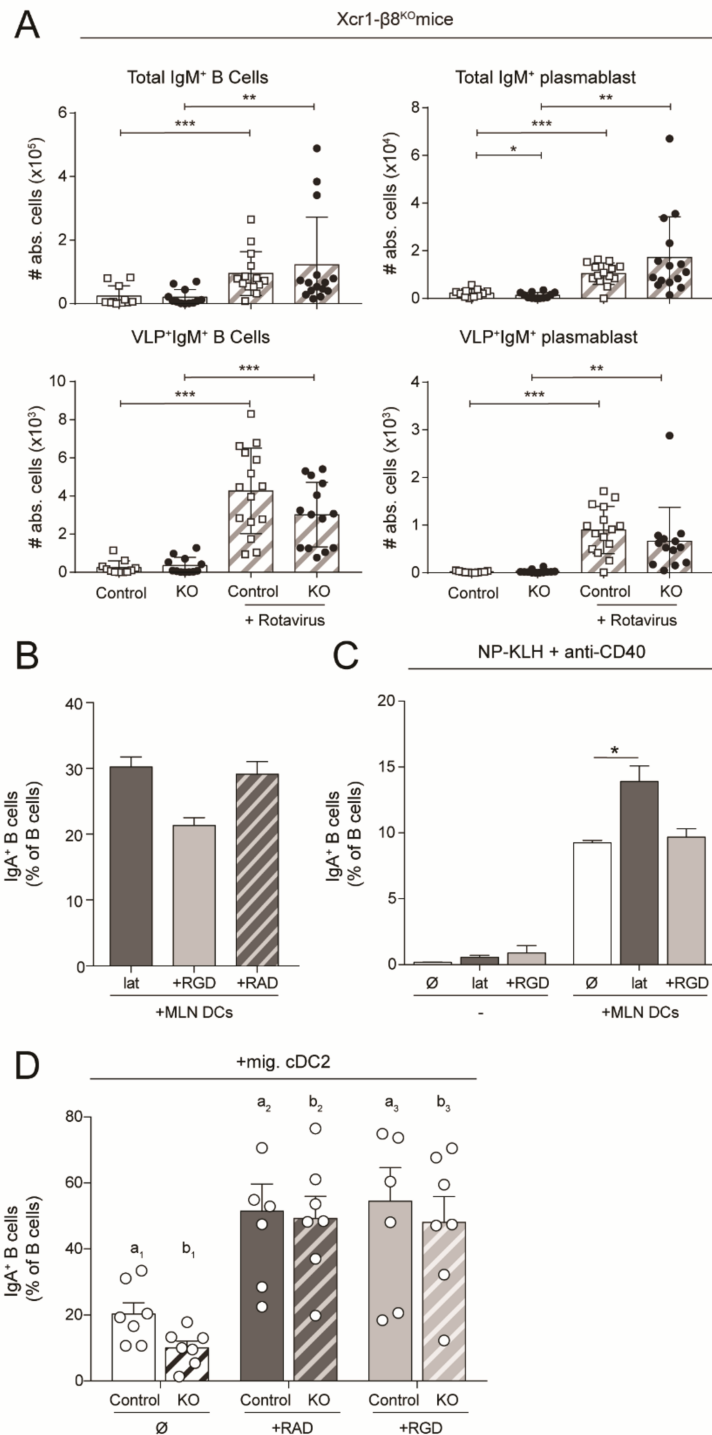


**Fig. S3. TLR3 stimulation preferentially induces *Itgb8* expression in spleen cDC1.** CD11c<sup>+</sup> MHC-II<sup>+</sup> spleen DCs from *Itgb8*-tdTomato mice were sorted by magnetic beads and then cultured in complete RPMI for 8h. Cells were either left untreated (untreated) or stimulated with (poly:I:C). **(A)** Splenic DC subsets are gated as XCR1<sup>+</sup> (cDC1) or CD172<sup>+</sup> (cDC2). Cells are pre-gated on live CD45<sup>+</sup> Lineage negative (CD3, CD19, B220) CD11c<sup>+</sup> MHC-II<sup>+</sup>. **(B)** *Itgb8*-tdTomato expression in each subset is assessed by flow cytometry (concatenated data from 2 mice). **(C)** % of *Itgb8*-tdTomato<sup>+</sup> cells for each DC subset. Data were collected from 2 independent experiments with 2 mice each. **(D)** *Itgb8*-tdTomato were orally infected with RV. The percentage (amongst total DCs) and total number of mLN DC subsets was monitored 3 days following infection. Two-way ANOVA (post hoc: Tukey) was performed for statistical analysis; \*p < 0.05, \*\*p < 0.001, \*\*\*p < 0.0001.



**Fig. S4. *In vivo*, αβ8 deficiency on all CD11c<sup>+</sup> DCs is associated with dysregulated humoral responses.** *Cd11c<sup>Cre</sup>.Itgb8<sup>flox/-</sup>* mice and their respective control littermates (*Cd11c<sup>Cre</sup>.Itgb8<sup>flox/+</sup>*) were sacrificed at mixed ages (8-30 weeks) and the proportion of IgA (A) or IgG (B) plasma cells (CD138<sup>+</sup>, B220<sup>-</sup>, IgA<sup>+</sup>), B cells (CD19<sup>+</sup>, B220<sup>+</sup>) or GC B cells (CD95<sup>+</sup>, GL7<sup>+</sup>) was analyzed by flow cytometry. Concentration of total IgA and

IgG antibodies from serum or intestinal washes was measured by ELISA. Data is normalized to the value of control littermates in each experiment. (C) Percentage of follicular Helper T cells (FoxP3<sup>-</sup> CXCR5<sup>+</sup> PD1<sup>+</sup>) and follicular regulatory T cells (FoxP3<sup>+</sup> CXCR5<sup>+</sup> PD1<sup>+</sup>) was measured by flow cytometry. Data pooled from 2-4 experiments (total n = 9-15). Two-way ANOVA (post hoc: Tukey) was performed for statistical analysis; \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001.



**Fig. S5. The TGFβ-activating αβ8 integrin on cDC1 DCs is redundant in the generation of total B cell numbers and IgM<sup>+</sup> cells during RV infection but, in contrast to its role on cDC2, required for the induction of IgA in DC - B cell co-cultures. (A)** XCR1-β8<sup>het</sup> and XCR1-β8<sup>KO</sup> mice were orally challenged with RV. Number of total (top) or VLP<sup>+</sup> (bottom) IgM<sup>+</sup> B cells (left) or plasmablast (right) at 7 days post-infection.

Dots represent data from individual mice. Data were collected from 3 independent experiments with 4-5 mice each. **(B-C)** Splenic QM B cells were isolated by magnetic purification and cultured alone (-) or with total CD11c<sup>+</sup>MHC<sup>+</sup> DCs isolated from mLN of C57/B6 mice in serum-free medium and in the presence of NP-Ficoll **(B)** or NP-KLH **(C)**, anti-CD40 Ab and RA. Latent TGFβ and/or a synthetic RGD (or RAD)-containing peptide were added as indicated. Data were collected from 1-2 independent experiments (total n=2-4). **(D)** CD103<sup>+</sup>CD11b<sup>+</sup> were sorted from XCR1-β8<sup>WT</sup> or XCR1-β8<sup>KO</sup> mice as previously described and cultured with FACS-sorted QM B cells (gated as CD19<sup>+</sup>B220<sup>+</sup>) in serum-free medium and in the presence of NP-Ficoll, anti-CD40 antibody and retinoic acid. Latent TGFβ and/or a synthetic RGD (or RAD)-containing peptide were added as indicated. Dots represent data from individual pools of mice. Data were collected from 4 independent experiments. **(A)** Two-way ANOVA (post hoc: Tukey) was performed for statistical analysis. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001. **(B, C)** Kruskal-Wallis Test was followed by post hoc Dunn test. \*p < 0.05, \*\*\*p < 0.001.

## Further consideration for the $\alpha_V\beta_8$ -mediated control of steady state IgA responses.

### $\beta_8$ expression by cDC2 is not required to control overall mucosal IgA responses

Our study shows that the conditional deletion of  $\beta_8$  on CD11c-expressing phagocytes induces an important dysregulation of the humoral responses (Fig. S4). However, restriction of  $\beta_8$  deletion to cDC1 ( $Xcr1-\beta_8^{KO}$ ) does not significantly impact steady state IgA responses (Fig. 2.1 below).

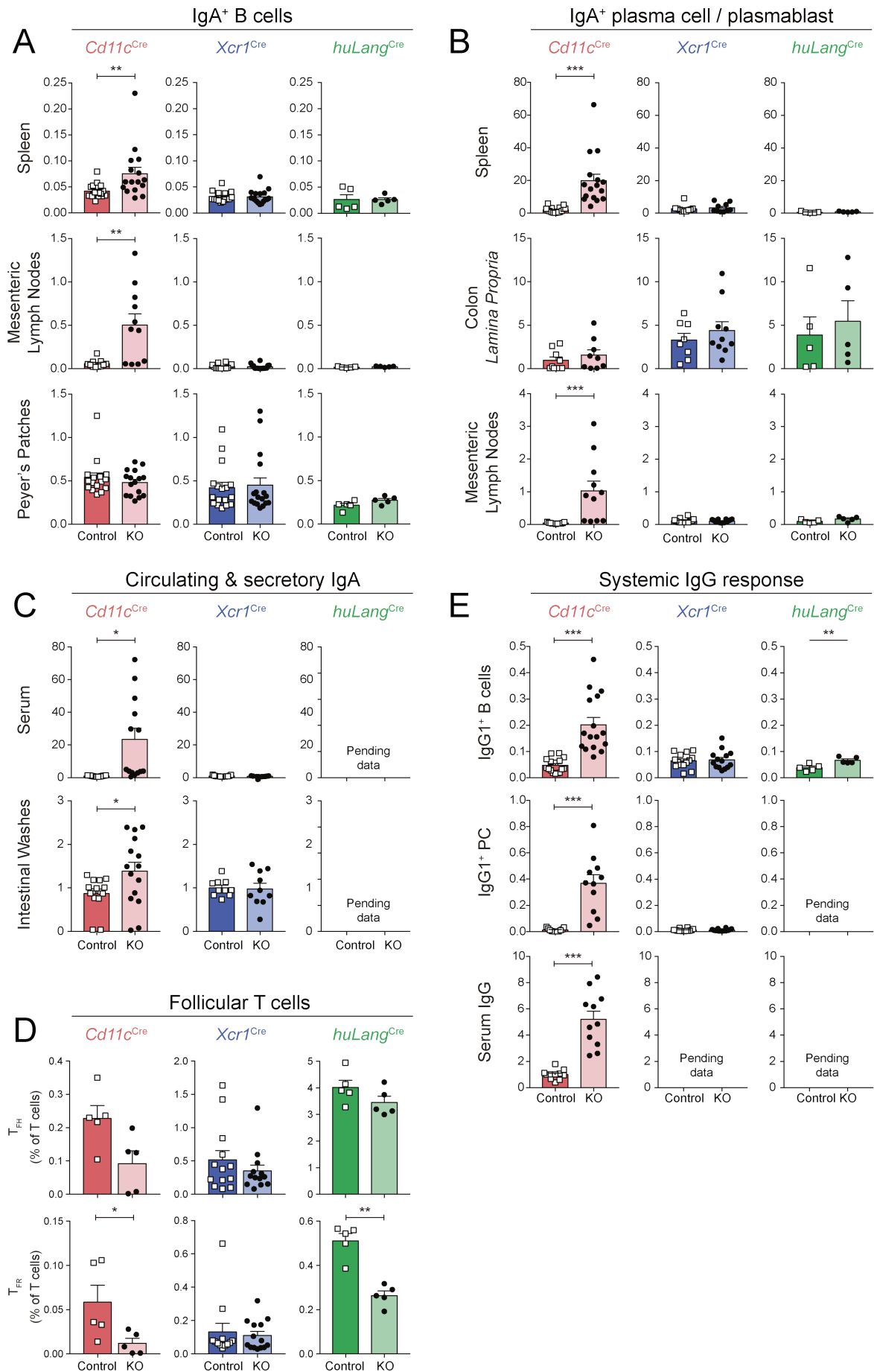
However, given the small but significant expression of  $\alpha_V\beta_8$  by MLN migratory cDC2 at steady state, we wanted to investigate the possibility that  $\beta_8$  expression by MLN cDC2 might license them for IgA CSR. We therefore analyzed in detail B cells responses of huLang- $\beta_8^{KO}$ , allowing the specific deletion of  $\beta_8$  in intestinal CD103<sup>+</sup>CD11b<sup>+</sup> MLN cDC2. We monitored IgA<sup>+</sup> B cells (Fig. 2.1A), plasma cells (Fig. 2.1B) and circulating Ig (Fig. 2.1C) and compared them to our previously shown on CD11c- $\beta_8^{KO}$  and  $Xcr1-\beta_8^{KO}$ .

We show here that huLang- $\beta_8^{KO}$  naïve mice have normal mucosal and systemic IgA response, for all the cells and compartment analyzed. The IgG1 B cell response however shows a small but significant increase compared to control littermates in huLang- $\beta_8^{KO}$  mice. This shows  $\beta_8$  on mucosal cDC2 is not required to promote the overall IgA response in the mucosal compartment.

cDC2 have been involved in the generation of follicular T cell (Krishnaswamy et al. 2018). Investigation of the follicular T cell compartment, namely follicular helper T cell ( $T_{FH}$ ) and follicular regulatory T cells ( $T_{FR}$ ) reveal that huLang- $\beta_8^{KO}$  mice, like CD11c- $\beta_8^{KO}$  have reduced  $T_{FR}$  and to some extent  $T_{FH}$  frequencies (Fig. 2.1D) in the MLN.

We show that overall mucosal IgA responses at steady state are not dependent on  $\alpha_V\beta_8$  expression by intestinal cDC2. CD11c- $\beta_8^{KO}$  and huLang- $\beta_8^{KO}$  display an impaired PP  $T_{FR}$  (and to some extent  $T_{FH}$ ) frequency. The process of  $T_{FR}$  generation and the requirement for DC and TGF $\beta$  is still unclear. Our results suggest that  $\alpha_V\beta_8$  expression by CD11c<sup>+</sup> phagocytes and cDC2 in particular, and likely their ability to activate and present active-TGF $\beta$ , could be one of the mechanisms promoting  $T_{FR}$  (and potentially  $T_{FH}$ ) generation. However, from these data we cannot say whether this phenotype is due to the impairment of *de novo*  $T_{FR}$  generation from naïve T cells, a consequence of the impairment of  $iT_{REG}$  in the mucosal organs or an impairment the ability of T cells to acquire the follicular phenotype.

Given the importance of  $T_{FR}$  in controlling the germinal center response (Fazilleau and Aloulou 2018), whether this impairment in  $T_{FR}$  frequency is responsible for the slightly dysregulated IgG (Fig. 2.1E) response in these mice still needs be fully investigated.



**Figure 2.1 - Mice with cDC1- or intestinal cDC2-specific  $\alpha_v\beta_8$  deletion display normal steady state mucosal IgA responses.**

(A-B) - Percentage of IgA<sup>+</sup> B cells (CD45<sup>+</sup>CD3<sup>+</sup>B220<sup>+</sup>CD19<sup>+</sup>CD138<sup>+</sup>IgA<sup>+</sup>) in (A) and number of plasma cells (SPL ( $\times 10^5$ ) & CLP ( $\times 10^3$ )) and frequency of plasmablast (MLN) (CD45<sup>+</sup>CD3<sup>+</sup>B220<sup>+</sup>CD138<sup>+</sup>IgA<sup>+</sup> as a % of CD45<sup>+</sup>) in (B) from indicated organs measured by flow cytometry.

(C) Serum and intestinal wash IgA levels in each mice model measured by ELISA. IgA absolute concentration is calculated from a standard and plotted as a fold-increase over the control.

(D) – Systemic IgG response in all mice model. Spleen IgG positive B cells and plasma cells were analyzed as described in (A-B) by flow cytometry. Circulating IgG were analyzed by ELISA.

(E) – T<sub>FH</sub> (CD3<sup>+</sup>CD4<sup>+</sup>FoxP3<sup>-</sup>CXCR5<sup>+</sup>PD1<sup>+</sup>) and T<sub>FR</sub> (CD3<sup>+</sup>CD4<sup>+</sup>FoxP3<sup>+</sup>CXCR5<sup>+</sup>PD1<sup>+</sup>) from PP analyzed by flow cytometry. Cell percentage are plotted as a percent of total CD4<sup>+</sup> T cells.

Data were collected from 1-4 independent experiments (n=7-15).

Statistical analysis: Mann-Whitney U test,  $\emptyset p > 0.05$ , \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001.



## Part 3 – Investigating the relevance of $\alpha_v\beta_8$ integrin expression by follicular T cell for the TGF $\beta$ -mediated control of B cell responses.

### Introduction

The development of a highly specific and high affinity antibody response is a critical component of the host immunity against pathogens. The differentiation of naïve B cells into plasma cells (PC) and memory B cells (mBC) is critical for the generation of an immune memory, protecting the host against re-infection. In their great majority, vaccines developed so far rely on production of PC and mBC to generate a long-lasting memory response. However, one of the challenges in vaccine development is the ability to generate an appropriate B cell memory response, i.e. not only conferring a long-lasting and broad-spectrum protection but also ensuring that antibodies (Ab) with the appropriate effector function (Ig class) are produced at the right anatomical site. Deeper understanding of the mechanisms involved in the generation of such a response is therefore critical to design new and more efficient vaccine formulation. It is also required to better understand how the pathological dysregulation of B cell response can lead to various pathologies such as lymphoma or autoimmune diseases. The Germinal Center Reaction (GCR). is a critical step of the T dependent B cell responses, which allows the generation of a highly amplified humoral response, of high affinity for the antigen and of an appropriate Ig-isotype. B cell interactions with follicular helper T cells ( $T_{FH}$ ) and follicular dendritic cells (FDC) respectively are critical for the regulation of the GCR. Recently, a new population of follicular T cells expressing Forkhead box P3 (FoxP3), called follicular regulatory T cells ( $T_{FR}$ ) was described.  $T_{FR}$  have been shown to exert both immunosuppressive function, as depletion of these cells leads to an exacerbated B cell response, and supportive functions, participating in the promotion of GC B cell responses (Fazilleau and Aloulou 2018). However to this day, no convincing consensus model has been proposed to describe  $T_{FR}$  origin, differentiation and the precise functions of these cells during the GCR (reviewed in (Stebegg et al. 2018)).

Cytokines presented by the GC 'helper cells' provide critical cues for B and T cell survival, activation and differentiation. The cytokine Transforming Growth Factor (TGF $\beta$ ) plays a key role in the regulation of immune responses. In the generation of humoral immunity, TGF $\beta$  is critical for the induction of IgA class-switch recombination (CSR) (Cazac and Roes 2000). However, TGF $\beta$  has also been involved in the regulation of other processes of B cell responses. For example, TGF $\beta$  has been shown to inhibit B cell proliferation and promote apoptosis *in vitro*. *In vivo*, TGF $\beta$ R<sup>KO</sup> B cells have reduced lifespan and display hyperresponsiveness to weakly immunogenic antigens (Lømo et al. 1995; Cazac and Roes 2000). Furthermore, within the scope of GC responses, it has been shown that pSMAD signaling is required for the migration of GC B cells from the light zone (LZ) to the dark zone (DZ) a cycle which is instrumental for improving their antigen-binding affinity (Albright et al. 2019).

The function of TGF $\beta$  in the biology of follicular T cells is still controversial. Conflicting evidence in mice report the inhibitory or stimulatory effect of TGF $\beta$  on  $T_{FH}$  differentiation and accumulation. *In vivo*, deletion of the TGF $\beta$ R2 in T cells leads to an accumulation of  $T_{FH}$ , suggesting that TGF $\beta$  exerts an immunosuppressive function on  $T_{FH}$  differentiation, proliferation or survival (McCarron and Marie 2014). On the contrary, a few studies report that TGF $\beta$  delivered by cDC2 and follicular macrophages could promote the generation of  $T_{FH}$  (Marshall et al.

2015; Schmitt et al. 2014; Durand et al. 2019). Additionally, conflicting evidence can be found regarding the requirement for TGF $\beta$  in the generation of T<sub>FR</sub>. McCarron and Marie show that T cell specific deletion of TGF $\beta$ R2 does not significantly impair the frequency of FoxP3<sup>+</sup>CXCR5<sup>+</sup>PD1<sup>+</sup> T<sub>FR</sub> while Miles and colleagues demonstrate the requirement of TGF $\beta$  signaling for the accumulation of T<sub>FR</sub> in the context of HIV infection (McCarron and Marie 2014; Miles et al. 2015). Overall, although all studies concur to show that TGF $\beta$  is a modulator of the GCR there is so far no unifying model explaining the apparently contradictory results reported in the literature regarding its function.

Unlike most cytokines, TGF $\beta$  is secreted in a latent form. This inactive TGF $\beta$  is encapsulated in a Latent Associated Peptide (LAP) that prevents binding to its receptor. Activation of latent TGF $\beta$  is therefore required to induce TGF $\beta$  signaling. In the immune system, alpha-v ( $\alpha_v$ ) integrins are key in this process (Worthington, Klementowicz, and Travis 2011; Worthington et al. 2012). Previously, it has been shown that  $\alpha_v\beta_8$  expression by dendritic cells (DC) and T<sub>REG</sub> is critical for the regulation of intestinal homeostasis (Travis et al. 2007; Lacy-Hulbert et al. 2007; Worthington et al. 2015).  $\alpha_v\beta_8$  expression by CD11c<sup>+</sup> phagocytes is involved in the generation of intestinal ROR $\gamma$ t<sup>+</sup> T<sub>REG</sub> and T<sub>H</sub>17 as well as helping the promotion of IgA responses in response to rotavirus infection (Acharya et al. 2010; Melton et al. 2010; Boucard-Jourdin et al. 2016; Nakawesi et al. 2020). Furthermore, T<sub>REG</sub> expression of  $\alpha_v\beta_8$  is critical for their ability to inhibit inflammatory T cell responses in the intestinal *lamina propria* (Worthington et al. 2015) (also see Part 1 of this manuscript).

The importance of TGF $\beta$ -activation in the regulation of the GCR has however been poorly studied. We hypothesized that mechanisms of TGF $\beta$ -activation and the spatiotemporal presentation of activated TGF $\beta$  to GC B cells or follicular T cells and the context in which it is presented (i.e. which cell is activating and presenting TGF $\beta$ ) might be critical to regulate the GCR and could underlie the pleiotropic functions of this cytokine on GC immune cells.

Focusing on the role  $\alpha_v\beta_8$  integrin in this study, we took advantage of our newly established reporter mouse model for *Itgb8* expression (*Itgb8*-tdTomato) to investigate the expression of  $\beta_8$  by GC lymphoid and stromal cells. We show that a proportion of T<sub>FH</sub> and virtually all T<sub>FR</sub> express the  $\alpha_v\beta_8$  integrin and that this expression is modulated throughout the GCR. However, the conditional KO of  $\alpha_v\beta_8$  on all T cells (*Cd4*<sup>Cre</sup>.*Itgb8*<sup>flox/flox</sup>) does not modify the breadth of the primary GCR to model antigens. Some preliminary experiments indicate that the generation of antigen specific PC might be blunted in  $\alpha_v\beta_8$  deficient animals leading to subsequent defects in the recall response to the same antigen. At the end of the study, we discuss our plan to further investigate the requirement for  $\alpha_v\beta_8$  expression by follicular T cells in the control of the GC.

## Methods

### Mice & Treatments

All animals were housed under specific pathogen-free conditions at Plateau de Biologie Expérimentale de la Souris (Lyon, France) under appropriate licenses within local and national guidelines for animal care. All mice were on C57Bl/6 background. Age- and sex-matched littermate controls were used for all experiments. Male and female mice were used.

Transgenic Tg(Cd4-cre)1Cwi mice (CD4<sup>Cre</sup>) were and *Itgb8*<sup>flox</sup> mice were obtained from Julien Marie (CRCL, France). B6.129(Cg)-Foxp3<sup>tm4(YFP/cre)Ayr/J</sup> (FoxP3<sup>Cre</sup>) and *Bcl6*<sup>flox</sup> (Hollister et al. 2013) mice were obtained from Nicolas Fazilleau (CPTP, France).  $\beta$ 8 integrin-reporter mice (*Itgb8-TdTomato*, (Nakawesi et al. 2020), Fig. 1A) were crossed with *C.Cg-Foxp3<sup>tm2Tch</sup>/J* mice (FoxP3-GFP) to generate *Itgb8*-tdTomato.Foxp3-GFP mice. Where indicated, mice were subcutaneously immunized with 100 $\mu$ g NP-KLH (LGC Biosearch Technologies) mixed with CFA (Sigma-Aldrich) at a 1:1 ratio. CT immunization was performed with 1 $\mu$ g CT (List Biological Labs, Inc.) in PBS.

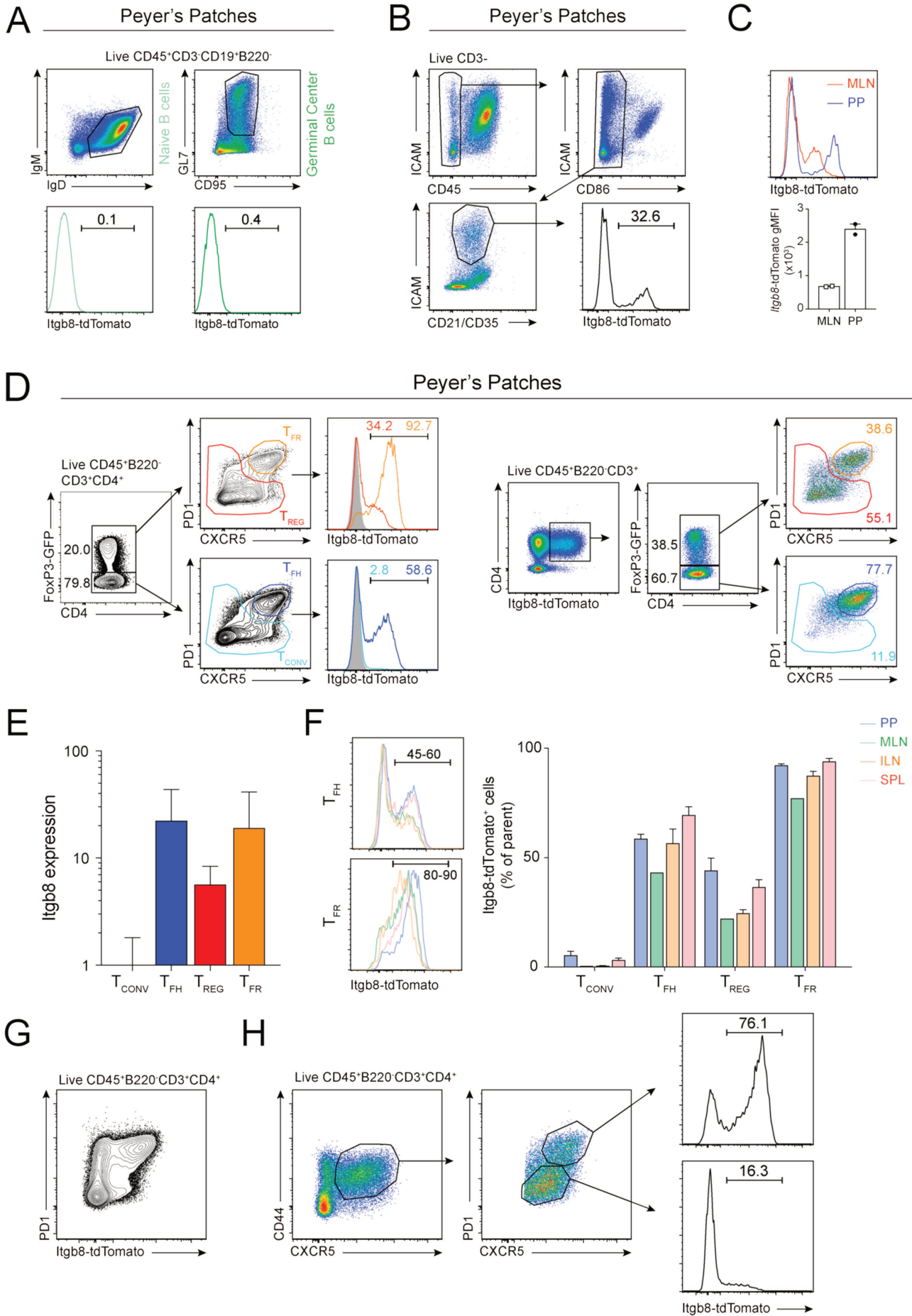
### Cell isolation & Flow Cytometry

For the analysis of lymphocyte population, single-cell suspensions from spleen, LN, and Peyer's Patches were generated by mechanical disruption through 70 $\mu$ m cell strainer in Complete RPMI buffer (10% FBS, 1% Pen/Strep (Gibco), 1% HEPES (Gibco) and 0.1%  $\beta$ -mercaptoethanol (Gibco)). Follicular DC were isolated by enzymatic digestion with 1mg/mL Collagenase IA (Sigma) and 100 $\mu$ g/mL DNase 1 (Roche) in FBS-free RPMI. NP-PE and NIP-FITC (LGC Biosearch Technologies) were used to detect NP-specific B cells and PC respectively. For the detection of CT-specific T cells, custom MHC-II tetramers were generated using the NIH Tetramer Core Facility. This peptides recognizes the highly immunodominant NNKTPHAIAAIS peptide from the CTB subunit (Cong, Bowdon, and Elson 1996; Nelson et al. 2015).

Data acquisition was performed using LSR-Fortessa cytometers while DC subsets were sorted using an BD FACSAriaII cytometer (BD Biosciences). Data analysis was performed using FlowJo software v9 (Treestar Inc).

### ELISA & ELISPOT

Serum was obtained by centrifugation of blood from intra-cardiac puncture following Ketamine & Xylazine anesthesia. ELISA was performed as previously described (Blanc et al. 2016). Briefly, 0.15 $\mu$ g/mL NP(25)-BSA (Biosearch Technologies) was coated on Maxisorp plates (ThermoFischer), serially-diluted serums were incubated for 2h at RT and revealed using anti-IgG (SouthernBiotech) antibody conjugated to AP and Alkaline Phosphatase Substrate (Sigma). For affinity ELISA, additional plates were coated with NP(2)-BSA (Biosearch Technologies) for all samples. The antibody affinity is defined as the ratio of the NP(2) over NP(25) titer. Antibody titers were calculated by modeling the measure OD405 of a sample dilution as a 4-parameter curve and mathematically extrapolating the concentration at which the curve exceeds a predefined threshold value. For ELISPOT, 2 $\mu$ g/mL NP-BSA was coated on Maxisorp plates (ThermoFischer) after activation of the plates according to the manufacturer instructions. BM cells were isolated by flushing with a complete RPMI solution. The Number of Live CD45<sup>+</sup> cells were counted using a C6 Accuri cytometer (BD Biosciences) and serially diluted cells were plated and incubated for 20-24 hours. Spots were then revealed using anti-IgG (SouthernBiotech) antibody conjugated to AP and BCIP/NBT substrate (Sigma).



### **Figure 3.1 - $\alpha_V\beta_8$ is expressed by a subset of follicular dendritic cells and follicular T cells.**

(A-D) – PP cells from *Itgb8*-tdTomato.*Foxp3*-GFP mice were isolated and analyzed by flow cytometry. Naïve and germinal center B cells (A), Follicular Dendritic Cells (B-C), and the various follicular T cell populations (D) were gated as indicated and analyzed for tdTomato fluorescence. Numbers represent the frequency of the gated population as a percent of the parent gate. Cells are pregated as indicated on the figure. Histogram in (C) overlays the fluorescence of FDC extracted from MLN or PP. Geometric mean fluorescence intensity of tdTomato in *Itgb8*<sup>+</sup> FDC is plotted in the graph below. n=2 from one experiment.

(E) PP T cells were sorted as indicated in (D) and *Itgb8* expression was analyzed by RT-qPCR. Data was normalized to *Gapdh* expression and to T<sub>CONV</sub> expression levels in each independent experiment. n=4 from 2 independent experiments

(F) – Overlaid histograms (left) and quantification (right) of tdTomato fluorescence in mucosal (PP & MLN) and systemic (SPL and inguinal LN) organs of *Itgb8*-tdTomato.*Foxp3*-GFP mice. Numbers represent the frequency of the gated population as a % of the parent gate. The bar graph represents the frequency of  $\beta_8$ -expressing cells in each population and each organ. n=3-5 from 2 independent experiment

(F-G) – Analysis of tdTomato fluorescence in total T cells (F) and CD4<sup>+</sup>FoxP3<sup>-</sup> T<sub>FH</sub> from PP of *Itgb8*-tdTomato.*Foxp3*-GFP mice. Cells are pregated as indicated

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### **Genomic PCR and RT-qPCR analysis of $\beta_8$**

Follicular T cells were sorted by flow cytometry. DNA and RNA were extracted using Proteinase K or Trizol/Chloroform (as previously described in (Boucard-Jourdin et al. 2016)) extraction respectively.

RT-qPCR of *Itgb8* expression was performed as previously described (Boucard-Jourdin et al. 2016) and normalized to *Gapdh* expression. Analysis of recombination at the *Itgb8* locus was performed by PCR of genomic DNA using the following set of primers: 5'-CCC ACT AAG ATA ACT GGC CGT ATC-3', 5'-GAG GGG TGG GGA AAT TTT TGT ATC-3', 5'-GTG GAT TCT ACA GGC AAG C-3'.

### **Statistics**

Statistical significance was evaluated as indicated in each figure legend using GraphPad Prism software.

## **Results**

### **$\alpha_V\beta_8$ is expressed by a subset of follicular dendritic cells and follicular T cells.**

To determine whether  $\alpha_V\beta_8$ -mediated TGF $\beta$  activation could be involved in the regulation of the GCR, we first analyzed  $\beta_8$  expression profile in Peyer's Patches (PP), where GC responses develop spontaneously due to chronic stimulation from the microbiota and other intestinal products. Using *Itgb8*-tdTomato, we first show that  $\beta_8$  integrin is expressed neither by naïve B cells (IgM<sup>+</sup>IgD<sup>+</sup>) nor by GC B cells (IgD<sup>-</sup>CD95<sup>+</sup>GL7<sup>+</sup>) (Fig. 3.1A). Any modulation of the GCR by GCB cells through  $\alpha_V\beta_8$ -mediated TGF $\beta$ -activation is therefore unlikely.

We next investigated  $\beta_8$  expression by CD45<sup>-</sup>CD86<sup>+</sup>ICAM1<sup>+</sup>CD21<sup>+</sup>/CD35<sup>+</sup> FDC. We show that 32% of FDC in PP express  $\alpha_V\beta_8$  integrin (Fig. 3.1B). Furthermore, the analysis of FDC isolated from Mesenteric Lymph Nodes (MLN) shows that this expression pattern is conserved in other intestinal lymphoid organs. Preliminary investigation of spleen FDC seems to indicate that they do not express  $\beta_8$  integrin suggesting that  $\beta_8$  expression is restricted to mucosal FDC.

Worthington *et al*, have previously demonstrated that  $\alpha_V\beta_8$  expression by T<sub>REG</sub> and especially activated T<sub>REG</sub> is required for suppressing active inflammation (Worthington et al. 2015). Other studies have further demonstrated that human T<sub>REG</sub> express  $\beta_8$  integrin which is required to activate GARP-bound latent-TGF $\beta$  and for T<sub>REG</sub> to

exert their TGF $\beta$ -dependent immunosuppressive function (Worthington et al. 2015; Edwards, Thornton, and Shevach 2014; Stockis et al. 2017). However,  $\beta 8$  expression pattern in other T cell subsets and in particular in follicular T cells is currently unknown. Here, using *Itgb8*-tdTomato.*Foxp3*-GFP mice, we analyzed expression of  $\beta 8$  integrin at the single cell level by flow cytometry in FoxP3<sup>+</sup>CXCR5<sup>+</sup>PD1<sup>+</sup> T<sub>FH</sub> and FoxP3<sup>+</sup>CXCR5<sup>+</sup>PD1<sup>+</sup> T<sub>FR</sub>, Foxp3<sup>+</sup>CXCR5<sup>+</sup>PD1<sup>-</sup> T<sub>REG</sub> and FoxP3<sup>-</sup>CXCR5<sup>+</sup>PD1<sup>-</sup> conventional T cells (T<sub>CONV</sub>).

We show that PP T<sub>REG</sub>, T<sub>FH</sub> and T<sub>FR</sub> but not T<sub>CONV</sub> express  $\beta 8$  (Fig. 3.1C). Indeed, while 38% of  $\beta 8$ <sup>+</sup> T cells are FoxP3<sup>+</sup> cells (comprising both T<sub>REG</sub> and T<sub>FR</sub>),  $\alpha_v\beta 8$  expression on FoxP3<sup>-</sup> cells is restricted to T<sub>FH</sub> (Fig. 3.1D). Importantly while 50% of T<sub>FH</sub> are *Itgb8*-tdTomato<sup>+</sup>, virtually all T<sub>FR</sub> (~90-95%) express  $\beta 8$  integrin. Preferential expression *Itgb8* in follicular T cells was confirmed by RT-qPCR on sorted cell populations, with T<sub>FH</sub> and T<sub>FR</sub> displaying a one-log higher expression level than T<sub>REG</sub> (Fig. 3.1E).

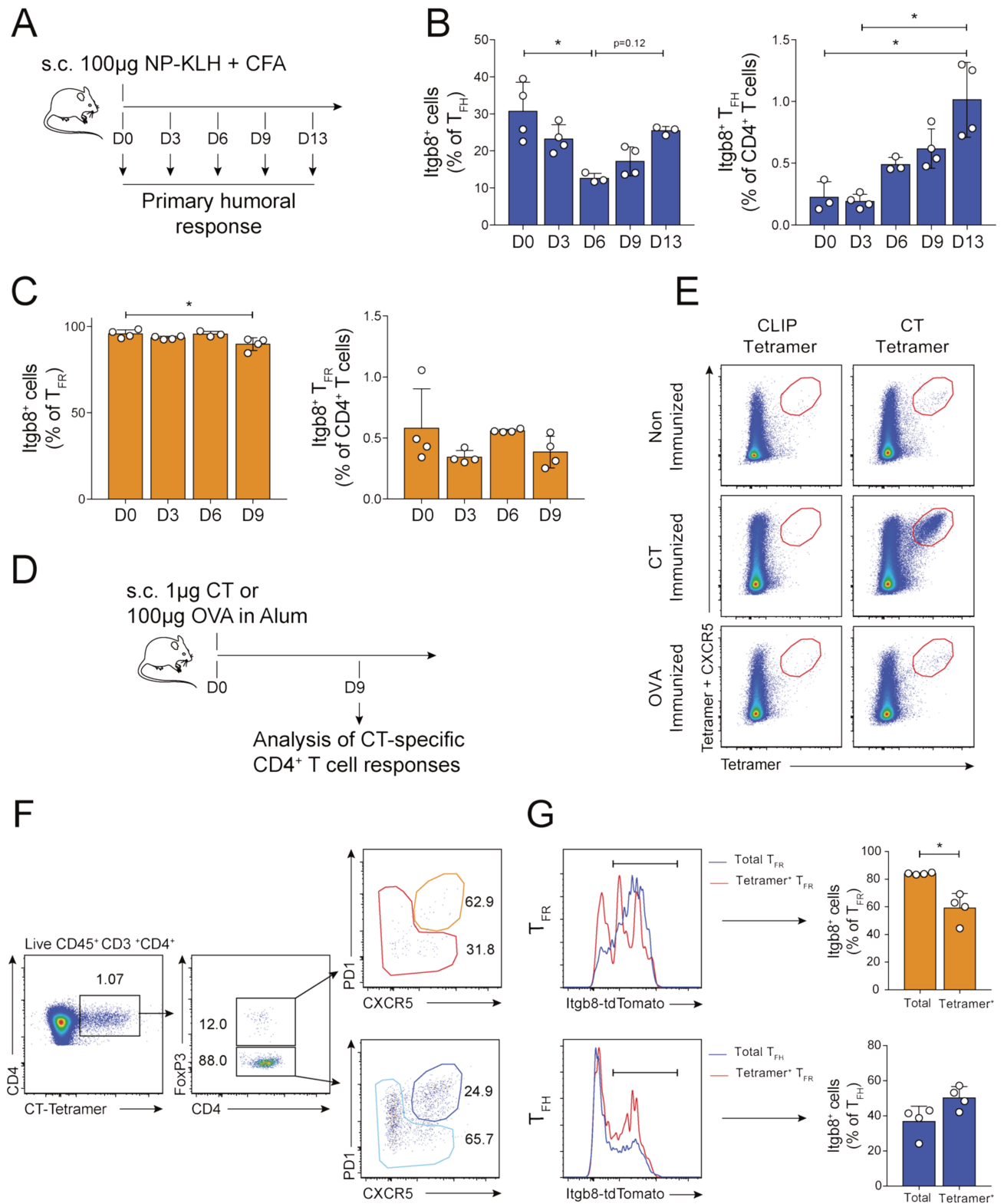
We next asked whether  $\beta 8$  was also expressed by T cells in MLN and non-mucosal SLO, such as the inguinal LN and spleen (SPL). Here, we show that in all SLO analyzed,  $\beta 8$  integrin is expressed by T<sub>FH</sub> and T<sub>FR</sub> population, with similar percentage of *Itgb8*-tdTomato<sup>+</sup> T<sub>FH</sub> and T<sub>FR</sub> in PP, MLN, inguinal LN and spleen (Fig. 3.1F). This strongly suggests that  $\alpha_v\beta 8$  on T<sub>FH</sub> and T<sub>FR</sub> might be important in regulating the GCR not only in the GALT but in all SLO, independently of mucosa specific cues.

### *$\alpha_v\beta 8$ is preferentially expressed by PD1<sup>hi</sup> T<sub>FH</sub> and is acquired throughout the GC reaction*

It has previously been shown that  $\beta 8$  is preferentially expressed by activated T<sub>REG</sub> and upregulated following *in vitro* activation (Worthington et al. 2015), We therefore hypothesized that  $\alpha_v\beta 8$  expression by T<sub>FH</sub> could be correlated to their activation or differentiation stage and therefore be modulated throughout the GCR, from the initial generation of T<sub>FH</sub> from naïve T cells to their terminal differentiation into memory T<sub>FH</sub>. In T cells, PD1 molecule has been characterized as an exhaustion markers on highly activated T cells (Wherry and Kurachi 2015), while it is crucial for T<sub>FH</sub> and T<sub>FR</sub> localization in the GC and GC affinity selection (Shi et al. 2018; Sayin et al. 2018; Trüb et al. 2017). In PP where there is chronic GCR, we show that *Itgb8*-tdTomato fluorescence is positively correlated with PD1 expression in follicular T cells (Fig. 3.1G). Furthermore, PD1<sup>hi</sup> T<sub>FH</sub> have a higher proportion of *Itgb8*-tdTomato expressing cells than their PD1<sup>low</sup> counterparts (Fig. 3.1H).

To explore the dynamics of  $\beta 8$  expression during the GC, we investigated  $\alpha_v\beta 8$ -expression pattern in follicular T cell populations following sub-cutaneous immunization with NP-KLH in Complete Freund's Adjuvant (CFA) (Fig. 3.2A). We show that NP-KLH immunization is associated with a transient 50% decrease in the proportion of  $\beta 8$ <sup>+</sup> cells among T<sub>FH</sub> (Fig. 3.2B – left). However, the frequency (and most likely the number) of  $\alpha_v\beta 8$ -expressing T<sub>FH</sub> increases with the development of the GC reaction (Fig. 3.2B – right). On the contrary, the total frequency of  $\beta 8$ <sup>+</sup> T<sub>FR</sub> is stable throughout the GCR (Fig. 3.2C – right), even though a small but significant 5% decrease in the proportion of  $\beta 8$ <sup>+</sup> cells in T<sub>FR</sub> can be observed (Fig. 3.2B – left).

One of the hypotheses to explain the transient decrease of the proportion of  $\beta 8$ <sup>+</sup> T<sub>FH</sub> while the GC develops, is that, in the early development of the GCR, newly differentiated T<sub>FH</sub> arising from naïve  $\beta 8$ <sup>-</sup> T cells progressively acquire  $\beta 8$  expression.



**Figure 3.2 -  $\alpha$ v $\beta$ 8 expression by T<sub>FH</sub> is modulated throughout the germinal center reaction**

Itgb8-tdTomato mice were immunized subcutaneously with NP-KLH in CFA (A-D) or CT (E-G) and tdTomato fluorescence by T<sub>FH</sub> and T<sub>FR</sub> was analyzed by flow cytometry at various times following immunization.

(A-C) – Analysis of T<sub>FH</sub> (B) and T<sub>FR</sub> (C) expression of  $\alpha$ v $\beta$ 8 in the draining LN at various timepoints following immunization. Data is either plotted as a percent of total T<sub>FH</sub> or T<sub>FR</sub> (left) or as a percent of CD4<sup>+</sup> T cells (right). n=3-4 from 1 experiment. Statistical analysis was performed using a Kruskal Wallis test with multiple comparison followed by a Dunn's multiple analysis correction.

(D-E) – Control mice were immunized with CT or OVA in Alum subcutaneously and analyzed with a custom generated MHC tetramer to detect CT-specific cells in the draining LN 10 days later. The CLIP tetramer is used as a negative control for non-specific binding of the MHC tetramer backbone.

(F-G) – Mice were immunized subcutaneously with CT and draining LN were analyzed 9 days later. Staining for T<sub>FH</sub> and T<sub>FR</sub> was realized and *Itgb8*-tdTomato fluorescence was analyzed. (G) – Representative dot plots and bar graphs indicating the frequency of total or CT-specific *Itgb8*<sup>+</sup> T<sub>FH</sub> or T<sub>FR</sub> among their parent population. Statistical analysis was performed using a Mann-Whitney U test.

Each data point represents an individual animal. <sup>o</sup>p>0.05, \*p < 0.05.

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In order to follow  $\beta 8$  expression during the differentiation and maturation, we choose to immunize *Itgb8*-tdTomato mice with cholera toxin (CT) for which we could use an MHCII-restricted tetramer specific for an immune-dominant CT peptide (Cong, Bowdon, and Elson 1996) to follow newly generated antigen-specific follicular T cells, rather than total polyclonal T<sub>FH</sub> responses (Fig. 3.2D). We confirm that this CT-specific tetramer allows the detection of *de novo* generated T cells from mice immunized by CT but not in non-immunized animals or mice immunized with another antigen such as OVA. The CLIP control tetramers confirm the absence of non-specific binding (Fig. 3.2E).

9 days after CT immunization of *Itgb8*-tdTomato mice, Tetramer<sup>+</sup> T cells can be found in the draining lymph nodes and represent around 1% of CD4<sup>+</sup> T cells. The vast majority (88%) of Tetramer<sup>+</sup>CD4<sup>+</sup> T cells are FoxP3<sup>-</sup> and among these 25% have committed to the T<sub>FH</sub> lineage (0.2% of total CD4<sup>+</sup> T cells) (Fig. 3.2F). We further show that the *Itgb8*-tdTomato fluorescence of these Tetramer<sup>+</sup> T<sub>FH</sub> is very similar to that of total T<sub>FH</sub>. Additionally, the frequency of  $\beta 8$ <sup>+</sup>Tetramer<sup>+</sup> T<sub>FH</sub> is similar to the frequency of total  $\beta 8$ <sup>+</sup> T<sub>FH</sub> (Fig. 3.2G).

Furthermore, we show that CT immunization allows the detection of CT-specific T<sub>FR</sub>. Although in low quantity at this particular timepoint, we show that *Itgb8*-tdTomato fluorescence can be detected in these cells albeit at a slightly lower frequency (60%) than total T<sub>FR</sub>.

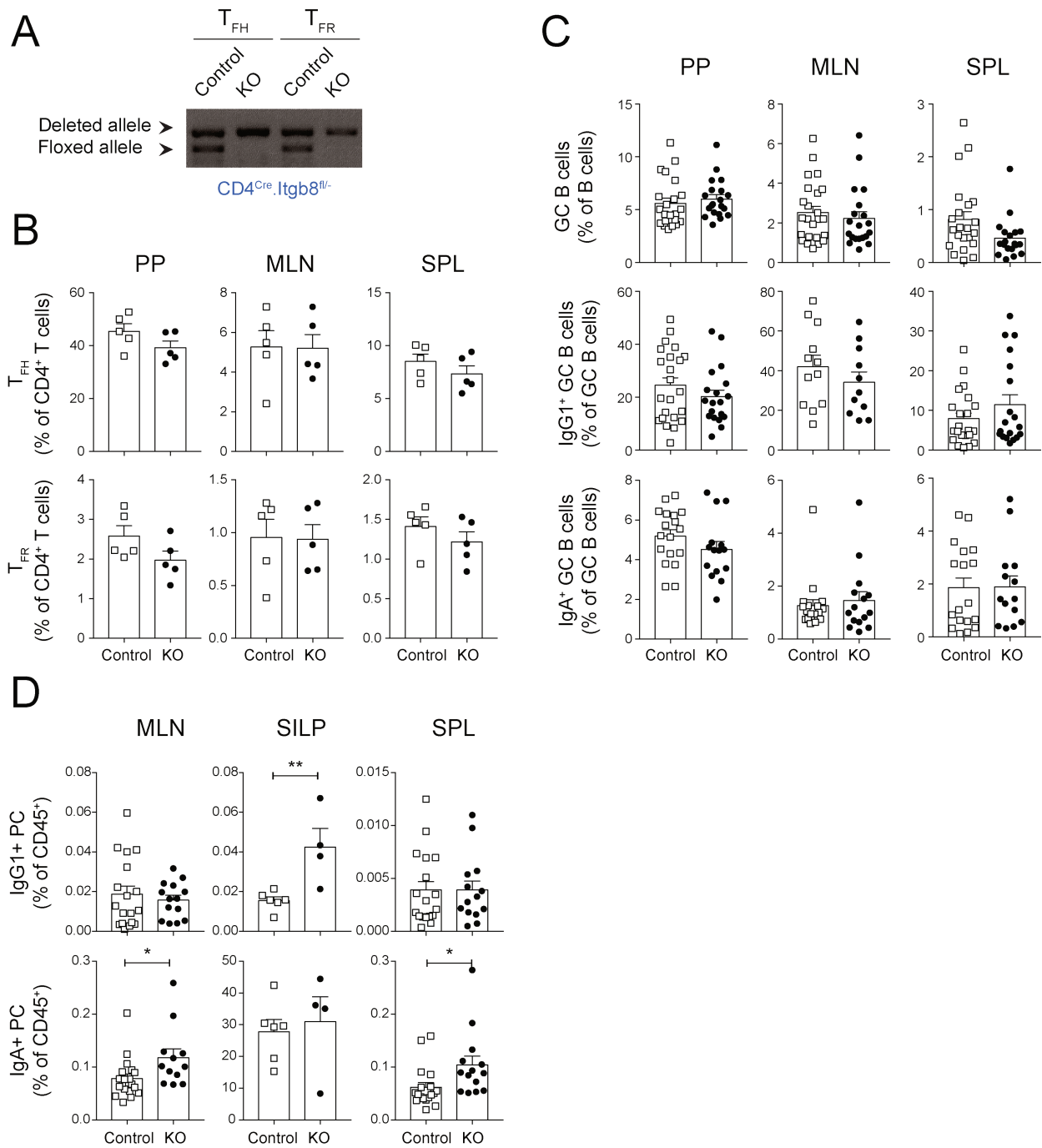
Altogether we describe for the first time 1/ the expression of  $\alpha \nu \beta 8$  by 40-50% of T<sub>FH</sub> during the GCR, 2/ the expression of  $\alpha \nu \beta 8$  by most T<sub>FR</sub> and 3/ the progressive accumulation of  $\alpha \nu \beta 8$ -expressing T<sub>FH</sub> throughout the GC. Furthermore, we bring the proof of concept that, following CT immunization, we are able to follow CT-specific T<sub>FH</sub> and T<sub>FR</sub>. Here CT, acts both as antigen and self-adjuvant.

The expression of TGF $\beta$ -binding and activating molecule by T<sub>FR</sub> had been previously described in the literature, showing that T<sub>FR</sub> generated in the MLN or following immunization express *Itgb8* and GARP (Wing et al. 2017; Sayin et al. 2018). However, the functional importance of  $\alpha \nu \beta 8$  expression on T<sub>FR</sub> has never been investigated to our knowledge.

### *T cell expression of $\alpha \nu \beta 8$ is not required for homeostatic B cell responses*

To investigate the relevance of  $\alpha \nu \beta 8$  expression by T<sub>FH</sub> and T<sub>FR</sub> *in vivo* for the control of GC B cell responses, we first analyzed natural B cell responses (i.e. in the absence of deliberate immunization) in mice with a selective ablation of  $\beta 8$  in T cells (CD4- $\beta 8$ <sup>KO</sup>). In this mouse model, the effective genomic deletion of *Itgb8* on T<sub>FH</sub> and T<sub>FR</sub> was verified by genomic PCR on sorted T<sub>FH</sub> and T<sub>FR</sub> (Fig. 3.3A).





**Figure 3.3 - T cell expression of  $\alpha_v\beta 8$  is not required for homeostatic B cell responses**

(A) – Genomic PCR analysis of the *Itgb8* locus in sorted T<sub>FH</sub> and T<sub>FR</sub> from CD4- $\beta 8^{\text{KO}}$  or CD4- $\beta 8^{\text{WT}}$  mice.  
 (B-E) – CD4- $\beta 8^{\text{KO}}$  antibody concentration (E) and mucosal immune responses (B-D) were monitored by ELISA or flow cytometry respectively. Frequency of T<sub>FH</sub> and T<sub>FR</sub> (B) and total, IgA<sup>+</sup> or IgG1<sup>+</sup> GC B cells (C) and PC (D) responses are plotted as a percentage of T cells, B cells, GC B cells or CD45<sup>+</sup> cells respectively in the indicated organs. Each data point represents an individual animal. Statistical analysis was performed using a Mann-Whitney U test.  $\emptyset p > 0.05$ , \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

TGF $\beta$  signaling is involved in the differentiation and accumulation of T<sub>FH</sub> and T<sub>FR</sub> (Schmitt et al. 2014; McCarron and Marie 2014; Marshall et al. 2015). We thus sought to first determine whether  $\alpha_V\beta 8$ -mediated activation by T cells could be involved in T<sub>FH</sub> and T<sub>FR</sub> development. In PP, MLN and SPL of CD4- $\beta 8^{KO}$  mice, we show that the proportion of T<sub>FH</sub> and T<sub>FR</sub> population is not significantly modified (Fig. 3.3B). Hence, any further effect of  $\beta 8$  deletion in T cell cannot be attributed to a defect in T<sub>FH</sub> or T<sub>FR</sub> population frequencies.

We next investigated the “spontaneous” B cell responses of CD4- $\beta 8^{KO}$  mice and we show that neither the frequencies of spontaneous GC in intestinal (MLN and PP) and non-mucosal (SPL) compartment nor the rate of Ig isotype switching in these GC (Fig 3.3C) is modified by deletion of  $\beta 8$  in all T cells. As a whole, IgG1<sup>+</sup> and IgA<sup>+</sup> PC response are not severely modified CD4- $\beta 8^{KO}$  mice (Fig 3.3D); although we found a small but significant increase in the frequencies of IgA<sup>+</sup> PC in MLN and SPL and of IgG1<sup>+</sup> PC in SILP of CD4- $\beta 8^{KO}$  mice as compared to control littermates.

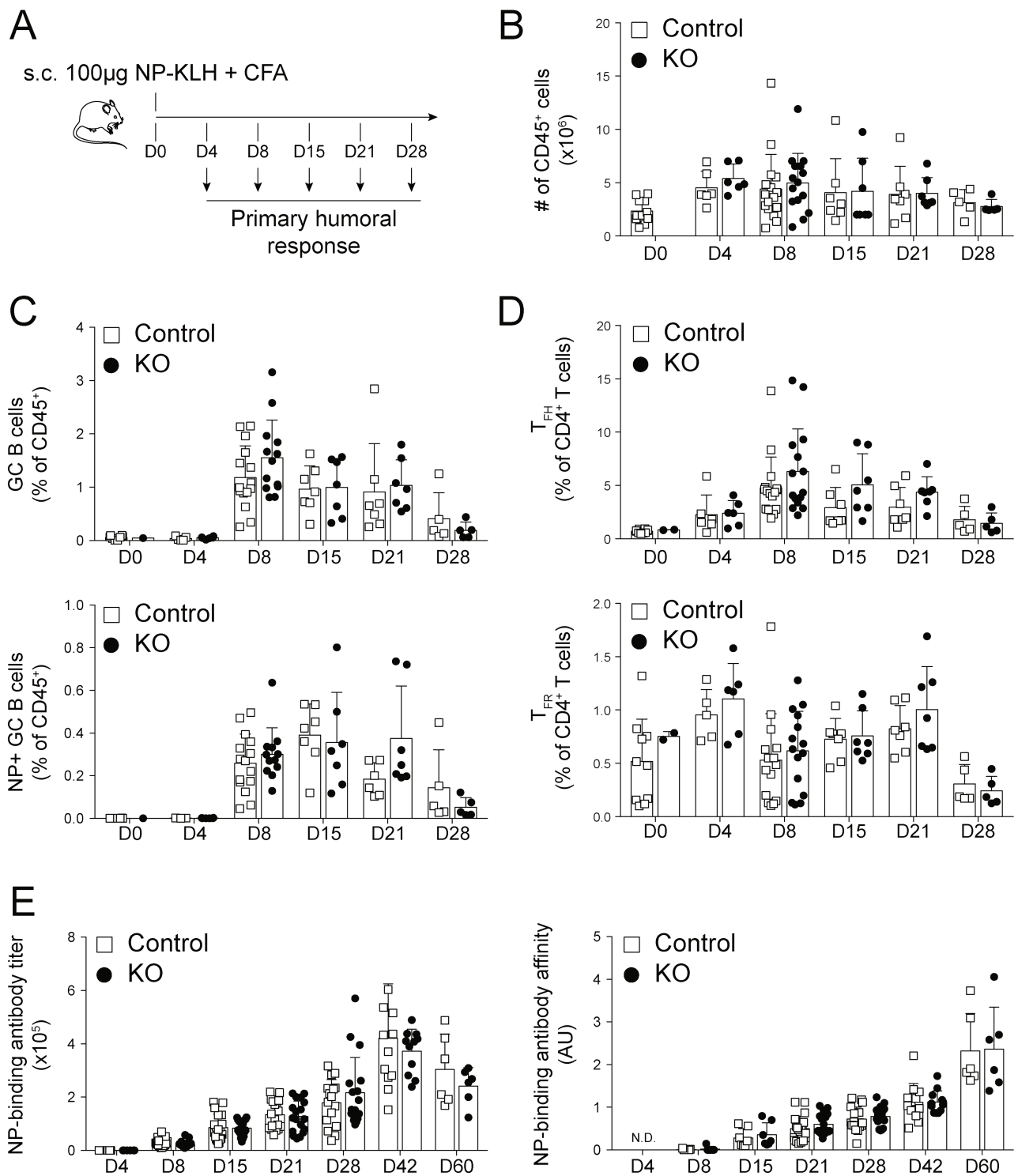
Overall, these data show that  $\beta 8$  expression by T cells is not required for homeostatic humoral responses which is consistent with the normal serum IgG, IgE and IgA levels found in these mice (Travis et al. 2007).

### *Itgb8 deletion in T cells does not impacts the primary GC reaction to model antigens*

Homeostatic mucosal IgA B cell responses are the result of both T-dependent and T-independent activation of B cells, generating both GC and extrafollicular responses. To specifically probe the role of  $\alpha_V\beta 8$  on T cells in the course of a T cell-dependent humoral response, we therefore chose to explore the outcome of T cell-targeted invalidation of  $\beta 8$  on the response to the prototypic hapten-carrier T-dependent antigen NP-KLH. This model immunogen, adjuvanted with CFA, was chosen for its ability to generate strong NP-specific B cell response for which many tools are available to detect their antigen-specificity (Wolniak, Noelle, and Waldschmidt 2006; Lalor et al. 1992).

CD4- $\beta 8^{KO}$  mice were subcutaneously immunized with NP-KLH and B cell responses were analyzed at different time points of the GCR (Fig. 3.4A). We show that the total number of CD45<sup>+</sup> cells is not affected by  $\beta 8$  deletion in T cells (Fig. 3.4B), indicating that there is no exacerbation of immune response in these mice. Similarly, and to our surprise, neither the total or the NP-specific GC B cell (Fig. 3.4C) nor the polyclonal T<sub>FH</sub> and T<sub>FR</sub> frequencies (Fig. 3.4D) are modified following immunization of CD4- $\beta 8^{KO}$  mice compared to the control littermates. Accordingly, the concentration of secreted antibodies and their affinity to the antigen are not disturbed by *Itgb8* deletion (Fig. 3.4E).

Overall, we show that deletion of  $\beta 8$  in all T cells does not significantly impact the amplitude or quality of the primary GC responses to a model antigen.



**Figure 3.4 - *Itgb8* deletion on T cells does not impacts the primary GC reaction to model antigens.**

CD4- $\beta$ 8<sup>KO</sup> mice and their control littermates were immunized subcutaneously with NP-KLH in CFA and analyzed according to the protocol in (A). Number of total Live CD45<sup>+</sup> cells (B), total and NP-specific GC B cells (C), T<sub>FH</sub> and T<sub>FR</sub> (D) and antibody concentration and affinity (E) were monitored by flow cytometry or ELISA at different timepoints after immunization. Each data point represents an individual animal. Statistical analysis was performed using a Kruskal Wallis test with multiple comparison followed by a Dunn's multiple analysis correction. \* $p > 0.05$

### Antigen-specific memory PC are slightly blunted in CD4- $\beta$ 8KO mice

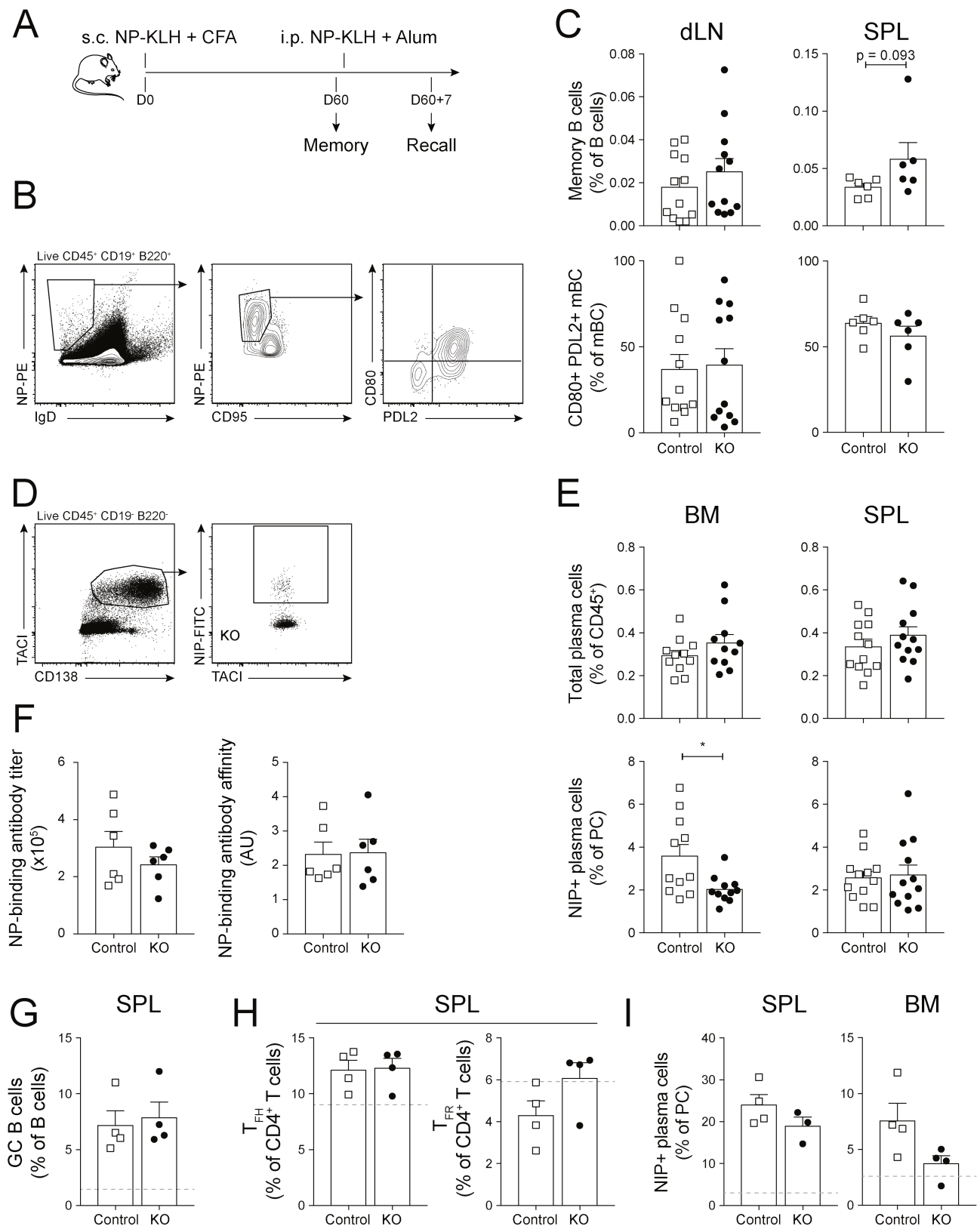
B cell interaction with follicular T cells in the GC is not only important for their selection and proliferation, it is also an important factor for B cell fate selection, and in particular the decision to differentiate into memory B cell (mBC) or PC. We therefore sought to investigate the ability of CD4- $\beta$ 8<sup>KO</sup> mice to generate functional memory responses to NP-KLH. To do so, we immunized mice with NP-KLH in CFA and measured the NP-specific memory response at day 60 following primary immunization or 7 day after recall by intra-peritoneal immunization with NP-KLH in Alum (Fig. 3.5A).

In our experimental setting NP-specific mBC were defined and gated as Ag-experienced NP-binding B cells, i.e. CD19<sup>+</sup>CD95<sup>low</sup>IgD<sup>-</sup>NP<sup>+</sup> B cells. We show that total mBC frequency at day 60 is not affected by  $\beta$ 8 deletion in T cells (Fig. 3.5B-C). Recent publications have established that the CD80 and PDL2 markers on mBC allows to distinguish between at least two functionally distinct mBC populations. CD80<sup>+</sup>PDL2<sup>+</sup> mBC have been shown to be poised for PC differentiation while CD80<sup>-</sup>PDL2<sup>-</sup> mBC are preferentially readdressed to the GC following reencounter with their antigen (Zuccarino-Catania et al. 2014). Here we show that the frequencies of the PC-committed (CD80<sup>+</sup>PDL2<sup>+</sup>) (Fig. 3.5C) and GC-committed (CD80<sup>-</sup>PDL2<sup>-</sup>) mBC (not shown) are not significantly modified in CD4- $\beta$ 8<sup>KO</sup> animals.

No differences were observed between control and  $\beta$ 8-deficient animals regarding the frequencies of polyclonal and NP-specific PC in the SPL. However, we show a 1.5-fold reduction of NP-specific (NIP<sup>+</sup>CD138<sup>+</sup>TACI<sup>+</sup>) Bone Marrow (BM) PC in CD4- $\beta$ 8<sup>KO</sup> mice as compared to their control littermates (Fig. 3.5D-E). Because of the very low number of antigen-specific memory cells, the detection of memory responses can be variable. This observation would therefore deserve to be repeated for further confirmation. The overall NP-specific antibody titers and affinity were however not modified at day 60 (Fig. 3.5F).

We next investigated the ability of previously immunized CD4- $\beta$ 8<sup>KO</sup> mice to generate a proper secondary GCR and PC response upon recall with the same antigen (Fig. 3.5A). Our preliminary results show that the GC response, as quantified by the frequency of GC B cells, T<sub>FH</sub> and T<sub>FR</sub> in draining lymphoid organs, was not modified in CD4- $\beta$ 8<sup>KO</sup> mice compared to littermate controls (Fig. 3.5G-H). On the contrary, the frequency of BM and to some extent SPL antigen-specific PC is decreased in  $\beta$ 8-deficient animals by 2-fold and 1.2-fold respectively (Fig. 3.5I). These experiments are currently being repeated to determine whether these changes are statistically significant.

Overall, these results suggest that generation of the PC response to a T-dependent model antigen is slightly blunted in CD4- $\beta$ 8<sup>KO</sup> animals. The functional relevance of this impairment still needs to be fully investigated.



**Figure 3.5 - Antigen-specific memory PC are slightly blunted in CD4- $\beta 8^{\text{KO}}$  mice**

CD4- $\beta 8^{\text{KO}}$  mice and their control littermates were immunized subcutaneously with NP-KLH in CFA and analyzed according to the protocol in (A).

(B-F) – Antigen specific and memory B cells (B-C) and memory PC (D-E) were monitored at day 60 post-immunization in the draining and non-draining lymphoid organs (SPL) and in the BM. Antibody concentration was measured by ELISA after 60 days.

(G-I) – Mice were rechallenged with NP-KLH in Alum intraperitoneally 8 weeks following primary immunization. GC B cell, polyclonal follicular T cells and NP-specific plasma cells were monitored after 7 days. The dotted line represents the value of the measured parameter at before rechallenge. Each data point represents an individual animal. Statistical analysis was performed using a Mann-Whitney U test. <sup>o</sup>p>0.05, \*p < 0.05

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## Discussion

The TGF $\beta$  cytokine plays a central role in the modulation of immune responses (Kelly et al. 2017). However, TGF $\beta$  is secreted under a latent form and requires activation to bind its receptor and induce signaling. In the regulation of immune responses,  $\alpha_v\beta_8$  integrin-mediated TGF $\beta$  activation is absolutely required (Nolte and Margadant 2020; McEntee, Gunaltay, and Travis 2019). The expression of the TGF $\beta$ -activating  $\alpha_v\beta_8$  integrin by DC and T<sub>REG</sub> is critical for the regulation of TGF $\beta$ -dependent T and B cell responses in the gut (Païdassi et al. 2011; Worthington et al. 2011; 2015; Acharya et al. 2010; Melton et al. 2010). Furthermore, TGF $\beta$  signaling during the GCR, both on T and B cells, is important to ensure the proper progression of this tightly regulated process (Tamayo, Alvarez, and Merino 2018). Thus, understanding how and when TGF $\beta$  is activated during the GCR is crucial to our understanding of the regulation of humoral responses. Here, we investigated the role of the TGF $\beta$ -activating  $\alpha_v\beta_8$  integrin in the regulation of the GCR.

On one hand, using a newly generated reporter mouse model for  $\beta_8$  integrin expression, we have been able to show that: 1/  $\alpha_v\beta_8$ -expressing T<sub>FH</sub> progressively accumulate during the GCR. In PP where there is chronic GCR, up to 50% of T<sub>FH</sub> express  $\alpha_v\beta_8$  integrin. 2/ 90-95% of T<sub>FR</sub> express  $\alpha_v\beta_8$ , 3/  $\alpha_v\beta_8$  expression on T<sub>REG</sub>, T<sub>FH</sub> and T<sub>FR</sub> is identical across all SLO we investigated, and not restricted to MALT as this is the case for  $\alpha_v\beta_8$  expression by DC.

On the other hand, the use of a conditional KO of  $\beta_8$  on all T cells reveals that: 4/ *In vivo* deletion of  $\beta_8$  on all T cells does not impair spontaneous T and B cell GC responses in naïve mice or following deliberate immunization and 5/  $\beta_8$  deficiency on T cell however seems to be associated with a decreased frequency of antigen-specific PC response in the BM compartment.

We show for the first time that 50% of T<sub>FH</sub> express  $\alpha_v\beta_8$  in a chronic GCR (in PP) and that the frequency of  $\alpha_v\beta_8^+$  T<sub>FH</sub> progressively increase as the GCR develops. This suggests that either 1/ a new previously undescribed  $\alpha_v\beta_8^+$  T<sub>FH</sub> population can arise, proliferate and be maintained throughout the GCR, 2/ that there is a dynamic and potentially transient expression of  $\alpha_v\beta_8$  on all T<sub>FH</sub> at a certain stage of the GCR where active TGF $\beta$  presentation is required or 3/ the proliferation and/or acquisition of CXCR5 and PD1 expression of a pre-existing  $\alpha_v\beta_8^+$  T cell population. The ability to follow antigen-specific response in the context of CT immunization using MHCII-CT-tetramers will allow us to investigate the kinetics of  $\beta_8$  expression on newly generated T<sub>FH</sub>, from early GC formation to the memory stage.

In their previous study, Worthington *et al.* described that FoxP3<sup>-</sup> T cells did not express the  $\alpha_v\beta_8$  integrin, whether resting or following *in vitro* activation (Worthington et al. 2015). We speculate that this discrepancy with our data stems from the fact that their study was performed on SPL and intestinal *lamina propria* of naïve mice where the frequencies of follicular T cell are extremely low. Furthermore, *in vitro* stimulation of T cells was

performed in conditions that do not favor the development of  $T_{FH}$ , thus preventing the observation of *Itgb8* expression on FoxP3<sup>-</sup> T cells by RT-qPCR.

We provide for the first time the proof that virtually all  $T_{FR}$  express  $\beta 8$  integrin. To our knowledge, this is the also first description of a cell population homogeneously expressing  $\beta 8$ . Although this had been suggested by RNA-sequencing on bulk  $T_{FR}$  (Wing et al. 2017), the single cell analysis was still missing. Furthermore, we show that upon stimulation with NP-KLH in CFA, the frequency of  $\alpha_v\beta 8$  expressing  $T_{FR}$  is not significantly modified. It has been previously shown that  $T_{FR}$  can be generated from thymic  $nT_{REG}$  ( $nT_{FR}$ ) or from naïve T cell ( $iT_{FR}$ ) (Aloulou et al. 2016). While  $nT_{FR}$  are specific for self-antigens,  $iT_{FR}$  are generated in the periphery and are specific for the immunizing antigen. Because the CFA adjuvant does not favor the generation of antigen specific  $T_{FR}$  but rather the proliferation of  $nT_{FR}$ , we hypothesize that in our NP-KLH in CFA immunization scheme, the large majority of observed  $T_{FR}$  are  $nT_{FR}$ . Following CT immunization, we have been able to detect a CT-specific population of  $T_{FR}$  generated upon immunization. These immunization protocols will allow us to investigate the potential differences in  $\beta 8$  expression by  $nT_{FR}$  and  $iT_{FR}$ .

Given the striking  $\beta 8$  expression pattern on  $T_{FH}$  and  $T_{FR}$ , the absence of phenotype in  $CD4-\beta 8^{KO}$  mice at homeostasis or after immunization has been surprising. We put forward several hypotheses:

- Despite the striking expression pattern of  $\beta 8$ , its expression does not have a functional relevance in the TGF $\beta$ -mediated regulation of the GCR. It is important to note that the detection of *Itgb8*-tdTomato fluorescence in a population does not imply that the  $\alpha_v\beta 8$  protein is actually present at the cell surface as additional translational or post-translational regulation of  $\beta 8$  expression could occur. Single-cell proteomics would be ideal to determine protein expression in  $T_{FH}$  and  $T_{FR}$  (Dou et al. 2019). Similarly, it will be crucial to properly demonstrate that  $\beta 8$  expression by these cell populations actually allows them to bind and activate TGF $\beta$ . The TLMC reporter cell line for TGF $\beta$  signaling, where the luciferase is transfected downstream of TGF $\beta$  activated promoter, should enable us to demonstrate that  $\alpha_v\beta 8$  expressing  $T_{FH}$  and  $T_{FR}$  actually activate TGF $\beta$  (Abe et al. 1994).
- As pointed previously, TGF $\beta$  has mostly been shown to promote IgA CSR and to inhibit proliferation. We therefore oriented our analysis towards the analysis of the size and isotype of the B cell responses mounted in  $CD4-\beta 8^{KO}$  mice. However, it has previously been shown that TGF $\beta$  is also required for class-switching towards IgG2b and IgG3, and can promote apoptosis (Lømo et al. 1995; Snapper et al. 1993). Furthermore, TGF $\beta$  is well known to prevent the development of auto-immunity (Sanjabi, Oh, and Li 2017). Investigation of these additional parameters of the GCR still need to be fully investigated in order to conclude our analysis of the  $CD4-\beta 8^{KO}$  mouse model.
- It is possible that other mechanisms of TGF $\beta$ -activation by T cells or TGF $\beta$ -activation by other cell populations could compensate for the loss of  $\beta 8$  on T cells.
- We suspect that deletion of *Itgb8* in  $T_{REG}$ ,  $T_{FH}$  and  $T_{FR}$  at the same time, as this is the case in  $CD4-\beta 8^{KO}$  mice, on which  $\alpha_v\beta 8$  could potentially have some opposite and counterbalancing functions, might mask the individual effects of targeted deletion of  $\beta 8$  on  $T_{FH}$  or  $T_{FR}$  on GC B cell response. New models of

specific deletion of  $\beta 8$  on  $T_{FH}$  or  $T_{FR}$  are currently being generated to precisely address this question. These efforts are reported later in the manuscript.

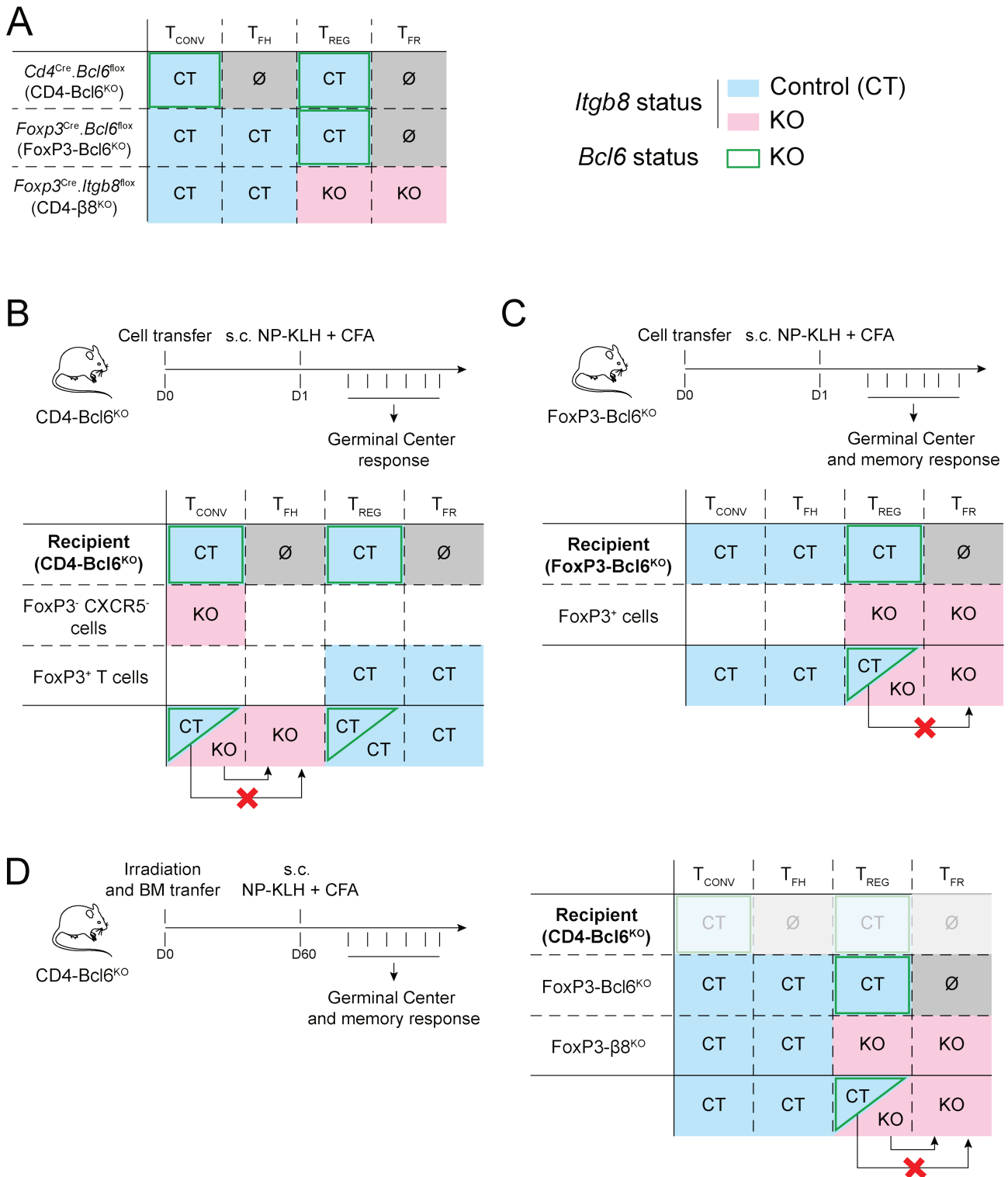
- It has been well demonstrated that the chosen adjuvant for immunization is critical in determining the type and amplitude of the resulting B cell response (Aloulou et al. 2016; S. G. Reed et al. 2009). It is reasonable to assume that the nature of the antigen, the adjuvant used, and the additional co-stimulant provided upon immunization will strongly influence the requirement for  $\alpha_v\beta 8$  expression by T cells. Investigating other combinations of antigen and adjuvant might highlight the requirement for  $\alpha_v\beta 8$  on follicular T cell in the control of the GCR.

The reduced frequency of PC in the BM niche of  $CD4-\beta 8^{KO}$  mice suggests that  $\alpha_v\beta 8$  is required for optimal induction of PC memory in primary and secondary responses. This could be due to an improper commitment of GC B cells or mBC to PC or, given that this phenotype is restricted to the BM compartment, the improper addressing of PC to their memory niche. However, the underlying mechanisms, whether this is mediated by  $T_{REG}$ ,  $T_{FH}$  or  $T_{FR}$  and whether this is mediated by the activation of  $TGF\beta$  will need further investigation.

A recent study has shown the involvement of  $\alpha_v$  integrins in the proper localization of  $T_{FH}$  to the GC (Schrock et al. 2019). The authors report in the case of  $\alpha_v$  conditional KO on T cells that long-lived PC but not mBC are improperly generated post-immunization. Although they suggest that the  $\alpha_v\beta 3$  integrin is more likely involved in this process, it is possible that  $\alpha_v\beta 8$  is also involved. Analysis of the localization of  $T_{FH}$  in  $CD4-\beta 8^{KO}$  mice would allow to investigate whether this effect might be due to  $T_{FH}$  localization rather than through  $TGF\beta$ -activation. Whether this impairment of BM PC population is of any functional relevance would also need to be investigated. To do so, the use of a challenge model of bacterial infection for example would allow to investigate the efficacy of the protection conferred by the memory immunity generated in  $CD4-\beta 8^{KO}$  mice would be very interesting.

Overall, we are so far unable to conclude on the function of  $\alpha_v\beta 8$  expression by  $T_{FH}$  and  $T_{FR}$  during the GCR. Further work is currently ongoing in the lab to continue investigating this interesting question. These efforts are summarized in the section below.





**Figure 3.6 - Generation of novel mice models to restrict *Itgb8* deletion to either T<sub>FH</sub> or T<sub>FR</sub>.**

Schematic representation of the new mice models developed in the lab to further question the requirement of  $\alpha_v\beta 8$  on follicular T cells for the control of GC B cell responses.

Color and text of each cell represent the status of each cell (columns) regarding *Itgb8* deletion in the recipient mouse model or transferred cells (row). The green border indicate that these cells are KO for *Bcl6* and cannot become follicular T cells.

## Further consideration for TGF $\beta$ , $\alpha_v\beta 8$ and the regulation of the GCR.

### Generation of novel mouse models to restrict *Itgb8* deletion to either T<sub>FH</sub> or T<sub>FR</sub>

Given the unspecific targeting of  $\alpha_v\beta 8$  deletion to all T cells in CD4- $\beta 8^{KO}$  mice, we are currently in the process of establishing other mouse models to restrict *Itgb8* deletion to either T<sub>FH</sub> or T<sub>FR</sub>. For this purpose, we are using Bcl6<sup>fllox</sup> mice crossed to either CD4<sup>Cre</sup> or FoxP3<sup>Cre</sup> mice to specifically delete the *Bcl6* gene, the transcription factor involved in follicular T cell development, in all or only in FoxP3<sup>+</sup> T cells (Hollister et al. 2013). As Bcl6 is required for the development of follicular T cells, CD4-Bcl6<sup>KO</sup> and FoxP3-Bcl6<sup>KO</sup> mice specifically lack all follicular T cells (T<sub>FH</sub> and T<sub>FR</sub>) or only T<sub>FR</sub> respectively (Fig. 3.6A). The phenotype of these new mouse models is illustrated in Fig. 3.6.

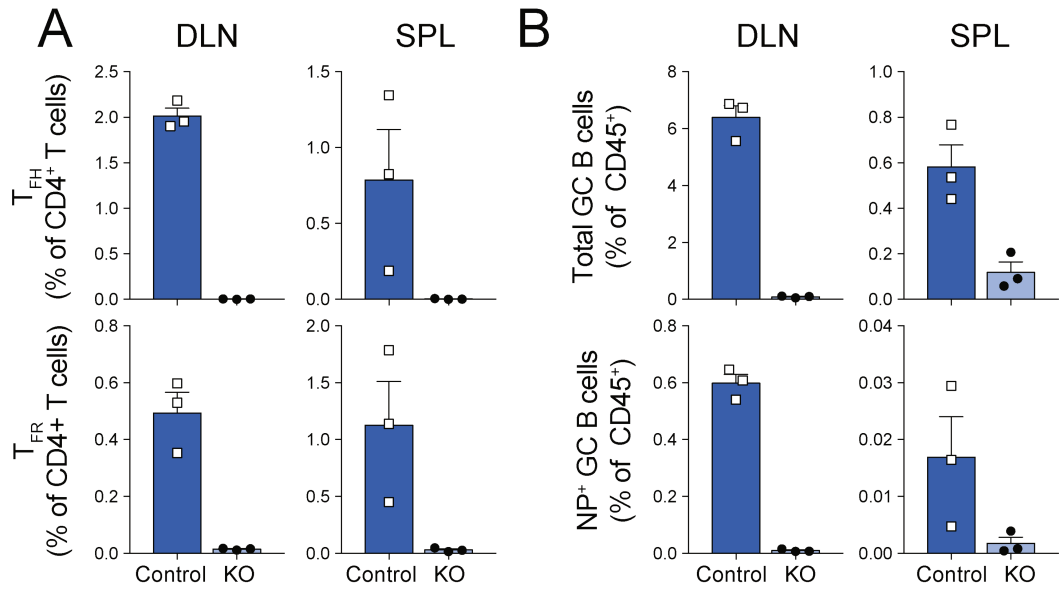
To specifically target *Itgb8* deletion in the T<sub>FH</sub> compartment, we plan to reconstitute CD4-Bcl6<sup>KO</sup> mice with 1/ control FoxP3<sup>+</sup> T cells allowing to reconstitute a normal T<sub>REG</sub> and T<sub>FR</sub> compartment and 2/ control or  $\beta 8$ -deficient FoxP3<sup>-</sup>CXCR5<sup>-</sup> naïve T cells. Upon immunization, only the transferred cells will be able to differentiate into T<sub>FH</sub> allowing the host to develop control or  $\beta 8$ -deficient T<sub>FH</sub> respectively, but  $\beta 8$ -competent T<sub>FR</sub> (Fig. 3.6B). The lack of CD62L expression by T<sub>FH</sub> impairs their ability to enter the LN from the blood once transferred, which is why we chose to inject non-T<sub>FH</sub> cells into the recipient mice.

To target *Itgb8* deletion to T<sub>FR</sub>, our first approach will likely be to use the FoxP3- $\beta 8^{KO}$  mice (Figure 3.6A) where  $\beta 8$  is deleted in T<sub>REG</sub> and T<sub>FR</sub> but not T<sub>FH</sub>. However, because T<sub>REG</sub> also express  $\beta 8$  and participate in the regulation of B cell responses, a model with  $\beta 8$  deletion only in T<sub>FR</sub> could be useful. To do so, FoxP3-Bcl6<sup>KO</sup> mice can be reconstituted with control or  $\beta 8$ -deficient Foxp3<sup>+</sup> cells. In these mice, T<sub>FH</sub> and most of the T<sub>REG</sub> will be WT for  $\beta 8$  expression while T<sub>FR</sub> will be the only  $\beta 8$ -deficient cell type (Fig. 3.6C). Alternatively, we plan to generate a cleaner model in which irradiated CD4-Bcl6<sup>KO</sup> mice are reconstituted with a 1:1 ratio of FoxP3-Bcl6<sup>KO</sup> and FoxP3- $\beta 8^{KO}$  BM. After reconstitution of immune compartment, T<sub>CONV</sub>, and T<sub>FH</sub> will be  $\beta 8$ -competent, T<sub>REG</sub> will be a 1/1 ratio of  $\beta 8^{KO}$  and  $\beta 8^{WT}$  while only  $\beta 8$ -deficient T<sub>FR</sub> will be allowed to develop (Fig. 3.6D). The use of CD4-Bcl6<sup>KO</sup> as a recipient ensures that potential residual host immune cells will not develop into T<sub>FR</sub>.

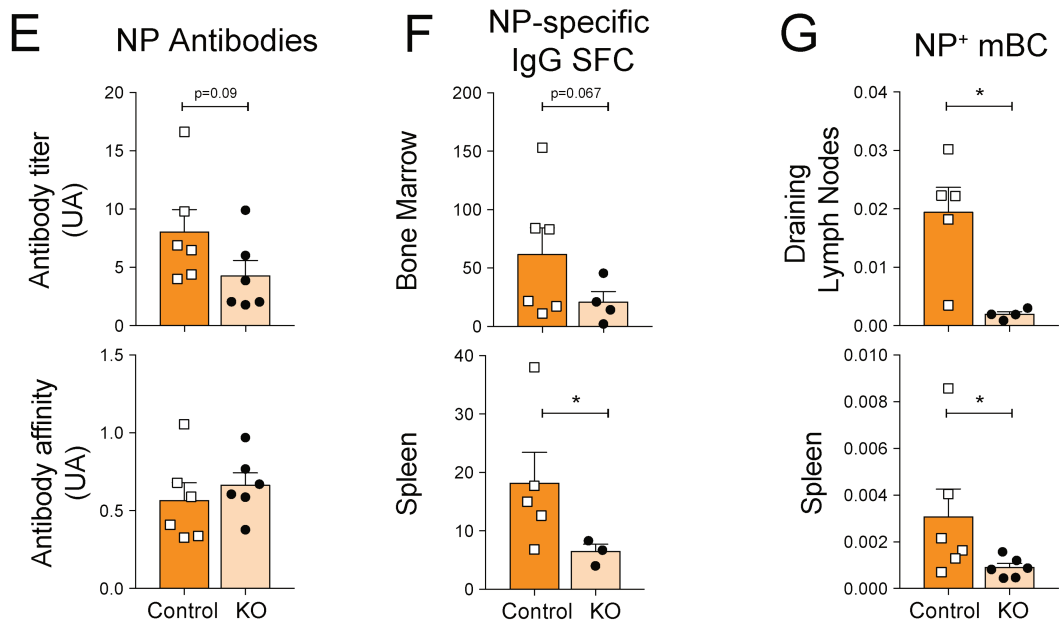
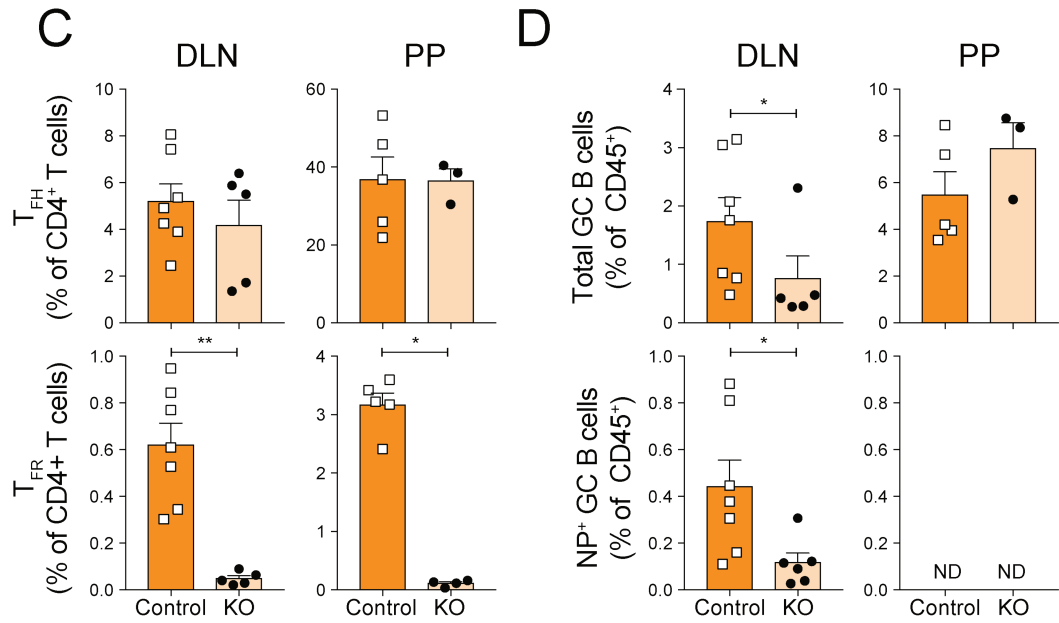
To assess the proper engraftment of the transferred cells in CD4-Bcl6<sup>KO</sup> and FoxP3-Bcl6<sup>KO</sup> hosts, we first investigated the GC phenotype of these mice. As expected, CD4-Bcl6<sup>KO</sup> mice lack both T<sub>FH</sub> and T<sub>FR</sub> (Fig. 3.7A) and do not develop GC following immunization (Fig. 3.7B). FoxP3-Bcl6<sup>KO</sup> mice also lack T<sub>FR</sub> but the frequency of T<sub>FH</sub> compartment is normal (Fig. 7C). These mice also display reduced GC response in the draining LN upon immunization with NP-KLH in CFA but seem to have normal GC response to the intestinal microbiota in PP (Fig. 3.7D). Similarly, although not significant, we see reduced antibody concentration in the serum of immunized animals at day 28 but no modification of antibody affinity (Fig. 3.7E). This is in accordance with previously published data on this model showing the role of T<sub>FR</sub> for promoting the GC (Fu et al. 2018; Wu et al. 2016; Xie et al. 2019). Furthermore, we show, to our knowledge, for the first time, that the memory response in FoxP3-Bcl6<sup>KO</sup> animals is also reduced, both regarding antigen-specific mBC (Fig. 3.7F) and long-lived PC compartment (Fig. 3.7G).

Altogether, these data should allow us to determine the functional impact on the GCR of  $\alpha_v\beta 8$  deletion on T<sub>FH</sub> and T<sub>FR</sub> using the mouse models presented in Fig. 3.6.

*Cd4<sup>Cre</sup>.Bcl6<sup>fllox</sup>*  
(*CD4-Bcl6<sup>KO</sup>*)



*Foxp3<sup>Cre</sup>.Bcl6<sup>fllox</sup>*  
(*FoxP3-Bcl6<sup>KO</sup>*)



### **Figure 3.7 - CD4-Bcl6<sup>KO</sup> and FoxP3-Bcl6<sup>KO</sup> have reduced primary and memory B cell responses**

(A-C) - CD4-Bcl6<sup>KO</sup> (A-B) or FoxP3-Bcl6<sup>KO</sup> (C-D) mice were immunized subcutaneously with NP-KLH in CFA and GC responses were analyzed at day 15. Bar plots quantify the follicular T cell population (A&C) and total or NP-specific GC B cell responses (B&D).

(E-G) – FoxP3- Bcl6<sup>KO</sup> mice were immunized subcutaneously with NP-KLH in CFA and memory responses were analyzed after 75 days. Bar plots quantify the titer and affinity of NP-specific antibodies measure by ELISA (E), NP-specific PC measured by ELISPOT (F) and NP+ mBC measured by flow cytometry (G).

Each data point represents an individual animal. Statistical analysis was performed using a Mann-Whitney U test.  $\emptyset p > 0.05$ , \* $p < 0.05$

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### **Adaptation of a coculture system for the study of $\alpha_V\beta_8$ involvement in the GCR.**

The T<sub>FH</sub>, T<sub>FR</sub> and B cell coculture system developed by the team of Arlene Sharp (Sage and Sharpe 2015) has so far been a reference to evaluate the immunomodulatory function of T<sub>FR</sub>. In order to test for the role of  $\alpha_V\beta_8$  expression by T<sub>FR</sub> and/or T<sub>FH</sub> on the regulation of the GCR, we adapted this protocol. One of the issues with working with TGF $\beta$  activation is the presence of active TGF $\beta$  in the Fetal Calf Serum used in most culture medium. I have therefore worked on adapting this coculture model in serum free medium.

This coculture system will then be set up for culture of  $\beta_8$ -competent or  $\beta_8$ -deficient T<sub>FH</sub> and or T<sub>FR</sub> in the presence of latent TGF $\beta$  and naïve B cells. Several parameters of the GCR – proliferation of B cell, acquisition of T<sub>FH</sub> markers and function, isotype switching, apoptosis, antibody secretion and affinity, etc. – will then be measured to assess the importance of  $\alpha_V\beta_8$ -mediated TGF $\beta$ -activation in this process.

# Discussion

The result section of this manuscript has investigated the expression pattern of  $\alpha_v\beta_8$  by innate and adaptive immune cells and their potential role in the control of TGF $\beta$ -dependent immune responses. We have shown that:

- Mucosal dendritic cells and in particular MLN migratory DC express the  $\beta_8$  integrin subunit. In terms of DC subsets, 60-80% of MLN mig. cDC1 and 10-20% of MLN mig. cDC2 express  $\beta_8$ .  $\alpha_v\beta_8$  expression by cDC1 is in part required for induction of intestinal iT<sub>REG</sub> (Part 1) and IgA B cell responses (Part 2) in the MLN. cDC2 restricted deletion of  $\beta_8$  leads to reduced iT<sub>REG</sub> and T<sub>H</sub>17 in the mucosal compartment indicating that  $\alpha_v\beta_8$  is also required cDC2 for T cell differentiation (Part 1).
- $\alpha_v\beta_8$  is expressed by follicular T cells. 80-90% of T<sub>FR</sub> present at immune homeostasis in the mucosal and systemic compartments express the  $\beta_8$  integrin subunit. Furthermore, around 50% of T<sub>FH</sub> also display  $\beta_8$  expression.  $\beta_8^+$  T<sub>FH</sub> accumulate during the course of the GCR. Conditional KO of  $\beta_8$  in all T cells has not revealed significant modification of primary GCR. However, memory PC generation is slightly reduced in  $\beta_8$ -deficient mice.

A number of questions have arisen from our results and additional data deserve to be commented. In this section, I will discuss our results that we currently cannot fully explain, present our current hypothesis to explain these data and present additional data to support them and finally try to integrate everything into the most recent literature and other field of  $\alpha_v\beta_8$  biology.

## Regulation of $\alpha_v\beta_8$ expression on DC

Unlike most integrin,  $\alpha_v\beta_8$  integrin is in a constitutionally active form (Mu et al. 2002, 1; Minagawa et al. 2014; Hu and Luo 2017).  $\alpha_v\beta_8$ -mediated TGF $\beta$  activation is therefore regulated at the level of  $\beta_8$  expression,  $\alpha_v$  being ubiquitously expressed. The understanding of how  $\beta_8$  expression is regulated is therefore a central objective of the team. I have been involved during my PhD in the better understanding of the mechanisms for *Itgb8* transcriptional regulation. The following paragraphs intent to present and discuss the mechanisms of regulation of *Itgb8* expression by DC. This analysis focuses on three major aspects of *Itgb8* expression by DC: 1/ the tissular restriction of  $\beta_8$  expression to MLN DC, 2/ the mostly subset specific restriction of  $\alpha_v\beta_8$  expression to cDC1 at steady state and 3/ the temporal regulation of *Itgb8* expression by DC in the context of immune or inflammatory stimulation.

## Tissue-specific expression of $\alpha_v\beta_8$ expression

At immune homeostasis, in the housing condition of our animal facility, we show that  $\beta_8$  expression in the GALT is mostly restricted to the MLN migratory DCs.  $\beta_8^+$  DC were however not found in the LP and in very low frequency in the PP, where it is restricted to Xcr1<sup>+</sup> cDC1. Additionally,  $\alpha_v\beta_8^+$  DC are not found in the spleen. As discussed later in this section, *Itgb8* expression is also found in other non-intestinal epithelial draining lymph nodes. In this paragraph, we solely focus on intestinal and GALT cDC.

It is important to note that the activation of LP DC by environmental cues induces the migration of these DC in a CCR7-dependent fashion to the MLN. In the CCR7 KO mice, the frequency of mig. DC is drastically reduced and expression of the *Itgb8* transcript by MLN cDC1 (mig. and res. cDC1) as measured by RT-qPCR also is. This suggests that the DC migrating from the LP are the major contributor to  $\beta 8$  expression by MLN cDC1. We have previously shown that a combination of TGF $\beta$ , RA and TLR stimulation (TLR9 mostly) is sufficient to induce *Itgb8* expression in the cDC1 lineage. These stimuli are found in very high quantities in the intestinal lamina propria and we have shown that they are required *in vivo* for  $\beta 8$  expression in MLN DC (Boucard-Jourdin et al. 2016). We believe this explains why spleen DC which are not exposed to the intestinal micro-environment, do not express  $\beta 8$ . However, this does not explain the absence of  $\alpha_v\beta 8^+$  DC in the LP and PP.

We propose that the mostly MLN-restricted expression of  $\beta 8$  by cDC1 is a combination of 3 factors:

- The induction of  $\beta 8$  expression upon stimulation by cDC1 is not immediate and will require some time. In that time, the activated cDC1 have already left the LP and migrated to the MLN. The investigation of  $\beta 8$  expression by CCR7<sup>KO</sup> LP cDC1 would be useful. If these LP cDC1 express the  $\beta 8$  integrin, this would suggest activation signals in the LP are sufficient to activate *Itgb8* expression by cDC1.
- The process of migration in the lymph duct imprints a particular transcription profile in mig. cDC1 instructing them to express the  $\beta 8$  integrin
- A specific micro-environment in the MLN, different from the PP micro-environment, also participates promoting  $\beta 8$  expression by cDC1.

### Subset specific bimodal expression of $\beta 8$ at steady state

The expression of *Itgb8* by MLN cDC is heavily skewed towards mig. cDC1 at steady state with less than 10% of MLN cDC2 expressing the  $\beta 8$  integrin. In this part, I will try to address the expression pattern of the  $\beta 8$  integrin subunit by DC subsets at steady state.

In regard to the preferential expression of  $\beta 8$  by MLN cDC1 (60-80% of cDC1) compared to cDC2 (10% of cDC2) we have previously proposed that epigenetic regulation of the *Itgb8* locus could explain the differential expression of  $\beta 8$  in response to environmental cues.

In a previous study, we have identified a putative regulatory enhancer for  $\beta 8$  located at -74kb upstream of *Itgb8* transcription start site. The locus is characterized by the presence of an IRF8 and BATF3 consensus binding site and the specific enrichment of H3K4me1 and H3K27ac histone modification (Boucard-Jourdin et al. 2016). These results suggest that this site is an active enhancer of *Itgb8* expression. This was further confirmed by ATACseq analysis of sorted MLN cDC indicating that the chromatin in this region is preferentially opened (data not shown). What is particularly interesting is the fact that the binding of IRF8 and BATF3, the enrichment of histone modification and the enhanced chromatin accessibility at this site was restricted to cDC1 and not cDC2. We have postulated that this epigenetic differences in the enhancer of *Itgb8* between cDC1 and cDC2 cell lineage plays a critical role in determining the subset specific expression of  $\beta 8$  in response to environmental conditioning factors. However, these experiments have not taken into account that a small subset of cDC2 can indeed express the integrin. It would be interesting to know if this enhancer is also in an active configuration in

those 10% of  $\alpha_v\beta_8$ -expressing MLN mig. cDC2 or if another pathway for the regulation of *Itgb8* expression is active.

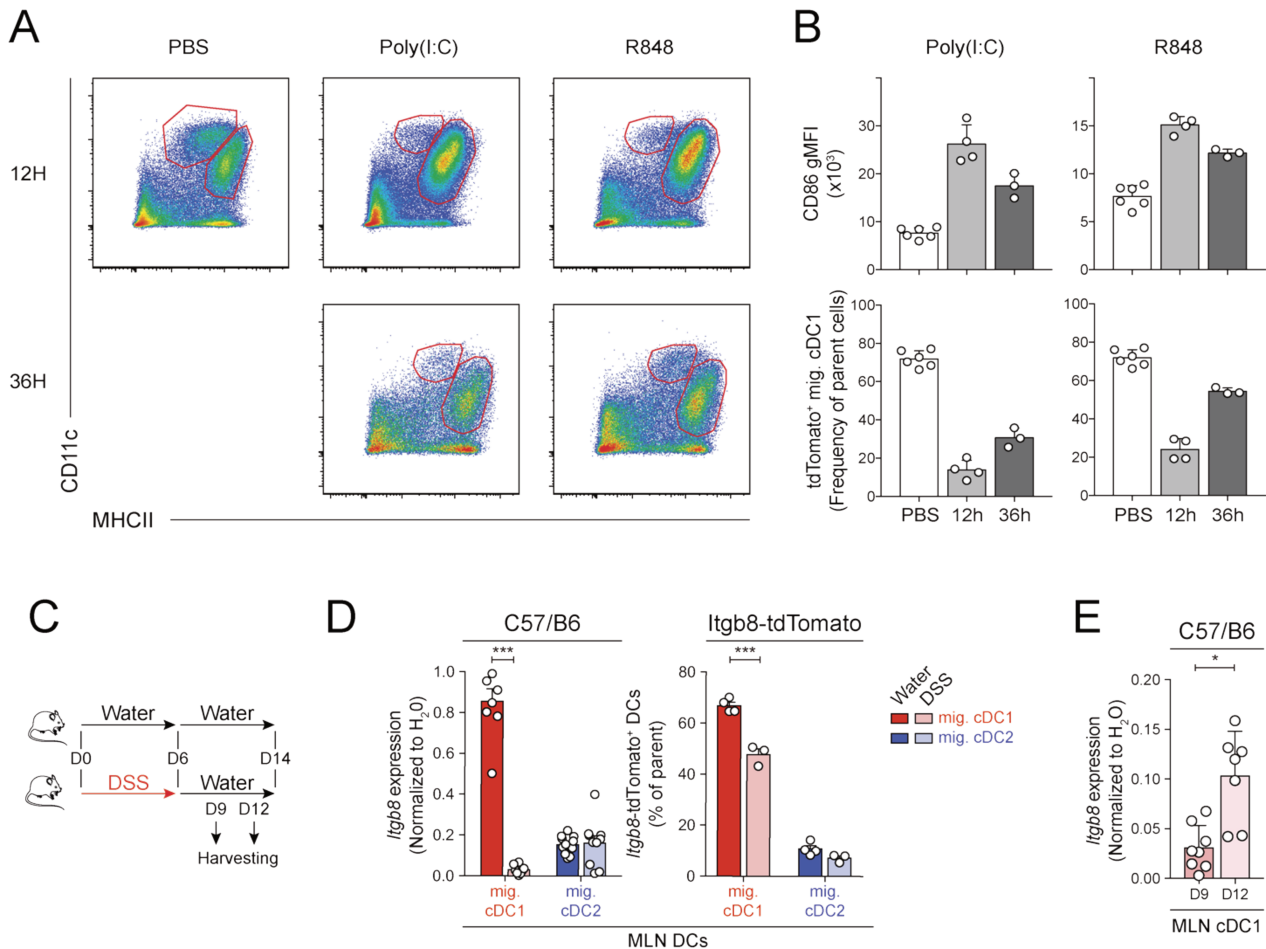
It has been reported that immune cells do not localize homogeneously along the gastro-intestinal track. LP cDC1 and cDC2 in particular occupy a distinct space of the intestine, with cDC1 being preferentially localized in the colon whereas cDC2 rather occupy the proximal small intestine (Mowat and Agace 2014). The different distribution of cDC1 (enriched in the colon) and cDC2 (enriched in the small intestine) in the intestinal tissues (Mowat and Agace 2014) could also participate in restricting the expression of  $\beta_8$  to the cDC1 subset because of the different signals perceived in both these organs.

Here, we described for the first time,  $\beta_8$  expression at the single cell level. Interestingly, in all  $\alpha_v\beta_8$ -expressing DC subsets (MLN cDC1, cDC2 and PP cDC1),  $\beta_8$  expression profile was bimodal. We currently cannot explain this observation but can propose a few hypotheses to explain this expression pattern.

- $\alpha_v\beta_8^+$  DC represent a new subset of cDC which are specialized for TGF $\beta$  activation and presentation. This would imply that developmental predisposition, possibly through the activation state of the -74kb putative enhancer, would govern the ability of mucosal DC to express the  $\beta_8$  integrin subunit. The analysis of the heterogeneity at the single cell level of Common DC Progenitor cells in regard to their -74kb site, and their ability to generate *Itgb8*-expressing DC could support this hypothesis.
- *Itgb8* expression by DC could be a marker of advanced differentiation. The death of these terminally differentiated DC and the migration of 'fresh',  $\alpha_v\beta_8$ -negative DC from the LP would create an equilibrium where a certain percentage of mig. DC express the integrin. An TLR-ligand feeding experiment described in the next section in the manuscript in partially supports this hypothesis.
- $\alpha_v\beta_8$  expression might reflect the initial origin of the migratory DC. The precise tissular origin of the DC (Small Intestine LP vs Colon LP) and the local differences in the activation and inflammatory status of the tissue (localized infection of intestinal microbiota) could potentially explain the difference in  $\alpha_v\beta_8$  expression. Whether this could explain the difference in  $\alpha_v\beta_8$  expression by both those subsets is an enticing hypothesis.

All these hypotheses are of course non-mutually exclusive and a midway between all of these is highly conceivable.

Using RNA sequencing of  $\alpha_v\beta_8^+$  and  $\alpha_v\beta_8^-$  cDC1, we have tried to investigate the differences in the transcriptional profile of these two subsets. Although these results are very preliminary and would deserve deeper analysis, very few genes were upregulated between those two subsets as compared to the difference between  $\alpha_v\beta_8^-$  mig. cDC1 and mig. cDC2 for example. Furthermore, a GEO analysis of the differentially expressed genes does not reveal any particular enriched regulatory pathway in the  $\alpha_v\beta_8^+$  cDC1 population (data not shown). This absence of striking differences between  $\beta_8^+$  and  $\beta_8^-$  cDC1 suggests that  $\beta_8$  is not a marker of a particular DC status but rather suggests that the regulation of  $\alpha_v\beta_8$  expression by a particular cDC subsets at steady state is probably regulated by the integration of environmental cues and antigen detected in the tissue from which they migrate.



**Figure 17 -  $\beta 8$  expression by cDC1 is downregulated in different models of intestinal inflammation**

*Itgb8*-tdTomato mice were stimulated with 100 $\mu$ g of Poly(I:C) or R848 by oral gavage. MLN were harvested at 12 and 36h after immunization and CD86 and  $\beta 8$  expression by cDC1 was analyzed by flow cytometry.

(A) – Representative flow cytometry plot of migratory (MHCII<sup>hi</sup>CD11c<sup>low</sup>) and resident (MHCII<sup>low</sup>CD11c<sup>hi</sup>) population in the MLN.

(B) – Quantification of CD86 MFI and the frequency of *Itgb8*-tdTomato in migratory cDC1 population as measured by flow cytometry.

A model of T-cell mediated colitis using 1.5% DSS in drinking water was induced on C57/B6, *Itgb8*-tdTomato and XCR1- $\beta 8^{\text{KO}}$  or huLang- $\beta 8^{\text{KO}}$  mice as indicated in (C).

(D) – *Itgb8* is downregulated following inflammation. *Itgb8* expression on sorted MLN DC of C57/B6 mice analyzed by RT-qPCR (left) and tdTomato fluorescence of mig. DC in *Itgb8*-tdTomato mice (right) at day 9 following DSS induction.

(E) – *Itgb8* expression on sorted MLN DC of C57/B6 mice analyzed by RT-qPCR at day 9 and day 12 post following DSS induction. qPCR data is normalized to GAPDH expression and to the H<sub>2</sub>O treatment for each independent experiment.



## Dynamic expression of $\alpha_v\beta_8$ by mucosal DC following activation

A few studies have now demonstrated or suggested the importance of  $\alpha_v\beta_8$  expressed by DC for the control of immune responses in experimental models of infectious or auto-inflammatory disease, mostly by the generation of  $T_H17$  and inhibition of Th2 responses (Acharya et al. 2010; Melton et al. 2010; Worthington et al. 2013; Steel et al. 2019; Zhou et al. 2019). These studies have often used the conditional KO of  $\beta_8$  on DC using the CD11c- $\beta_8^{KO}$  mouse model. However, none of these studies have investigated the expression levels of *Itgb8* by the various DC subsets in their inflammatory experimental models. It is therefore difficult to speculate on the specific regulation of  $\alpha_v\beta_8$  expression. As indicated previously, we suspect that cDC1 and cDC2 differentially integrate the environmental cues for the regulation of  $\beta_8$  expression.

Questioning the regulation of  $\beta_8$  by cDC1 in an inflammatory setting, we submitted our *Itgb8*-tdTomato mice to Rotavirus infection, TLR-ligand stimulation *in vivo* and DSS-induced colitis.

Using a model of rotavirus infection, we have demonstrated that the number of *Itgb8*+ cDC1 seemed to increase although not significantly in the MLN of infected animals 3 days post infection (Results – Fig. 2.5G&H). Using a model of TLR ligand feeding to *Itgb8*-tdTomato mice, we have observed at early timepoints (12h) that the frequency of  $\alpha_v\beta_8$ -expressing cDC1 among the recent CD86<sup>hi</sup> mig. DC immigrant was drastically reduced. After 36h, the frequency of  $\alpha_v\beta_8$ -expressing cDC1 is increased, not yet reaching the level of steady state cDC1 (Fig. 17A-B). Similarly, in a DSS-induced colitis model, we show that  $\beta_8$  expression by MLN cDC1 and the frequency of  $\beta_8$ + cDC1 is decreased at the peak of inflammation and later increases during the recovery phase (Fig. 17C-E).

We have developed two non-mutually exclusive hypothesis to explain these data:

- The inflammatory status of the LP during cDC1 activation determines their ability to express  $\beta_8$  integrin. Upon strong TLR stimulation or inflammation in the LP, cDC1 migrating in the MLN cannot express the integrin which reduces their ability induce iT<sub>REG</sub> in the MLN. Whether this is a detrimental consequence of the inflammation or an evolutionary-selected mechanism to allow inflammatory responses to get rid of a potential pathogen, is an interesting question.

During the resolution of the intestinal inflammation, the cDC1 newly migrating from the LP to the MLN regain the ability to express the  $\beta_8$  integrin, allowing them to participate in the further resolution of the inflammatory condition.

This hypothesis is quite well supported by the modulation of  $\beta_8$  expression on MLN cDC1 in DSS-induced colitis and TLR feeding described above.

- The expression of  $\beta_8$  on a particular cell is temporally regulated and  $\beta_8$  is expressed at a later time.  $\beta_8$ -immature DC in the LP are activated by the inflammatory signals and migrate into the MLN where they mature and later express  $\beta_8$  as a marker of advanced differentiation. This hypothesis is also consistent with the increase of  $\beta_8$ + MLN cDC1 relatively quickly (36h) after oral TLR stimulation.

Discerning the relative contribution of both these mechanisms of  $\beta_8$  regulation by cDC1 in response to inflammation might prove complex. A main parameter that would allow to favor one mechanism or the other is the relative difference between the length of the inflammation symptoms and the turnover of individual cDC1 in the MLN following activation in the LP. In a model of acute inflammatory stimulation such as TLR ligand feeding,

the temporal regulation of  $\beta 8$  expression on migratory cDC1 might be predominant. In the more prolonged inflammatory induction of the DSS colitis, the evolution of the inflammatory state of the LP might be the main determinant for  $\beta 8$  expression by MLN cDC1.

As indicated earlier, the mechanisms of regulation of  $\beta 8$  expression by MLN cDC2 might be different than in cDC1. In our model of DSS-induced colitis, we have indeed shown that the decreases of the frequency and of the level of *Itgb8* expression by MLN mig. cDC1 is not found in MLN mig. cDC2.

The study of *Itgb8* induction by TLR signaling has been published before and indicate that CpG oligonucleotides (TLR9 agonist) is a strong inducer of *Itgb8* expression by cDC in vitro (Boucard-Jourdin et al. 2016). Furthermore, it has been reported that the stimulation of LP cDC2 with ATP, induces their expression of *Itgb8* (Atarashi et al. 2008). The authors suggest that this ATP might originate from dead epithelial cells or directly from the microbiota and represent a danger signal recognized by cDC2. Fenton and colleagues have identified that LPS was able to induce *Itgb8* expression by human LP cDC2 (Fenton et al. 2016). Additionally, Durand et al, have demonstrated that R848 and CD40L+IFN $\gamma$  stimulation can induce  $\beta 8$  expression by cDC2 (Durand et al. 2019). Overall, these results suggest that inflammatory cues and Danger Associated Molecular Pattern that might be presented in an infectious or inflammatory or infectious context have the ability to induce *Itgb8* expression on cDC2 in the gut LP and lymphoid tissues.

The signaling cascade leading to *Itgb8* expression is still unknown. However, recent publications in the field of *Itgb8* expression however give some leads as to the potential upstream regulators of  $\beta 8$  expression. Markovics and colleagues have shown that *Itgb8* expression in human cell lines and primary stromal cells was regulated by the AP-1 transcription factors family and the p38 signaling pathway (Markovics et al. 2010). Interestingly, these signaling molecules and transcription factor are known to be downstream of the TLR signaling (J. Brown et al. 2011). Furthermore, these same author have demonstrated that  $\beta 8$  is also induced by IL1 $\beta$ , a well-known inflammatory cytokines signaling through Myd88 among other (Markovics et al. 2011). These studies thus suggest a potential pathway positively regulating *Itgb8* expression in response to TLR signalization and inflammatory cytokines. Whether this is indeed the case in intestinal cDC2 is worth investigating.

On the other hand, the proteasome activator REG $\gamma$  has also recently been shown to be involved in the degradation of the IRF8 transcription factor and thus represses the expression of the  $\beta 8$  integrin (Zhou et al. 2019). It has also been shown that REG $\gamma$  expression is induced by TLR signaling. In turn REG $\gamma$  potentiate pro-inflammatory cytokines (J. Sun et al. 2016). Whether this could be part of a pathway for negative regulation of *Itgb8* and induction of pro-inflammatory immune program would be worth investigating. However, the expression of *Itgb8* in the distinct DC subsets has not been made in the REG $\gamma$ <sup>KO</sup> mice, preventing any further conclusion. The balance between both those pathway and others yet to be identified is most likely responsible for the differential regulation of *Itgb8* in inflammatory settings between cDC1 and cDC2.

## Addressing the phenotypes of $Xcr1\text{-}\beta8^{\text{KO}}$ and $huLang\text{-}\beta8^{\text{KO}}$ .

The deletion of  $\beta8$  or  $\alpha_v$  on CD11c-expressing phagocytes leads the development of a severe spontaneous colitis associated with a loss of intestinal  $iT_{\text{REG}}$  and  $T_{\text{H}}17$  response and promotion of  $T_{\text{H}}1$  and  $T_{\text{H}}2$  responses in the intestine (Travis et al. 2007; Lacy-Hulbert et al. 2007). Further *ex vivo* characterization of DC subsets in mouse GALT first revealed that MLN  $CD103^+$  DC and later  $CD103^+CD11b^-$  cDC1 but not  $CD103^+CD11b^-$  cDC2 expressed the  $\beta8$  integrin and were able to induce TGF $\beta$ -mediated  $T_{\text{REG}}$  generation (Worthington et al. 2015; Païdassi et al. 2011; Boucard-Jourdin et al. 2016). Further studies have implicated the importance of  $\alpha_v\beta8$  on CD11c-expressing phagocytes for the generation of  $T_{\text{H}}17$  in various inflammatory contexts (Acharya et al. 2010; Melton et al. 2010; Zhou et al. 2019; Steel et al. 2019). It has been shown that induction of  $T_{\text{H}}17$  responses is associated with upregulation of *Itgb8* expression by cDC2 following Damage-Associated Molecular Pattern (DAMP) signaling (Atarashi et al. 2008). It's been hypothesized that  $\alpha_v\beta8$  upregulation by cDC2 in an inflammatory environment which would license them for  $T_{\text{H}}17$  generation (Fig. 18)

The restriction of  $\alpha_v\beta8$  deletion to individual DC subsets, namely cDC1 and cDC2, using  $Xcr1\text{-}\beta8^{\text{KO}}$  and  $huLang\text{-}\beta8^{\text{KO}}$  mouse models respectively, intended to be the first formal *in vivo* demonstration of the distinct function of  $\alpha_v\beta8$  on cDC1 and cDC2 for T cell differentiation. The results of our experiments show that:

- Deletion of the  $\beta8$  integrin on cDC1 or on cDC2 is not associated with spontaneous inflammation
- $\alpha_v\beta8$  on cDC1 and cDC2 is at least partially required for the generation of intestinal  $iT_{\text{REG}}$
- Deletion of  $\alpha_v\beta8$  on cDC2 might slightly impair their ability to generate MLN  $T_{\text{H}}17$
- $\alpha_v\beta8$  on cDC1 and cDC2 is not required for homeostatic IgA responses in the gut.

### Deletion of $\beta8$ integrin on cDC1 or on cDC2 is not associated with spontaneous inflammation

First, it is first interesting to note the absence of inflammatory phenotype in both  $Xcr1\text{-}\beta8^{\text{KO}}$  and  $huLang\text{-}\beta8^{\text{KO}}$ . In  $Xcr1\text{-}\beta8^{\text{KO}}$  mice in particular, we expected that deletion of  $\beta8$  in the highest expressing cDC subsets to mimic  $CD11c\text{-}\beta8^{\text{KO}}$  mice. However, this is not the case as  $Xcr1\text{-}\beta8^{\text{KO}}$  mice do not develop any wasting syndrome or clinical and cellular markers of inflammation (Results – Fig. 1.2B-D). As briefly overviewed in the Results section, a few hypotheses have been put forward to explain these observations:

Compensatory or redundant mechanisms, further detailed below, could be at play to overcome the loss of  $\beta8$  expression by cDC1 or cDC2:

- Given that both migratory cDC1 and cDC2 express  $\alpha_v\beta8$  at steady state, we first suspected that compensatory expression of  $\beta8$  expression between cDC1 and cDC2 might counteract for the loss of  $\beta8$  in one or the other subset. We are currently investigating *Itgb8* expression in MLN cDC1 and cDC2 in both mouse models. Although very preliminary, our data seem to indicate that this is not the case in  $Xcr1\text{-}\beta8^{\text{KO}}$  mice. However,  $\beta8$  expression at the single cell level would be warranted, maybe through the use of new anti- $\beta8$  antibodies currently being generated for mice (mAb ADWA-11) (Stockis et al. 2017; Reboldi et al. 2016).

- Although TGF $\beta$ -activation by other  $\alpha_v$ -associated integrin have not been associated to the regulation of TGF $\beta$ -dependent immune process, MLN DC express significant amount of  $\beta_1$ ,  $\beta_3$  and  $\beta_5$  integrin ((Païdassi et al. 2011) and ImmGen MNP OpenSource project). It is possible that redundant mechanisms of TGF $\beta$  activation, by which other integrins or integrin-independent mechanisms and even by other cell type, are in place to compensate the loss of function of one or the other thus preventing tissue inflammation. The development of redundant mechanisms to regulate tissue homeostasis is very likely from an evolutionary standpoint.
- CD11c- $\beta_8^{KO}$  mice have a strong inflammatory phenotype. If these mice are generally used to target DC the CD11c<sup>Cre</sup> construction is not specifically targeting conventional DC as CD11c can be expressed by other phagocytes and some lymphocytes (Abram et al. 2014). It is therefore likely that  $\beta_8$  expressed by other CD11c-expressing phagocytes participates in the maintenance of intestinal homeostasis. To question this hypothesis, we crossed CD11c<sup>Cre</sup> line with the ROSA-lox-stop-lox-EYFP reporter mouse in order to fate-map CD11c<sup>Cre</sup> expression by immune cells. In parallel, we are analyzing the expression of  $\beta_8$  in these same cells using the *Itgb8*-tdTomato reporter mice. Preliminary results obtained so far seem to show that the non-cDC CD11c-expressing cell population of the GALT (moDC, macrophages, B cells, etc.) in naïve adult mice do not significantly express  $\beta_8$  integrin (data not shown). It is therefore unlikely that these cells could participate in the maintenance of the gut homeostasis. However, sign of immune activation in CD11c- $\beta_8^{KO}$  mice can be found as early as 3 weeks of age (unpublished data). We believe that a transient expression of  $\beta_8$  integrin by CD11c-expressing cells in neonates could be critical in maintaining tissue homeostasis. One likely candidate is neonatal DC. Indeed, neonatal DC display a more anti-inflammatory phenotype than their adult counterparts (Papaioannou, Pasztoi, and Schraml 2019). It is possible that the impairment of  $\beta_8$  expression by neonatal CD11c<sup>+</sup> DC at the neonatal stage might be the trigger for this early-onset spontaneous colitis of CD11c- $\beta_8^{KO}$  mice which is then amplified by the lack of T<sub>REG</sub> and T<sub>H</sub>17 generation.

## cDC1 and cDC2 are able to drive TGF $\beta$ -dependent generation of iT<sub>REG</sub>

Second, we describe for the first time the ability of both cDC1 and intestinal cDC2 to generate T<sub>REG</sub> through  $\alpha_v\beta_8$ -dependent mechanisms.

The expression of  $\beta_8$  by MLN cDC2 and the 2-fold decrease of iT<sub>REG</sub> in the huLang- $\beta_8^{KO}$  mice have been unexpected. Indeed, we have previously shown that  $\beta_8$  expression by MLN mig. cDC2 was much (5-fold) lower than MLN mig. cDC1. Furthermore, these cells were unable to induce T<sub>REG</sub> in an  $\alpha_v\beta_8$ -dependent manner *in vitro* (Boucard-Jourdin et al. 2016). However, we show that only a small proportion of cDC2 in the MLN (~10%) express the  $\beta_8$  integrin subunit. In that regards, the effect of  $\alpha_v\beta_8$ -mediated T<sub>REG</sub> activation by cDC2 could have been missed.

This strongly suggests that the few  $\beta_8$ -expressing cDC2 cells (10% of total mig. cDC2) in the MLN are able to promote T<sub>REG</sub> differentiation through  $\alpha_v\beta_8$ -dependent mechanisms which contributes to about half of the total ROR $\gamma$ <sup>t</sup>FoxP3<sup>+</sup> iT<sub>REG</sub> pool. Importantly, we have shown that the penetrance of the  $\beta_8$  deletion of cDC2 is not

entirely complete and that some cDC2 might retain  $\beta 8$  expression. The  $\alpha_v\beta 8$ -dependent induction of  $iT_{REG}$  by cDC2 might therefore account for more than half of the steady state  $iT_{REG}$  population.

Interestingly,  $Cd11c^{Cre}.Irf4^{flox/flox}$  mice, allowing the specific deletion of intestinal  $CD103^+CD11b^+$  cDC2 does not exhibit this defect in the frequency of total  $ROR\gamma t^+FoxP3^+$   $iT_{REG}$  (Persson et al. 2013). This highlights the difference between the depletion of a cell and the loss of function of that cell.

Further studies are required to determine if 1/ the 10-15% of *Itgb8*-expressing cDC2 are specialized for  $T_{REG}$  generation, 2/ *Itgb8* deletion in cDC2 can impact other immune cell development or function and subsequently impacts  $T_{REG}$  generation or 3/ the Cre-recombinase under the *huLang*<sup>Cre</sup> promoter can be expressed in other  $\alpha_v\beta 8$ -expressing immune cells throughout the immune development through physiological mechanisms or because of a leaky Cre-recombinase expression.

The decrease of MLN and LP  $ROR\gamma t^+$   $iT_{REG}$  in  $Xcr1-\beta 8^{KO}$  mice, although significant, is low (Results – Fig. 1.3C). Furthermore, in the case of DSS-induced colitis, these small decrease in  $iT_{REG}$  frequency does not have a significant impact on disease susceptibility, progression or remission. Given that 70-80% of MLN cDC1 express the integrin, this was very unexpected. This suggests that  $\alpha_v\beta 8$  expression by cDC1 is dispensable for  $iT_{REG}$  induction. While the frequency of  $iT_{REG}$  are reduced, investigating the functionality and immunosuppressive functions of the  $T_{REG}$  generated in this model is warranted to make this assertion. However, this does not mean that MLN cDC1 are not able to induce  $iT_{REG}$  by  $\alpha_v\beta 8$ -dependent  $TGF\beta$ -activation in the GALT.

Given the *in vitro* evidence that intestinal cDC1 can induce  $T_{REG}$  through  $\alpha_v\beta 8$ -dependent  $TGF\beta$  activation (Boucard-Jourdin et al. 2016), we propose that cDC1 can induce  $iT_{REG}$  in the MLN at steady state but that  $\beta 8$ -deletion on cDC1 in the  $Xcr1-\beta 8$  mice is rescued by the expression of  $\alpha_v\beta 8$  by cDC2 or by other  $TGF\beta$ -activating mechanisms.

Previously published data have already demonstrated a cooperation between cDC1 and cDC2 in the generation of  $T_{REG}$  at steady state. Indeed, it has been observed that the conditional deletion of intestinal cDC1 (*Batf3*<sup>-/-</sup> or *CD11c*<sup>Cre</sup>.*Irf8*<sup>flox</sup>) or cDC2 (*CD11c*<sup>Cre</sup>.*Irf4*<sup>flox</sup> or *huLangherin*-DTA) in mice does not drastically reduce intestinal  $T_{REG}$  (Luda et al. 2016a; Arnold et al. 2019b; Persson et al. 2013; Welty et al. 2013). However, in their study, Welty *et al.* observed that the double transgenic *huLangherin*-DTA.*Batf3*<sup>-/-</sup> mice resulting in the loss of both cDC1 and cDC2 present a significant reduction of LP  $T_{REG}$  (2-fold) and of expression of the gut-homing CCR9 chemokine receptor (Welty et al. 2013).

To test this hypothesis, we are in the process of generating a simultaneous cDC1 and cDC2-specific deletion of  $\beta 8$  by crossing  $Xcr1-\beta 8^{KO}$  and *huLang*- $\beta 8^{KO}$ . In this model the development of a severe spontaneous colitis and a specific loss of  $T_{REG}$  and  $T_H17$  mimicking the *CD11c*- $\beta 8^{KO}$  model would confirm our hypothesis.

### **huLang- $\beta 8^{KO}$ and $T_H17$ induction**

The study of  $T_H17$  differentiation in both mouse model reveals that  $\alpha_v\beta 8$  expression by cDC1 is dispensable for  $T_H17$  generation. In the *huLang*- $\beta 8^{KO}$  the lack of data for LP  $T_H17$  frequency and the relatively low decrease in MLN  $T_H17$  prevents us from fully concluding on the requirement of  $\alpha_v\beta 8$  for the specialization of cDC2 to induce  $T_H17$  by  $TGF\beta$ -dependent mechanisms. The specialization of cDC2 but not cDC1 for the generation of  $T_H17$

responses and the ability of CD11c<sup>+</sup> DC to induce T<sub>H</sub>17 by  $\alpha_v\beta_8$ -dependent TGF $\beta$ -activation however supports this hypothesis.

T<sub>H</sub>17 are well known for their dual nature, both supporting homeostatic intestinal protection but also being an important player in inflammatory disorder. Therefore, we would like to investigate the requirement for  $\alpha_v\beta_8$  expression by cDC2 in a T<sub>H</sub>17-driven inflammatory disorder. In particular the Experimental Autoimmune Encephalomyelitis (EAE) model seem particularly adapted as  $\alpha_v\beta_8$  expression by DC is absolutely required in generating inflammatory T<sub>H</sub>17 responses. In the context of EAE, it has been shown that CD103<sup>-</sup>Sirp $\alpha^+$ CD11b<sup>+</sup> cDC2 but not CD103<sup>+</sup>CD11b<sup>-</sup> cDC1 are producing IL6 and presenting it to T cells for T<sub>H</sub>17 induction (Heink et al. 2017). We hypothesize that  $\alpha_v\beta_8$  expression by cDC2 and their restricted expression of IL6 could be the mechanisms by which they are specialized for T<sub>H</sub>17 induction as compared to cDC1.

Alternatively, the double mutated (R192G/L211A) *Escherichia coli* derived heat labile toxin (dmLT) has been shown to induce a robust T<sub>H</sub>17 and T<sub>H</sub>1 driven inflammatory response in the gut following oral administration (Clements and Norton 2018). Hence, investigating the requirement for  $\alpha_v\beta_8$  expression by cDC2 for the generation of T<sub>H</sub>17 responses following dmLT gavage might also be an interesting approach to probe our hypothesis.

## $\beta_8$ expression by cDC1 and cDC2 is not required to control homeostatic mucosal IgA responses

The measure of steady state IgA response in CD11c- $\beta_8^{\text{KO}}$  mice revealed a much higher IgA response in all regards in  $\beta_8$ -deficient animals compared to their control littermates (peripheral IgA<sup>+</sup> B cells, MLN and SPL IgA<sup>+</sup> PC, antibody titer among many other). Of course, given the critical importance of TGF $\beta$  in inducing IgA response (Stavnezer and Kang 2009) and the well described role of  $\alpha_v\beta_8$  for TGF $\beta$  activation (Worthington, Klementowicz, and Travis 2011), we had expected that  $\beta_8$ -deficiency would lead a reduction rather than an increase in IgA responses. Evidence has been collected indicating that this dysregulation of IgA responses increases with age and correlates with the degree of clinical and cellular inflammation. Although this has not been formally proven, we believe that the highly inflammatory immune system, coupled with the loss of iT<sub>FR</sub> drives an exaggerated IgA response, possibly even to self-antigens. Whether this is mediated by increased IgA induction or the uncontrolled proliferation of preexisting IgA B cells would further need to be studied. In the case of *de novo* IgA generation, the source of TGF $\beta$  and of its activation would need to be investigated. It is likely that FDC, follicular T cells or other stromal cells could activate TGF $\beta$  in  $\beta_8$ -dependent or independent mechanisms.

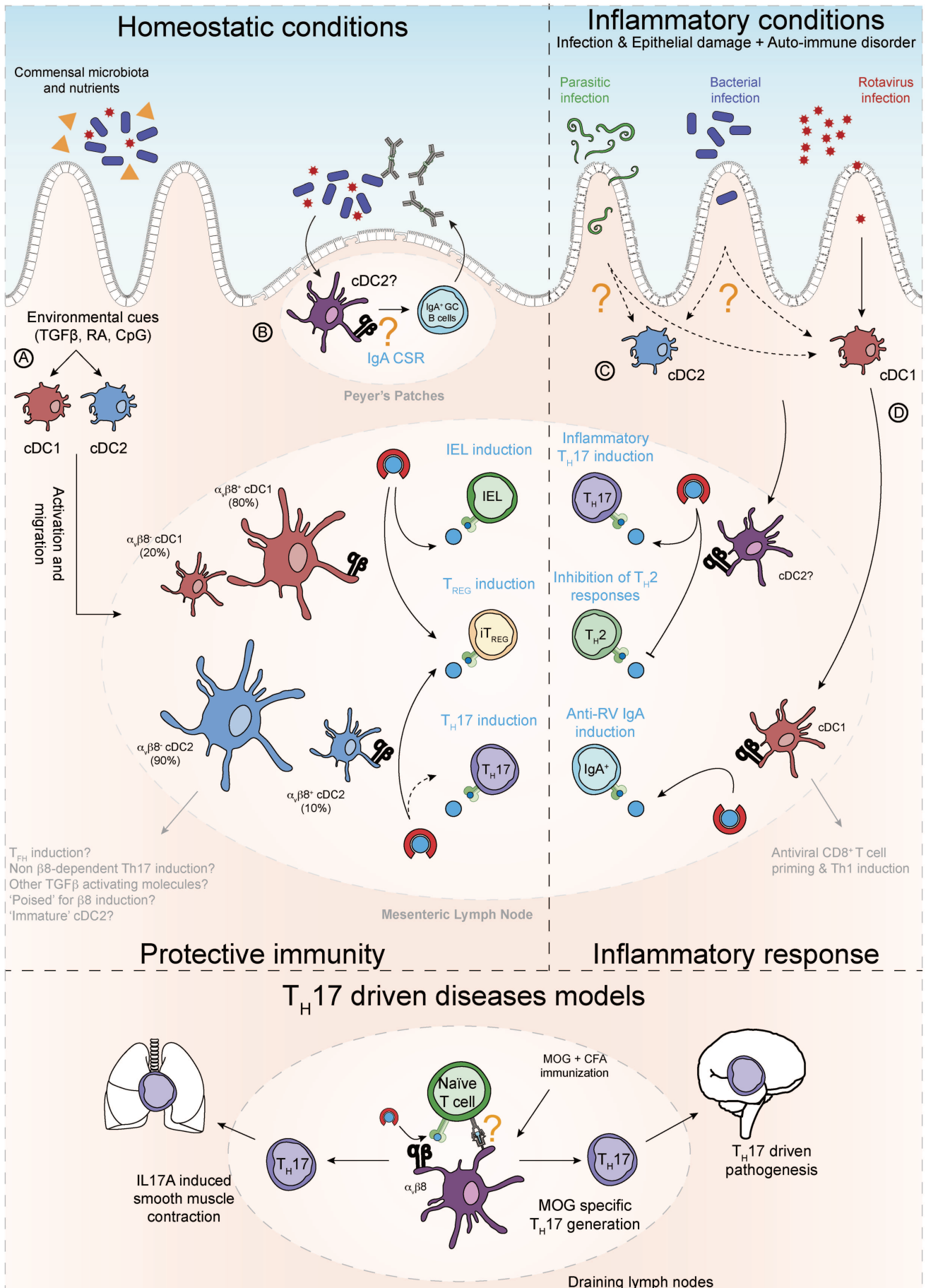
The ability to restrict  $\beta_8$  deletion to specific DC subsets and the discovery these models do not develop colitis has given us the ability to dissect the relative importance of  $\alpha_v\beta_8$ -mediated TGF $\beta$  activation of cDC1 and cDC2 in the control of IgA response. Surprisingly, in neither mouse model significant changes to steady state IgA responses, in terms of number and proportion of IgA<sup>+</sup> B cells and LP PC, were observed, suggesting that overall mucosal IgA responses at steady state are not dependent on  $\alpha_v\beta_8$  expression by cDC1 and intestinal cDC2. Other parameter of IgA B cells responses, in particular the repertoire and specificity of the mounted response, could be investigated to confirm or infirm our previous claim.

A few studies have however provided some clues that  $\alpha_v\beta_8$  could promote IgA through TGF $\beta$  activation.

- Reboldi and colleagues have shown that  $\alpha_v\beta_8$  deletion of CD11c-expressing phagocytes results in a reduction of GC IgA<sup>+</sup> population in PP. They further suggest that  $\alpha_v\beta_8$  expression by PP cDC2 could be responsible for TGF $\beta$  activation and presentation of active TGF $\beta$  to B cells in the Sub-Epithelial Dome (Reboldi et al. 2016). Our investigation of PP IgA<sup>+</sup> GC B cells in CD11c- $\beta_8^{\text{KO}}$  or huLang- $\beta_8^{\text{KO}}$  does not reveal any defects (data not shown). It is however important to note that: 1/ Their experiment involved the use of a bone marrow transplant of CD11c- $\beta_8^{\text{KO}}$  cells into  $\beta_8$ -competent host, limiting the development of inflammation and 2/ preliminary investigation of the  $\beta_8$  genomic deletion of PP DC in our huLang- $\beta_8^{\text{KO}}$  mice indicate that PP DC are not targeted by the huLang<sup>Cre</sup> promoter. Unfortunately, our current data and experimental models does not allow to confirm or infirm the hypothesis developed in this study.
- Ruane and colleagues have shown a lung draining cDC1 and cDC2 but not macrophages are able to induce IgA responses in the lung which home to the intestinal compartment. They also show that these cells express the  $\beta_8$  integrin at higher levels than macrophages (Ruane et al. 2016). Although this is just correlative, it would be interesting to focus our analysis of IgA responses in the lung, provided that the huLang<sup>Cre</sup> transgene allows to target  $\beta_8$  deletion to lung draining cDC2.

Given the critical role of cDC1 in the control of viral infection, we therefore explored the role of  $\alpha_v\beta_8$  on this subset in the context of intestinal viral infection and the host IgA response towards it. At this time, Katharina Lahl had described that cDC1 was required for IgA response to RV infection. We therefore started a collaboration with the team of Dr. Katharina Lahl in Lund, Sweden. Infecting the huLang- $\beta_8^{\text{KO}}$  mice with RV, we have now showed the importance of  $\alpha_v\beta_8$  on cDC1 for optimal IgA responses to RV infection.

A schematic summarizing the proposed model for the regulation of  $\alpha_v\beta_8$  expression on cDC in the gut and its function in the induction of T<sub>REG</sub> and T<sub>H</sub>17 is shown in Fig. 18.





### **Figure 18 - Proposed model for the role of $\alpha_V\beta_8$ expressed by DC in the regulation of intestinal immunity.**

(A) – Under homeostatic conditions, stimulation from the intestinal microenvironment induces the activation and migration of intestinal LP DC to the mesenteric Lymph Nodes (MLN). Upon migration,  $\alpha_V\beta_8$  expression is acquired by 80-90% of mig. cDC1 and 10% of cDC2. Expression of  $\alpha_V\beta_8$  by DC1 and cDC2 is partially required to generate steady state iT<sub>REG</sub> and potentially T<sub>H</sub>17 in response to the commensal flora stimulation.

(B) – At steady state, Reboldi and colleagues have suggested that *Itgb8* expression by Peyer's Patches (PP) cDC2 was required for the induction of IgA response to the commensal flora in the PP (Reboldi et al. 2016). In vivo conditional deletion of  $\beta_8$  on cDC2 however does not affect overall IgA response in the gut.

(C) – In the context of intestinal parasitic infection, it has been shown that  $\alpha_V\beta_8$  expression by intestinal cDC was required to generate T<sub>H</sub>17 and inhibit T<sub>H</sub>2 responses (Steel et al. 2019; Worthington et al. 2013). The specific subsets involved in this response was not investigated although the high capacity of cDC2 to induce T<sub>H</sub>17 irrespective of  $\alpha_V\beta_8$  make them likely candidates.

(D) – In the context of intestinal rotavirus (RV) infection, MLN cDC1 are required to facilitate early IgA RV-specific response in part through  $\alpha_V\beta_8$ -mediated mechanisms (Nakawesi et al. 2020).

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## Considerations on the role of $\alpha_V\beta_8$ expression by follicular T cells

Using the *Itgb8*-tdTomato mouse model, we have been able to report for the first time the expression of the  $\beta_8$  integrin at the single cell level on follicular T cells. We have shown that 40-50% of T<sub>FH</sub> and most T<sub>FR</sub> (80-90%) express  $\beta_8$  integrin at homeostasis. Furthermore, although still preliminary, we show that, upon NP-KLH in CFA immunization,  $\beta_8$  expression by T<sub>FH</sub> is progressively acquired throughout the GCR, while  $\beta_8$  expression by T<sub>FR</sub> does not seem modified (% of  $\beta_8^+$  T<sub>FR</sub>). With a CT subcutaneous immunization however, preliminary data suggest that  $\beta_8$  integrin is expressed by only ~60% of antigen-specific T<sub>FR</sub>.

Additionally, we have shown that  $\beta_8$  expression is conserved on human tonsil T<sub>FH</sub> at a frequency that is close to that of SLO murine T<sub>FH</sub> (data not shown). This suggest that the mechanisms of regulation of the GCR found in mice potentially should be translatable to human applications.

## Spatio-temporal expression of $\alpha_V\beta_8$ on T<sub>FH</sub>

Similar to what is observed in mucosal,  $\beta_8$  expression on T<sub>FH</sub> is bimodal, suggesting that a subset of T<sub>FH</sub> that is specialized for  $\alpha_V\beta_8$ -mediated TGF $\beta$  activation. We have so far been unable to decipher the biological phenomenon behind this observation. We've so far developed 3 main non-mutually exclusive hypotheses:

- $\beta_8$  expression on T<sub>FH</sub> identifies a new sub-population of T<sub>FH</sub>, defined by a specific transcriptional signature and licensed by  $\alpha_V\beta_8$  expression to present activated TGF $\beta$  to T cells. RNA-sequencing of  $\beta_8^+$  and  $\beta_8^-$  T<sub>FH</sub> might shed some light on this hypothesis by highlighting transcriptional differences in the expression of particular transcription factors and/or signaling pathways. Alternatively, transferring  $\beta_8^+$  T<sub>FH</sub> into a congenic host and analyzing their ability to generate  $\beta_8^-$  T<sub>FH</sub> following re-immunization might be a way to demonstrate the existence of a potential new T<sub>FH</sub> identity
- Physical localization in the follicle of T<sub>FH</sub> might determine their expression of  $\beta_8$ . We have shown that  $\beta_8$  expression positively correlates with PD1 expression. Levels of PD1 expression by T<sub>FH</sub> has been linked to their localization and activity (Shi et al. 2018, 1; Trüb et al. 2017). It is entirely possible that the requirement of  $\beta_8$  expression is different in the different compartments of B cell response (T:B border, GC, LZ:DZ border, ...). Using the *Itgb8*-tdTomato mouse model, we will be able to precisely localize of

$\beta 8^+$  T<sub>FH</sub> in the B cell follicle using confocal microscopy. This technique is currently being developed in the lab.

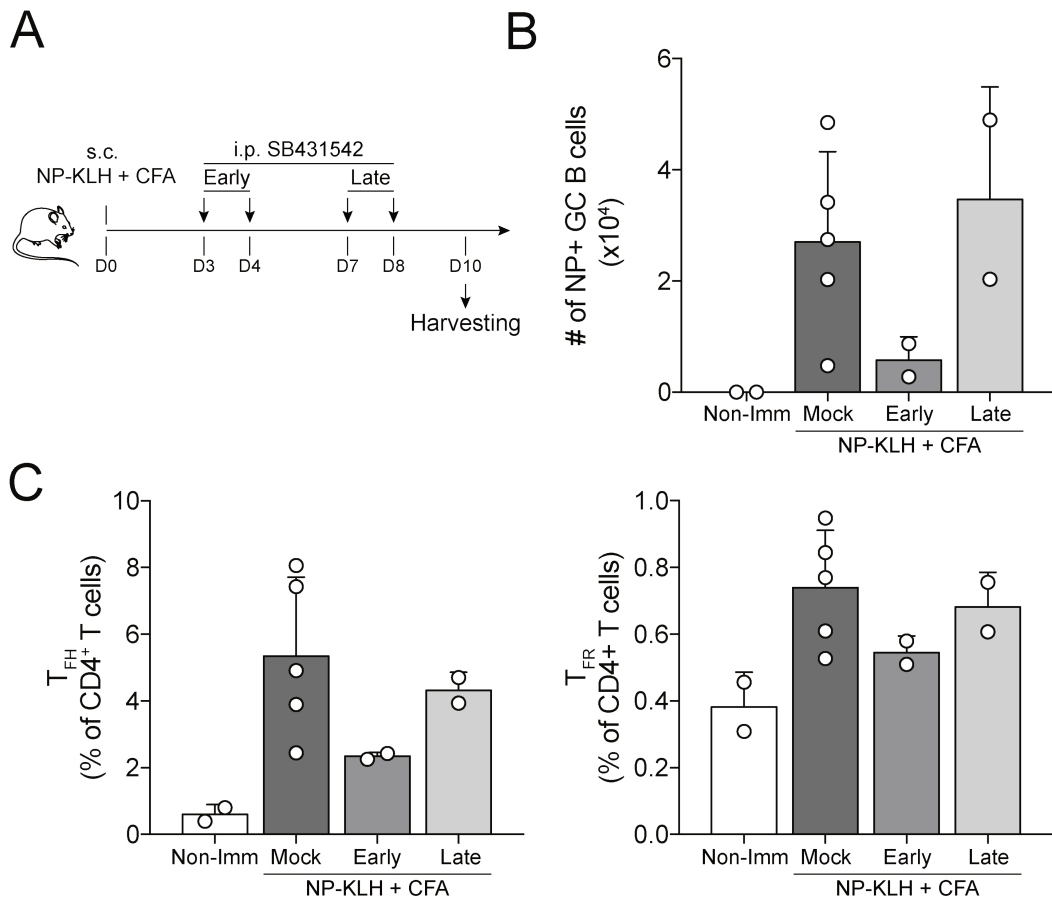
- Third, much like for DC, it is likely that *Itgb8* expression is upregulated in activated cells, upon TCR or cytokine stimulation during the GCR, at a certain stage of T<sub>FH</sub> lifetime when TGF $\beta$  presentation is required. Whether this expression would then be downregulated or persists on T<sub>FH</sub> until their death or into the memory phase still needs to be investigated. It is unfortunately difficult with our current tools to follow a single cell to assess its temporal expression of  $\beta 8$  throughout the GCR. Intravital confocal microscopy coupled with transfer of *Itgb8*-tdTomato mice into a congenic host might reveal some insight in this complex question.

Regardless of the mechanisms regulating  $\alpha_v\beta 8$  expression on T<sub>FH</sub>, it is interesting to wonder about the potential role that  $\alpha_v\beta 8$ -mediated TGF $\beta$  activation could have on T<sub>FH</sub> function. In the absence of a pronounced phenotype on our CD4- $\beta 8^{\text{KO}}$  mice, it is tricky for us to formerly conclude on this matter but critical review of the literature and of the previously described function of TGF $\beta$  on B cells might give an insight into this matter.

It has been shown that  $\alpha_v\beta 8$ -mediated activation of TGF $\beta$  by DC to naïve T cells for the induction of T<sub>H17</sub> required cell-cell contact (Acharya et al. 2010). Furthermore, a recent publication has proposed that latent TGF $\beta$  can be activated by  $\alpha_v\beta 8$  integrins without the need to be released from the GARP/ $\alpha_v\beta 8$  complex and thus can be directly presented to TGF $\beta$  receptor (Campbell et al. 2020). These data suggest that cell-cell interaction and probably cognate interaction of  $\alpha_v\beta 8^+$  T<sub>FH</sub> is required for the activity of the  $\alpha_v\beta 8$  integrin to happen. In that sense, it is very likely that active TGF $\beta$  presentation by T<sub>FH</sub> would be made to GC B cells of the same antigen-specificity during their cognate interaction in the GC. The potential presentation of active TGF $\beta$  from T<sub>FH</sub> to GC B cells raises the question of the potential role of T<sub>FH</sub>-activated TGF $\beta$  in the control of the GC.

TGF $\beta$  signaling on B cells has mostly been described to limit B cell survival (inducing apoptosis and limiting B cell responsiveness) and promote CSR (IgA, IgG2b or IgG3 CSR) (McIntyre et al. 1993; Snapper et al. 1993; Cazac and Roes 2000; Lebman and Edmiston 1999; Stavnezer and Kang 2009).

- To imagine that  $\alpha_v\beta 8$ -mediated TGF $\beta$  delivery to B cell by T<sub>FH</sub> in a context where it limits B cell proliferation and survival is incoherent with the multiple description throughout the literature that T<sub>FH</sub> promote B cell survival and proliferation in the GC B cell selection process.
- It is appealing to imagine that  $\alpha_v\beta 8$  expression could help T<sub>FH</sub> to deliver active TGF $\beta$ , leading to the promotion of B cells CSR. However, it has recently been shown that CSR occurs infrequently during the GCR but rather is predetermined before the GC is fully developed (Roco et al. 2019). Furthermore, Reboldi and colleagues describe a mechanism in the PP where B cells are instructed for CSR by Sub-Epithelial Dome cDC at the pre-GC stage after antigen encounter and before entering the GC.
- Lastly, one could imagine that presentation of TGF $\beta$  by T<sub>FH</sub> to B cells could be one of the mechanism that allow them to cycle back to the LZ as proposed by Albright and colleagues (Albright et al. 2019). The authors however suggest that TGF $\beta$ -signaling in GC B cells is independent of T cell help but their one-day antibody depletion of T cells does not very convincingly make that point.



**Figure 19 - Early but not late administration of the SB431542 TGF $\beta$ -signaling inhibitory molecule prevents the proper development of the GCR.**

(A) – Protocol used for the immunization and administration of SB431542.

(B & C) – Quantification of the number of NP-specific GC B cells (B) and frequency of the follicular T cell population in the draining lymph nodes at day 10.

Each point represents an individual mouse.

These reflections suggest that a role for  $\alpha_v\beta_8$  expression by  $T_{FH}$  for the direct control of B cell is unlikely and raise the questions of whether  $\alpha_v\beta_8$ -mediated TGF $\beta$  activation by  $T_{FH}$  could impact directly T cell responses and in particular  $T_{FH}$  development in an autocrine manner. The role of TGF $\beta$  on  $T_{FH}$  development is still controversial. In the context of viral infection, TGF $\beta$  signaling has been shown to be critical for the development of  $T_{FH}$  in the lung mucosa (Marshall et al. 2015). However, attenuated TGF $\beta$  signaling on T cells induces an accumulation of  $T_{FH}$  in response to peripheral immunization (McCarron and Marie 2014). It is therefore once again hard to speculate on the potential role of  $\alpha_v\beta_8$  in the control of GC T cell response. The new methods currently developed in the lab, in particular the restriction of  $\beta_8$  KO to  $T_{FH}$  only and *in vitro* co-culture of KO  $T_{FH}$  with GC B cells, should shed some light on those issue.

As suggested in the introduction, the timing of TGF $\beta$  delivery, early or later in the GCR, might greatly influence the output of TGF $\beta$  signaling on T or B cells. We are currently in the process of developing an assay, using the synthetic SB431542 TGF $\beta$  inhibitor to inhibit TGF $\beta$  signaling at different timepoint of the GCR and investigate the potential effect on the GCR. Preliminary experiments with this compound suggest that early (D3&D4 post-immunization) but not late (D7&D8 post-immunization) administration of the molecule highly inhibits the generation of the GC response, inhibiting GC,  $T_{FH}$  and to some extent  $T_{FR}$  development. (Fig. 19).

### Integrated model for $\alpha_v\beta_8$ expression on $T_{FR}$

The field of follicular regulatory T cells ( $T_{FR}$ ) is recent, with the first observations of  $T_{REG}$  being able to suppress  $T_{FH}$  and GC response in the 2000's and explosion of the field in the 2010's. The study of these particular cells has led to the development of several models of  $T_{FR}$  depletion and *in vitro* culture models. Overall, these studies have demonstrated very different functions for  $T_{FR}$  cells, showing both a negative (suppressive) and positive regulation on the GCR. More recently, it has been shown by Aloulou et al. that  $T_{FR}$  can arise from both self-reactive thymic natural  $T_{REG}$  (natural  $T_{FR}$ ,  $nT_{FR}$ ) and from naïve T cells (induced  $T_{FR}$ ,  $iT_{FR}$ ) in which case the  $T_{FR}$  is specific for the immunizing antigen (Aloulou et al. 2016). It has been proposed that the different observed functions of  $T_{FR}$  can be attributed to the involvement of  $nT_{FR}$  and/or  $iT_{FR}$  in a particular experimental setting (extensively reviewed in (Fazilleau and Aloulou 2018)).

In our study of  $\beta_8$  expression by  $T_{FR}$ , we have not investigated the antigen specificity and origin of  $\alpha_v\beta_8$  expressing  $T_{FR}$ . Although a large majority of  $T_{FR}$  express the  $\beta_8$  integrin subunit, one cannot exclude the possibility that  $\alpha_v\beta_8$  expression might be slightly different on  $nT_{FR}$  and  $iT_{FR}$ . When observing follicular T cells at immune homeostasis in the PP, MLN or the systemic compartment, it is still unknown what is the specificity of the  $T_{FR}$ . It is reasonable to assume that most of the  $T_{FR}$  would be of natural origin, being generated by the stimulation of thymic  $T_{REG}$  in the mucosa by stimulation with self or self-associated peptides and recirculating to peripheral SLO. However, in our immunizing scheme (See Results - 3.2), the adjuvants used in NP-KLH immunization and the ability to detect CT-specific  $T_{FR}$  allow us to have an insight into this issue. It is important to note that these results are preliminary and the interpretation and the potential role of  $\alpha_v\beta_8$  on  $T_{FR}$  developed below is mostly speculative but develops an interesting model on which to continue this study.

The use of CFA adjuvant with the NP-KLH immunization is a poor inducer antigen specific T<sub>FR</sub> (Aloulou et al. 2016). It is therefore likely that most of the T<sub>FR</sub> observed in the axillary lymph nodes before and following immunization are nT<sub>FR</sub> (Results - Fig 3.2F). In this experiment, the percentage of  $\beta 8^+$  total T<sub>FR</sub> is not modified (in contrast to the CT-specific T<sub>FR</sub>) suggesting a tight regulation of  $\alpha_v\beta 8$  expression by these cells.

It has recently been shown that T<sub>FR</sub> play an important role in the prevention of autoimmune disease. Using the FoxP3-Bcl6<sup>KO</sup> mouse model, the author show that the lack of T<sub>FR</sub> induces the development of late-onset spontaneous autoimmune disease (Fu et al. 2018; Xie et al. 2019). Given the self-reactivity of nT<sub>FR</sub>, it is possible to imagine that the  $\alpha_v\beta 8$ -expressing nT<sub>FR</sub> can interact in a cognate fashion with autoreactive B cells, present TGF $\beta$  and prevent their proliferation and development (Fig. 20). Furthermore, it is entirely possible that cross-reactivity of naïve T cell to self- or self-associated peptide might lead to the induction of iT<sub>FR</sub> with a self-reactivity which could also participate in the prevention of autoimmunity. However, the positive selection of high self-affinity clone during the thymic selection probably limits this possibility.

In our CD4- $\beta 8^{\text{KO}}$  mouse model, we are currently investigating if these mice develop any auto-immune symptoms. Preliminary analysis of the auto-antibodies of 2 year-old mice indicated that  $\beta 8$  deletion on T cells does not impact age-related development of auto-antibodies, as a similar frequency of  $\beta 8$ -deficient mice displayed anti-nuclear antibodies (measured on Hep2 cells) compared to the control littermate population (data not shown).

In the case of 'induced' T<sub>FR</sub> such as those generated following CT immunization (Results – Fig. 3.2F), we show that at day 9 post-immunization the percentage of  $\beta 8^+$ Tetramer<sup>+</sup> T<sub>FR</sub> is much lower (~60%) than it is on total (bulk) T<sub>FR</sub> (80-90%). We hypothesized that iT<sub>FR</sub> newly generated from  $\alpha_v\beta 8$ -negative naïve T cells will likely be  $\alpha_v\beta 8$ -negative while they acquire *Foxp3* and *Bcl6* expression and upon activation throughout the GCR will progressively acquire the  $\beta 8$  integrin.

The signals, through which T<sub>FR</sub> might acquire  $\alpha_v\beta 8$  expression, are still unknown but TCR stimulation (as is the case for T<sub>REG</sub>) by antigen presenting B cells is a likely candidate. Additionally, it has been shown that the deletion of the mTORC1 complex lead to the complete extinction of *Itgb8* expression in T<sub>FR</sub>, suggesting that  $\beta 8$  expression might also be regulated by various metabolic pathways. (Xu et al. 2017).

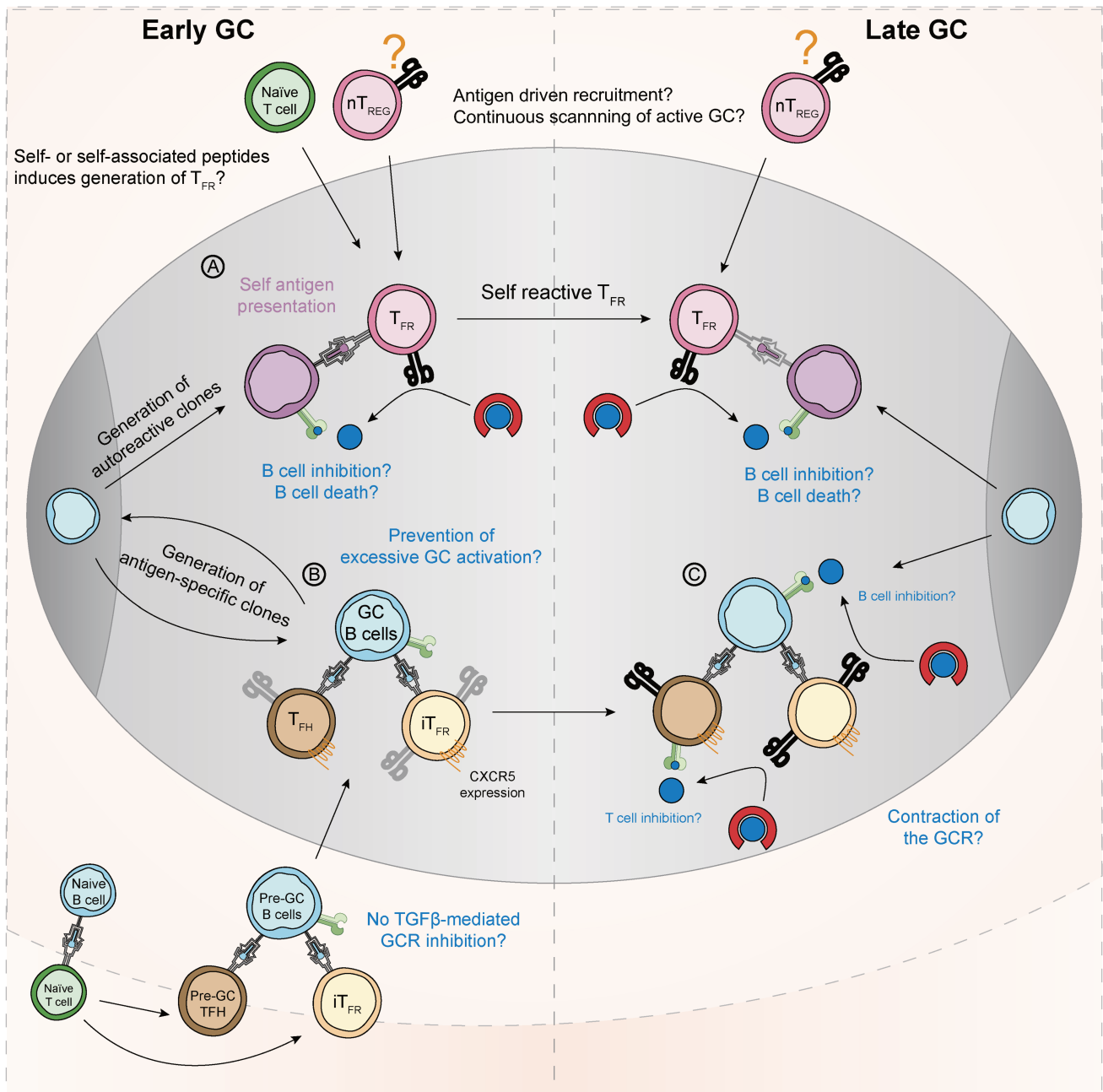
The initial absence of  $\alpha_v\beta 8$  on T<sub>FR</sub> and the later acquiring through activation might be one of the mechanisms which might initially allow the GCR to take place but later participate in the retraction and prevents the over-activation of the GCR (Fig. 20).

In the paradigm developed above,  $\alpha_v\beta 8$  expression on nT<sub>FR</sub> would be required to prevent the development of autoimmune B cell clone, while  $\alpha_v\beta 8$  expression by iT<sub>FR</sub> would participate in the contraction of the GCR or preventing the over-reaction of the GC after the primary encounter of the antigen. At this moment, we cannot hypothesize if the presentation of  $\alpha_v\beta 8$ -activated TGF $\beta$  would be directly to B cells or to antigen-specific T<sub>FH</sub>. It has been shown, in the case of DC presentation of TGF $\beta$  to T cells, that cell-cell contact is required of  $\alpha_v\beta 8$  to present activated TGF $\beta$  (Acharya et al. 2010; Campbell et al. 2020). In the case of direct presentation of TGF $\beta$  by T<sub>FR</sub> to B cells, it is possible to imagine the cognate interaction of nT<sub>FR</sub> with auto-reactive and of iT<sub>FR</sub> with B presenting the immunizing antigen peptide. In the context of T<sub>FR</sub> presentation of TGF $\beta$  to T<sub>FH</sub>, how close contact

interaction is made possible remains an open question. Here, it is possible to imagine that  $\alpha_v\beta_8$ -activated TGF $\beta$  is presented to T<sub>FH</sub> during a ménage-à-trois interaction between B cells, T<sub>FH</sub> and T<sub>FR</sub>.

A schematic summarizing the proposed model for the role of  $\alpha_v\beta_8$  on T<sub>FR</sub> can be found in Fig. 20. It is important to note that this schematic is not a representation of demonstrated  $\alpha_v\beta_8$ -mediated function of T<sub>FR</sub> but is simply one model among multiple possible given our current knowledge of  $\alpha_v\beta_8$  function on T<sub>FR</sub> and TGF $\beta$  function on the GCR.

Furthermore, this hypothetical model does not imply that  $\alpha_v\beta_8$ -mediated TGF $\beta$  activation is the main mechanism through which T<sub>FR</sub> can control the GC. It is clear that many other mechanisms (CTLA/PD1-dependent) are at play. In our model of conditional  $\alpha_v\beta_8$  KO (CD4- $\beta_8^{KO}$ ), other regulatory mechanisms could also potentially and probably do compensate the lack of  $\alpha_v\beta_8$ -mediated TGF $\beta$  activation. On top of this,  $\alpha_v\beta_8$  mediated TGF $\beta$ -activation by T<sub>FR</sub> might not be solely involved in the negative regulation of the GC. Indeed, recent evidence points towards a role of T<sub>FR</sub> for the positive control of the GCR development (Xie et al. 2019; Wu et al. 2016). T<sub>FR</sub>-mediated control of T<sub>FH</sub> by  $\alpha_v\beta_8$ -activated TGF $\beta$  could be a pathway to improve the GCR response.



**Figure 20 - Proposed model for  $\alpha_v\beta_8$  function in the biology of T<sub>FR</sub>.**

Schematic representation of the role of  $\alpha_v\beta_8$ -mediated TGF $\beta$  activation in the biology of follicular regulatory T cells (T<sub>FR</sub>). T<sub>FR</sub> can arise from both nT<sub>REG</sub> with self-specificity (nT<sub>FR</sub>) or from naïve T cells with antigen-specificity (iT<sub>FR</sub>)

(A) – We have shown that most T<sub>FR</sub> (80-90%) found at immune homeostasis in peripheral organs (likely nT<sub>FR</sub>) express the  $\alpha_v\beta_8$  integrin. T<sub>FR</sub> have been shown to be critical in the control of antibody-mediated autoimmune symptoms development. nT<sub>FR</sub> are a likely candidate for the control of self-specific B cell clone by their ability to recognize self-peptide through their TCR molecule. We hypothesized that these nT<sub>FR</sub> express the  $\alpha_v\beta_8$  integrin independently of the progression of the GCR which will help them inhibit the development of these autoimmune clones. The recruitment of nT<sub>FR</sub> into the GC might be due to a constant scanning of active GC or to the presentation of self-antigen to nT<sub>REG</sub> at the T/B border inducing the transcription of *Bcl6* and the follicular T cell transcription pathway.

(B-C) – Upon interaction with antigen-presenting B cells at the T/B border, naïve T cells can differentiate into T<sub>FH</sub> and iT<sub>FR</sub>. Initially  $\alpha_v\beta_8$ -negative, we hypothesize that newly generated iT<sub>FR</sub> will not express the  $\alpha_v\beta_8$  integrin (B).  $\alpha_v\beta_8$  expression will then progressively be acquired upon TCR stimulation by antigen-specific GC B cells (C). We hypothesize that this progressive acquiring of  $\alpha_v\beta_8$  is one of the mechanisms through which T<sub>FR</sub> can exert their inhibition of the GCR, prevent over-activation of the GC and eventually participate in the contraction of the GCR.

## $\alpha_v\beta_8$ expression is not restricted to CD4<sup>+</sup> T cells and intestinal DC

The development of the *Itgb8*-tdTomato mouse model has been a huge help in further characterizing the expression profile of the  $\beta_8$  integrin. Further studies will allow us to investigate further the relevance of  $\alpha_v\beta_8$  expression by the following populations.

### TGF $\beta$ and TGF $\beta$ activation in Follicular Dendritic Cells (FDC) biology.

We have shown that around 30% of PP and MLN but not SPL FDC express the  $\beta_8$  integrin. A few studies have already studied the importance of TGF $\beta$  in the biology of FDC.

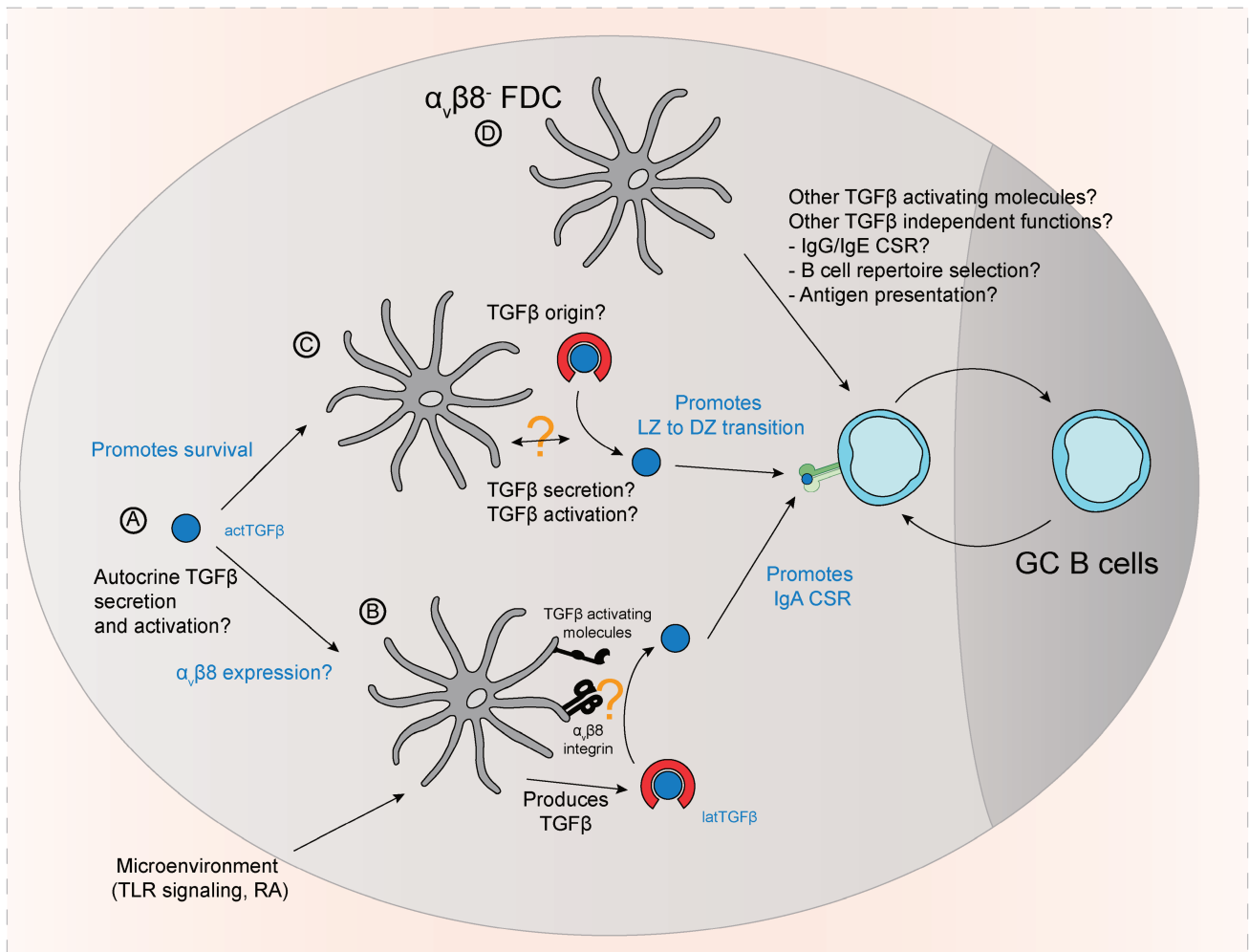
In the study of Follicular non-Hodgkin's lymphomas (FL), Munoz *et al.* provide evidence that FDC-like cells can provide TGF $\beta$  which induces Smad1 phosphorylation in FL-derived cell lines (Munoz *et al.* 2004). Although this study did not investigate the specific biological relevance of FDC-secreted TGF $\beta$  on FL B cell biology, this was to my knowledge the first description of TGF $\beta$  secretion by FDC.

In the gut, several studies have highlighted the ability of FDC to sense the microenvironment through TLR signaling (Deshane and Chaplin 2010; Garin *et al.* 2010; Suzuki *et al.* 2010). Furthermore, Suzuki *et al.* provide evidence that TLR and RA signaling in the gut allows FDC to acquire a 'gut signature' (Suzuki *et al.* 2010). The authors elegantly showed that mucosal FDC supported IgA CSR by through secretion of BAFF and TGF $\beta$ . Furthermore, the 'mucosal signature' of FDC is characterized by the upregulation of TGF $\beta$  and TGF $\beta$ -activating molecules (*Mmp2*, *Mmp9*, *Itgav*) further suggesting that TGF $\beta$  activation might be achieved by FDC themselves. Our observation that some PP and MLN FDC express the  $\beta_8$  subunit reinforce the concept that mucosal FDC can promote TGF $\beta$ -dependent responses, such as IgA CSR, thanks to their expression of TGF $\beta$  activating molecules. Further studies investigating the importance of this expression of  $\beta_8$  would be highly interesting. A conditional KO of  $\alpha_v\beta_8$  on FDC could potentially be achieved using the Cr2<sup>Cre</sup> mice. In Cr2<sup>Cre</sup> mice, the Cre-recombinase is expressed in mature B cells and FDC. Irradiation and reconstitution of the Cr2<sup>Cre</sup>.*Itgb8*<sup>flox</sup> immune compartment with WT BM could generate FDC-specific deletion of *Itgb8*.

Finally, a very recent paper by Albright and colleagues has demonstrated that PP FDC are essential to provide TGF $\beta$  to GC B cells. In turn they show that the Smad2-mediated TGF $\beta$ R signaling in B cells is essential to promote the LZ to DZ transition in the GC and subsequently improves antigen affinity. Whether FDC-mediated TGF $\beta$  signaling in GCB is mediated by TGF $\beta$  secretion and/or TGF $\beta$  activation and whether presentation of active TGF $\beta$  is made directly by FDC or through another cell type still needs to be investigated (Albright *et al.* 2019).

Overall, the authors of the previous studies and our data suggest that FDC have a gut-specific expression pattern, the preferential expression of TGF $\beta$ -activating molecule being one of them (Suzuki *et al.* 2010). It is interesting to note that this resembles more the expression pattern of  $\alpha_v\beta_8$  by DC, which is restricted to the MLN DCs, rather than by follicular T cells with ubiquitous expression pattern in mucosal and non-mucosal SLP. Restriction of  $\alpha_v\beta_8$  expression to the GALT is in agreement with the presentation of activated TGF $\beta$  by FDC to promote IgA responses. However, how the regulation of the LZ to DZ transition might fit into this tissue specific gene signature and expression of TGF $\beta$ -activating molecules still needs to be further studied.





**Figure 21- TGFβ and TGFβ activation in Follicular Dendritic Cells (FDC) biology.**

Schematic representation of the role of TGFβ and TGFβ activation in the biology of FDC. TGFβ has been shown to both promote FDC survival while at the same time being part of the molecular toolkit used by FDC for the control of the GCR.

(A) – TGFβ signaling on FDC promotes their survival by preventing Fas-mediated apoptosis (Park et al. 2005). The origin of this active TGFβ is still unknown. Given their ability to produce and activate TGFβ (see point 2), an autocrine secretion and activation of TGFβ could be hypothesized. Furthermore, whether this TGFβ signaling could induce αvβ8 expression by FDC is an enticing hypothesis but would require further demonstrating.

(B) – TLR and RA signaling in PP FDC promotes their expression of the TGFβ cytokine as well as TGFβ activating molecules (Mmp2, Mmp9, Itgav) which licenses them to promote IgA CSR in the GALT (Suzuki et al. 2010). Whether their expression of the αvβ8 TGFβ activating molecule is involved in this process is still unknown.

(C) – When studying the importance of TGFβ signaling on GC B cells, Albright et al. show that Cr2 expressing stromal cells are important to promote TGFβ signaling on GC B cells independently of CD4+ T cells. Whether FDC are important for TGFβ secretion and/or activation and whether presentation of active TGFβ is made directly by FDC or through another cell type has not been investigated.

(D) – Single cell analysis of β8 expression reveals that about 70% of FDC do not express the integrin. Whether this is indicative of a different function (regulation of TGFβ-independent mechanism), of a different maturation state or that other TGFβ activating molecules are expressed by this particular subset is still unknown.

The bimodal expression of  $\alpha_v\beta_8$  by FDC is also intriguing. To our knowledge, different FDC subsets have never been described. This raises the question to whether some FDC could be specialized to induce IgA CSR as opposed to other specialized for GCB selection, whether this is a function of their localization within the GC or whether this is a function of their differentiation status.

Lastly, it has been shown that TGF $\beta$ -induced Smad2 signaling on FDC decreases Fas expression (Park et al. 2005) which prevents the TNF-induced apoptosis of FDC and thus to maintain a function FDC network throughout the GCR. Furthermore, we have shown that TGF $\beta$  signaling coupled with RA and TLR ligand stimulation, of which there is plenty in the gut, induces *Itgb8* expression of cDC. Investigating if TGF $\beta$  signaling is also responsible for *Itgb8* expression by FDC would be interesting. Conversely, one can wonder if *Itgb8* expression by FDC and their activation of TGF $\beta$  could be one of their mechanism for self-sustenance in the gut lymphoid organs.

A model combining the different role for TGF $\beta$  and TGF $\beta$  activation for FDC biology can be found on Fig. 21.

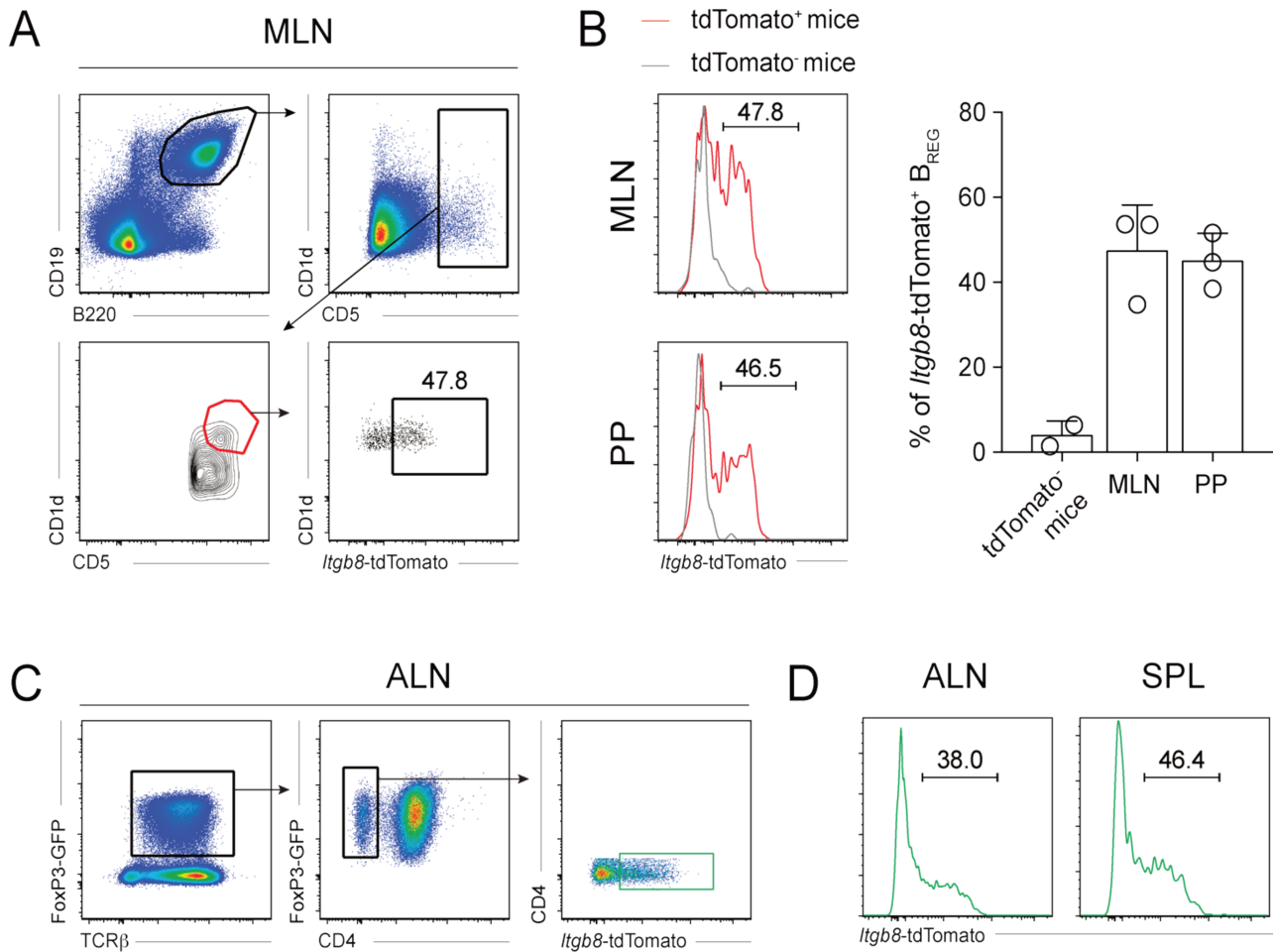
### $\alpha_v\beta_8$ expression by B<sub>REG</sub> and CD8<sup>+</sup> T<sub>REG</sub>

The immunomodulatory role of TGF $\beta$  is now well established with a critical role in the function and differentiation of regulatory T cells (T<sub>REG</sub>). However, numerous studies have shown that TGF $\beta$  is also critical in the function of other regulatory cells, namely CD8<sup>+</sup> T<sub>REG</sub> and B<sub>REG</sub>.

B<sub>REG</sub> are a subsets of B cells that arise in a wide variety of pathologies and inflammatory conditions and participate to the resolution of this inflammation by various mechanism. B<sub>REG</sub> have been found in different mouse models or human infectious disease (*Schistosoma*, *Brucella abortus*), allergic disease (allergic airway disease, allergy-induced intestinal inflammation), anti-tumoral immunity (breast tumor model, glioma), autoimmune disease (Rheumatoid Arthritis, experimental autoimmune encephalitis, collagen induced arthritis) and transplantation (islet or kidney transplantation) among others. In all those pathologies, the secretion of TGF $\beta$  has been suggested to be central to their immunomodulatory functions (reviewed in (Dai, Zhong, and Xu 2017; Rosser and Mauri 2015; van de Veen et al. 2016; Wortel and Heidt 2017; Y. Zhang, Gallastegui, and Rosenblatt 2015)). Although this varies according to the pathology studied, TGF $\beta$  secreted by B<sub>REG</sub> has been mostly involved in the regulation of T cell immunity, through the generation of T<sub>REG</sub> and inhibition of CD8<sup>+</sup> T cell or T<sub>H1</sub>/T<sub>H2</sub> responses.

Similarly, CD8<sup>+</sup> T<sub>REG</sub> have been shown to have an immunosuppressive function in a wide variety of pathological conditions. TGF $\beta$  is important for the immunomodulatory function of CD8<sup>+</sup> T<sub>REG</sub>. When studying Hepatitis C infection and Colorectal cancer or Systemic Lupus Erythematosus, secretion of TGF $\beta$  by CD8<sup>+</sup> T<sub>REG</sub> has been associated with decrease effector T cell and B cell responses respectively (Vieyra-Lobato et al. 2018).

However, the study of TGF $\beta$  activation in the biology of these regulatory cells has so far been overlooked. As part of my PhD, we have investigated the expression of  $\alpha_v\beta_8$  by B<sub>REG</sub> and CD8<sup>+</sup> T<sub>REG</sub>. Using the *Itgb8*-tdTomato mouse model, we show that close to 50% of steady state PP and MLN CD19<sup>+</sup>B220<sup>+</sup>CD5<sup>hi</sup>CD1d<sup>hi</sup> B<sub>REG</sub> express  $\beta_8$  integrin (Fig 22A-B). Similarly, around 40% of SPL and Axillary LN CD3<sup>+</sup>FoxP3<sup>+</sup>CD4<sup>-</sup> T<sub>REG</sub>, largely constituted of CD8<sup>+</sup> T<sub>REG</sub>, also express  $\beta_8$  (Fig. 22C-D). It is important to note that the population investigated here are steady state population and that this might not reflect the biology of these cells in inflammatory conditions.



**Figure 22 - A subset of regulatory B cells (B<sub>REG</sub>) and CD8<sup>+</sup> T<sub>REG</sub> express the  $\beta 8$  integrin.**

MLN, PP, Axillary LN (ALN), and Spleen (SPL) *Itgb8*-tdTomato.FoxP3<sup>GFP</sup> mice were analyzed for  $\beta 8$  expression in B<sub>REG</sub> (A-B) and CD8<sup>+</sup> T<sub>REG</sub> by flow cytometry.

(A) - Representative gating strategy employed to analyze B<sub>REG</sub>. Cells are pre-gated to exclude T cells

(B) - Representative histogram of *Itgb8*-tdTomato fluorescence (B – left). Quantification of the frequency of  $\beta 8^+$  B<sub>REG</sub> in the MLN and PP. *Itgb8*-tdTomato<sup>-</sup> non-transgenic mice were used as control to evaluate autofluorescence.

(C) - Representative gating strategy employed to analyze CD8<sup>+</sup> T<sub>REG</sub>. Cells are pre-gated to exclude B cells

(D) – *Itgb8*-tdTomato fluorescence in CD8<sup>+</sup> T<sub>REG</sub> of the ALN and SPL.

The expression of  $\alpha_V\beta_8$  by both those regulatory subsets warrants further investigations. It is interesting to note that in previously published work, the conditional deletion of  $\beta_8$  in T cells or  $T_{REG}$  has been achieved using  $CD4^{Cre}$  and  $FoxP3^{Cre}$  animals (Worthington et al. 2015). In both these mouse models,  $\beta_8$  is deleted in total or  $FoxP3^+CD4^+$  but also  $CD8^+$  T cells. This is worth noting as the *in vivo* effect of  $\beta_8$  deletion might be partially due to the loss of  $\alpha_V\beta_8$  on  $CD8^+$   $T_{REG}$ . In their study, Lacy-Hubert et al. have generated the  $CD19^{Cre}.Itgav^{flox}$  mouse model in which the  $\alpha_V$  subunit is deleted in all B cells (Lacy-Hulbert et al. 2007). These mice do not develop spontaneous colitis indicating that  $\alpha_V$  expression by B cells does not appear to have any role in maintaining mucosal homeostasis. However, the study of  $\beta_8$  deletion in other inflammatory conditions in which the requirement for  $B_{REG}$  activation of  $TGF\beta$  is higher might reveal the functional relevance of  $\alpha_V\beta_8$  expression by  $B_{REG}$ .

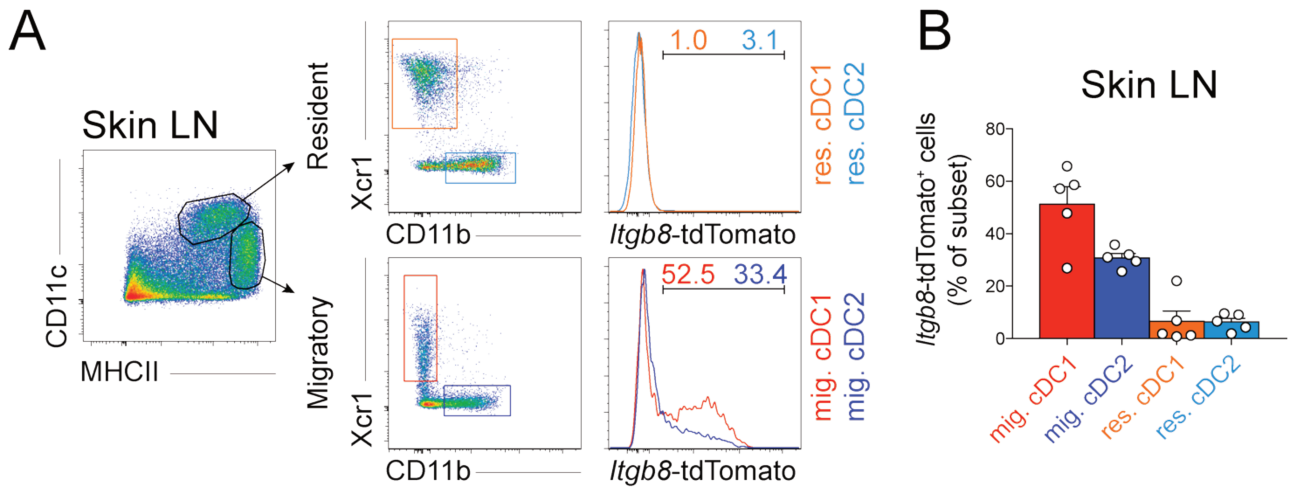
During my PhD, the study of the *Itgb8*-tdTomato mouse model has brought into light that most of the regulatory lineages, namely  $CD4^+$   $T_{REG}$ ,  $T_{FR}$ ,  $CD8^+$   $T_{REG}$  and  $B_{REG}$ , can express the  $\alpha_V\beta_8$  integrin. All these cell populations (except for  $T_{FR}$ ) have been shown to require  $TGF\beta$  for their proper functions. These expression data suggest that a subset (from 35% to 90%) of these cells might be also be specialized for  $TGF\beta$  activation by their expression of the  $\beta_8$  integrin. Alternatively, it is entirely possible that other mechanisms of  $TGF\beta$  activation (thrombospondin-1, ROS, pH, ...) might be expressed by  $\alpha_V\beta_8^-$  regulatory cells to achieve  $TGF\beta$  activation.

### $\alpha_V\beta_8$ expression by DC is not restricted to the intestinal mucosa

When investigating DC expression of  $\alpha_V\beta_8$ , this study has mostly focused on the gut associated compartment (PP, MLN, SILP). However, other mucosal tissues and more broadly epithelial barrier have very similar immune responses.

In the skin, dendritic cells and Langerhans cells (LC) are the major antigen presenting cells. Various studies have shown that all these APC subsets can promote  $T_{REG}$  differentiation or proliferation (Kashem, Haniffa, and Kaplan 2017; Whibley, Tucci, and Powrie 2019). Furthermore, a recent paper has shown that migratory cDC in the skin draining LN express *Itgb8*. The authors of this study show that the exposure of resting naïve  $CD8^+$  T cells to  $\alpha_V$ -expressing DC and  $TGF\beta$  preconditioned them for effective formation of eTRM cells upon activation (Mani et al. 2019). Additionally, it has been suggested that  $\alpha_V\beta_8$  expression by LC could be one of the mechanisms through which they activate  $TGF\beta$  and induced  $T_{REG}$  in the skin (Dioszeghy et al. 2018).

Using our *Itgb8*-tdTomato mouse model, we investigated the tdTomato fluorescence of cDC in the skin draining LN. Consistent with this literature, we found that both mig. cDC1 and mig. cDC2 display tdTomato fluorescence and thus  $\beta_8$  expression at the immune homeostasis (Fig. 23). The expression of  $\alpha_V\beta_8$  by both cDC1 and cDC2 is interesting as  $\beta_8$  expression profile differs from what has been described in the gut compartment. This might be due to the differences in micro-environment and microbiota between the intestine and the skin, although formal studies would be required to investigate this issue. Whether this expression is relevant for the generation of  $T_{REG}$  or other  $TGF\beta$  dependent immune responses in the skin during homeostasis or in the context of tolerance or allergy induction would be very interesting.



**Figure 23 - Skin draining migratory cDC1 and cDC2 express *Itgb8*.**

Inguinal lymph nodes from *Itgb8*-tdTomato mice were harvested and processed for FACS analysis.

(A) – Gating strategy and representative histogram of DC from skin draining lymph nodes as analyzed by FACS analysis.  
 (B) – Frequency of tdTomato+ DC is plotted for each DC subpopulation as gated in (A). Frequency is expressed as a percent of the parent population. Cells are pre-gated on Live CD45<sup>+</sup>CD3<sup>-</sup>B220<sup>-</sup> cells

These previous results suggest that  $\alpha_v\beta_8$  expression by DC is not restricted to the intestinal mucosal but rather is a hallmark of epithelial barrier DC. The expression of this integrin also seems to specialize the epithelial DC for the control of TGF $\beta$ -dependent immune responses.

Overall, we show that the expression of the  $\beta_8$  integrin subunit is expressed by a wide variety of cells, namely cDC, FDC, follicular T cells, CD4<sup>+</sup> and CD8<sup>+</sup> T<sub>REG</sub> and B<sub>REG</sub>. In all of these cell types, apart maybe for T<sub>FR</sub>,  $\beta_8$  is only expressed in a particular subpopulation, possibly specialized for  $\alpha_v\beta_8$ -mediated TGF $\beta$  activation. Ever since the discovery of the TGF $\beta$  molecule in the late 1970's, early 1980's (Moses, Roberts, and Derynck 2016b), the published biological effects of the TGF $\beta$  molecule have been extremely diverse, ranging from inhibition of proliferation, to T cell differentiation, to B cell CSR, all the way to wound healing and carcinogenesis. This highly pleiotropic nature of TGF $\beta$  can be attributed to the differences in the TGF $\beta$ -secreting cell type and to the individual downstream signaling and gene activation in each particular TGF $\beta$ -activated cell type. However, we believe that the study of which cell type activates and present active-TGF $\beta$  in each context can be of great importance when trying to reconcile the pleiotropic nature of this cytokine. The development of the *Itgb8*-tdTomato tool has been and will further be of great use to study the  $\alpha_v\beta_8$ -mediated TGF $\beta$ -activation in the regulation of the immune processes.

The field of  $\alpha_v\beta_8$ -mediated (and other  $\alpha_v$  integrin) TGF $\beta$  activation has recently gained a large attention and the next couple of years might bring into light more and more function of  $\alpha_v\beta_8$ -mediated TGF $\beta$  activation for the regulation of immune responses. In particular, this field has gained a popularity in the study of  $\beta_8$  integrin as a potential therapeutic target in a few TGF $\beta$ -mediated disease. This field is reviewed in the next section.

## Therapeutics

In a few pathologies such as many forms of cancer, airway pathologies and tissue fibrosis, the immunomodulatory functions of TGF $\beta$  have been shown to promote disease progression. In pathologies such as autoimmune or autoinflammatory syndromes, strategies aiming at restoring immunoregulatory pathways are warranted to limit disease progression. Therapeutics approached based on TGF $\beta$  and TGF $\beta$  activation are in the process of being developed. In this section, we review these therapeutics efforts while proposing potential improvement based on the current research being done in the lab.

In many auto-immune diseases such as Multiple Sclerosis (MS), Rheumatoid Arthritis (RA) or Inflammatory Bowel Disease (IBD), the balance between the pro-inflammatory response and the anti-inflammatory T<sub>REG</sub> response is highly critical. In all these disease, T<sub>REG</sub> generation is blunted, therefore skewing the balance towards the inflammatory pathway. Amplification of this imbalance through innate and adaptive pathway then lead to the characteristic high inflammation of the affected tissues (intestine, joints, nervous system, etc.) (Lee 2018; Noack and Miossec 2014). In an effort to contain these disease, inhibition of the inflammatory cytokines (anti-TNF, anti-IL6 or anti-IL17) is often employed as a therapeutic option. Attempts at amplifying the

immunomodulatory side of the balance, TGF $\beta$  signaling and T<sub>REG</sub> generation in particular, have however proven difficult and are still under development.

Although the concentration of the TGF $\beta$  cytokine is high in the intestinal mucosal of IBD patient, TGF $\beta$  signaling has been shown to be deficient, because of an elevated level of the inhibitory protein Smad7. Targeting TGF $\beta$  signaling using a Smad7 antisense oligonucleotide (Mongersen) improves TGF $\beta$  signaling and disease progression in a mouse model of colitis. Mongersen has also shown to induce clinical response and induce some clinical remission, making it a promising TGF $\beta$ -directed therapeutic options for IBD. Additionally, preliminary therapeutic approaches using adoptive T<sub>REG</sub> or tolerogenic DC transfer have been investigated. While DC transfer has shown some promising results, adoptive T<sub>REG</sub> transfer has failed to bring promising results in IBD treatment. The antigen-specificity of transferred T<sub>REG</sub> is likely very important and the ex-vivo generation or expansion of T<sub>REG</sub> might not be the most promising way of boosting the T<sub>REG</sub> response in IBD patients. These therapeutic options are extensively reviewed in (Giuffrida et al. 2019).

The ability to promote the *in vivo* generation of iT<sub>REG</sub> by DC is appealing as this would generate physiological T<sub>REG</sub> with an appropriate antigen-specificity. The ability to manipulate DC subsets to induce T<sub>REG</sub>, even in the particular inflammatory environment of IBD, RA or MS patient, would be a great therapeutic option. In this way, we believe that a better understanding of the mechanisms by which DC subsets can activate and present TGF $\beta$  to T cells and generate T<sub>REG</sub> is critical.

As we and other have shown,  $\alpha_v\beta_8$  expression by mucosal DC is critical to license them to induce peripheral T<sub>REG</sub>. It has been shown that DC of IBD patients have an increased *Itgb8* expression on intestinal CD1c<sup>+</sup> cDC2 (Fenton et al. 2016). We have suggested that cDC2 are able to generate both T<sub>REG</sub> and T<sub>H17</sub> through  $\alpha_v\beta_8$ -mediated TGF $\beta$  activation. We have furthermore hypothesized that the environmental cues detected by those cDC2 might determine the utilization of  $\alpha_v\beta_8$ -activated TGF $\beta$  for the generation of T<sub>REG</sub> or T<sub>H17</sub>. In the inflamed environment of the IBD intestine, it is likely that the high abundance of IL6 rather drives the generation of T<sub>H17</sub> rather than T<sub>REG</sub> by  $\alpha_v\beta_8^+$  cDC2. A recent Genome Wide Association Study (GWAS) on IBD patients has shown that a variant of the *Itgb8* locus, inducing an increase in *Itgb8* expression, was associated with increased IBD risk (de Lange et al. 2017). This further suggests the negative outcome of *Itgb8* expression by LP cDC2 in IBD patients. However, given the ability of  $\alpha_v\beta_8$  expressing cDC1 to induce iT<sub>REG</sub> and the poor ability of this subset to induce T<sub>H17</sub>, the ability to induce  $\beta_8$  expression on this particular subset of mucosal DC, through bio-engineered or pharmaceutical means, might improve the generation of T<sub>REG</sub> over T<sub>H17</sub>.

Alternatively, downregulating *Itgb8* expression by DC in patients with such inflammatory diseases might also decrease their ability to generate T<sub>H17</sub> in response to the environmental cues. In the EAE model, Acharya and colleagues show that injection of cyclic RGD peptides, preventing the binding of  $\alpha_v\beta_8$  to its ligand, TGF $\beta$  in particular, inhibits the development of T<sub>H17</sub> responses and delays the onset of the clinical signs (Acharya et al. 2010). Furthermore, AP-1, SP3 transcription factors and the p38 signaling pathway are critical in the control of *Itgb8* expression (Markovics et al. 2010). Furthermore, REG $\gamma$  (*Psmc3*) was recently described as a negative regulator of *Itgb8* expression. These studies provide clues as to the potential target for a therapeutic approach to control  $\beta_8$  expression.

The anti-inflammatory properties of TGF $\beta$  can have some adverse effect and promote disease progression. In pulmonary fibrotic conditions, TGF $\beta$  signaling on stromal cells induces myofibroblast accumulation, epithelial cells apoptosis and extracellular matrix remodeling all participating in the increase of lung fibrosis (Yue, Shan, and Lasky 2010). In this context, TGF $\beta$  activation is mainly mediated by  $\alpha_v$ -integrins ( $\alpha_v\beta_5$ ,  $\alpha_v\beta_6$  and  $\alpha_v\beta_8$ ) (Tatler and Jenkins 2012). Similarly, in IBD patients, TGF $\beta$  signaling is responsible for increased fibrotic scarring (Yun, Kim, and Kim 2019). In the study of tumorigenesis, TGF $\beta$  has been shown to induce extracellular matrix (ECM) deposition, myofibroblast differentiation, and angiogenesis, and to suppress both the innate and adaptive immune systems, hence promoting disease progression (Akhurst 2017). In both these contexts, the activation of TGF $\beta$  by  $\alpha_v$  integrins and in particular  $\alpha_v\beta_8$  has been well documented (reviewed in (Nolte and Margadant 2020; Goodman and Picard 2012)).

As a therapeutic option, blocking of the TGF $\beta$  molecule or the TGF $\beta$  signaling have been tested as potential therapeutic target to reduce disease progression (Yue, Shan, and Lasky 2010; Akhurst 2017). However, the manipulation of TGF $\beta$  activation by integrins, and in particular by  $\alpha_v\beta_8$  in the treatment of these conditions have already been documented. In particular, the use of an  $\alpha_v\beta_8$  blocking antibody to prevent disease progression has been demonstrated in multiple studies in the context of fibroinflammatory airway diseases, tumorigenesis (Minagawa et al. 2014; Takasaka et al. 2018). These studies have demonstrated that this antibody can inhibit TGF $\beta$  activation by  $\alpha_v\beta_8$  by lung stromal cells and DC, cancer cells, and T<sub>REG</sub>. These studies suggest the clinical potential of  $\alpha_v\beta_8$  activity inhibition in the treatment of TGF $\beta$ -mediated disease.

Alternative targeted inhibition of  $\alpha_v\beta_8$  expression could also be achieved. Recent descriptions of a highly specific cyclic peptide for the targeting of  $\alpha_v\beta_8$  activity and other small molecule  $\alpha_v$  integrin inhibitors could also improve cost-effectiveness of anti- $\beta_8$  treatment (Reichart et al. 2019; Hatley et al. 2019).

However, these methods will most likely not provide lasting inhibition of  $\alpha_v\beta_8$  functions, requiring prolonged treatment. Our team has recently identified a putative active enhancer near the *Itgb8* locus (-74kb) which we believe to be directly responsible for *Itgb8* expression. The chromatin at this locus is preferentially opened in MLN cDC1 compared to cDC2 as measured by ATAC-seq (data not shown). Studies are under way to determine if the genetic or epigenetic manipulation of this locus could regulate *Itgb8* expression. In particular, Crispr-Cas9 inactivation of this locus is being performed to evaluate if  $\beta_8$  expression could be shut down efficiently. If effective, targeted mutation of the -74kb locus could be a method to specifically and permanently decrease *Itgb8* expression in tumor cells or tumor infiltrating T<sub>REG</sub> for example and provide long lasting inhibition of  $\alpha_v\beta_8$ -mediated TGF $\beta$ -activation.

## Mucosal vaccination

Mucosal vaccination and the induction of proper protective IgA responses have proven to be challenging. Apart from a few existing vaccines, the development of novel mucosal (oral, nasal, ...) based vaccination strategies have been hard to develop. A lot of hurdle, may they be physical and chemical (stomach acid, proteolytic enzymes, ...) or immunological (mechanisms of oral tolerance) have hindered the development of oral vaccine. A lot of strategies are developed to overcome those hurdles such as next generation adjuvant or delivery



system. But one approach which we find particularly interesting is to try to induce mucosal protection through systemic immunization (all reviewed in (Lycke 2012; Vela Ramirez, Sharpe, and Peppas 2017; Azegami, Yuki, and Kiyono 2014)).

The regulation of IgA CSR by mechanisms of TGF $\beta$  activation ( $\alpha_v\beta_8$  among other) has now been demonstrated in a few studies (Suzuki et al. 2010; Ruane et al. 2016; Reboldi et al. 2016; Nakawesi et al. 2020). All these studies suggest that the expression of TGF $\beta$  activating integrins by either DC or FDC specializes these cells for IgA production. We and other have hypothesize and/or demonstrated that the sensing of environmental cues by DC and FDC could induce the expression of these TGF $\beta$ -activating integrins and according to the stimulus promote various TGF $\beta$  dependent responses. We therefore speculate that given the right combination of environmental cues in conjunction with the desired antigen, DC or FDC or other B cell supporting cells (FDC, stromal cells,...) could upregulate TGF $\beta$ -activating integrins ( $\alpha_v\beta_8$  in particular) and orient B cell CSR to the IgA class.

It has already been shown that cytokine (TGF $\beta$  itself), metabolites (RA) and TLR stimulation (LPS, ATP or Pam2CSK4) can induce *Itgb8* on DC or TGF $\beta$ -activating molecules on FDC. Further characterization of the precise environmental cocktail required for IgA induction, and the targeting of such adjuvants to the proper helper cells could be one of the mechanisms by which IgA protection in the gut could be induced by systemic immunization. A recent study by Pavot and colleagues describe an antigen-delivery method allowing to simultaneously deliver Nod ligand encapsulated in a Poly(Lactic Acid) (PLA) particle. PLA are efficiently taken up by DC and induce a strong upregulation of activation markers. Furthermore, this adjuvant is much more efficient to generate humoral responses to the encapsulated antigen. Encapsulating TGF $\beta$ , RA or mucosal specific metabolites in the particle could be a way to induce *Itgb8* expression on DC and promote IgA response.

## Other research output.

During the course of my PhD, I have had the opportunity to be involved in additional studies through my collaboration with other teams in Lyon. These studies have led to the publication of 2 additional peer-reviewed papers ((Boucard-Jourdin et al. 2016; Webster et al. 2017) see below for details).

In addition, although not included in this manuscript, my collaboration with Dr. Thierry Defrance on PC biology during the reviewing process of Dr. Pascal Blanc work led to the publication an article in the *Nature Communications* journal (Blanc et al. 2016). In parallel, the project I had been involved in during master internship also came to fruition during the course of my PhD with the publication of two studies in *Gastroenterology* and *Nature Medicine* journals in which I was included (Hegazy et al. 2017; West et al. 2017). Finally, Helena and I have had the opportunity to take part in a recent project of the Immunological Genome consortium (ImmGen), the 'ImmGen MNP OpenSource project'. This project aimed at generating RNAseq data from various mononuclear phagocytes (MNP) in mice. We participated by isolating MLN and PP DC subsets for their analysis by RNAseq. The data generated is available on the ImmGen project website (<http://rstats.immgen.org/Skyline/skyline.html>). A paper using these data for the comparative analysis of MonoNuclear Phagocytes metabolic profile and for which I am a co-author is currently under review in *Nature Immunology*.

## $\beta 8$ Integrin Expression and Activation of TGF $\beta$ by Intestinal Dendritic Cells Are Determined by Both Tissue Microenvironment and Cell Lineage.

When I first came into the lab, the study of the regulation of  $\alpha_v\beta 8$  expression by intestinal dendritic cells and the importance of microenvironment cues and cell lineage was coming to an end. I contributed to this study by performing some molecular analyses of  $\alpha_v\beta 8$  expression by MLN DC subsets, which allowed me to be first immersed in the world of  $\alpha_v\beta 8$  biology for T<sub>REG</sub> generation. This study, to which I am a co-author was published in the *Journal of Immunology* (Boucard-Jourdin et al. 2016).

## Plasmacytoid dendritic cells control dengue and Chikungunya virus infections via IRF7-regulated interferon responses.

A collaboration with the team of Dr. Marlène Dreux in Lyon lead me from the beginning of my PhD to participate in the setup of an *in vivo* murine model for Dengue and Chikungunya virus infection. In collaboration with two post-doctoral fellows from Dr. Dreux Lab, I took part in this study all along my PhD. Although the study is currently still ongoing, part of this study was accepted in the *eLife* journal and is also attached below (Webster et al. 2017).

## Other publications.

### [Itgb8<sup>pos</sup> regulatory T cells promote cancer cell-immune-escape by activating TGFβ present in the tumor-microenvironment](#)

Lainé A, Labiad O, Hernandez-Vargas H, **THIS S**, Sanlaville A, Léon S, Dalle S, Sheppard D, Travis MA, Paidassi H, Marie JC.

*Submitted to Cancer Cell*

### [Open Source ImmGen: network perspective on metabolic diversity among mononuclear phagocytes](#)

Gainullina A, Yng LS, Kent A, Jia B, Seddu K, Yan Y, Krchma K, Wu J, Crozat K, Tomasello E, Narang V, Dress R, See P, Todorov H, Scott C, Gibbings S, Bajpai G, Desai JV, Maier B, **THIS S**, Kim K, Wang P, Aguilar SV, Poupel L, Dussaud S, Zhou TA, Angeli V, Blander JM, Choi K, Dalod M, Dzhagalov I, Gautier EL, Jakubzick C, Lavine K, Lionakis MS, Paidassi H, Sieweke MH, Ginhoux F, Guilliams M, Randolph GJ, Benoist C, Merad M, Sergushichev A, Artyomov MN, ImmGen Consortium

*Submitted to Nature Immunology*

### [Circulating and Tissue-Resident CD4+ T Cells With Reactivity to Intestinal Microbiota Are Abundant in Healthy Individuals and Function Is Altered During Inflammation](#)

Hegazy AN, West NR, Stubbington MJT, Wendt E, Suijker KIM, Datsi A, **THIS S**, Danne C, Champion S, Duncan SH, Owens BMJ, Uhlig HH, McMichael A; Oxford IBD Cohort Investigators, Bergthaler A, Teichmann SA, Keshav S, Powrie F

*Gastroenterology, 2017*

### [Oncostatin-M signaling is increased in inflammatory bowel disease and promotes intestinal inflammation by activating gut-resident stromal cells](#)

West NR, Hegazy AN, Owens BMJ, Bullers SJ, Linggi B, Buonocore S, Coccia M, Görtz D, **THIS S**, Stockenhuber K, Pott J, Friedrich M, Ryzhakov G, Baribaud F, Brodmerkel C, Cieluch C, Rahman N, Müller-Newen G, Owens RJ, Kühl AA, Maloy KJ, Plevy SE, Oxford IBD Cohort Investigators, Keshav S, Travis SPL, Powrie F

*Nature Medicine, 2017*

### [Mature IgM-expressing plasma cells sense antigen and develop competence for cytokine production upon antigenic challenge](#)

Blanc P, Moro-Sibilot L, Barthly L, Jagot F, **THIS S**, de Bernard S, Buffat L, Dussurgey S, Colisson R, Hobeika E, Fest T, Taillardet M, Thauinat O, Sicard A, Mondière P, Genestier L, Nutt SL, Defrance T

*Nature Communications, 2016*

# $\beta$ 8 Integrin Expression and Activation of TGF- $\beta$ by Intestinal Dendritic Cells Are Determined by Both Tissue Microenvironment and Cell Lineage

Mathilde Boucard-Jourdin,<sup>\*,†,‡,§,¶,1</sup> David Kugler,<sup>||,1</sup> Marie-Laure Endale Ahanda,<sup>\*,†,‡,§,¶</sup> Sébastien This,<sup>\*,†,‡,§,¶</sup> Jaime De Calisto,<sup>#</sup> Ailiang Zhang,<sup>\*\*</sup> J. Rodrigo Mora,<sup>††</sup> Lynda M. Stuart,<sup>||</sup> John Savill,<sup>\*\*</sup> Adam Lacy-Hulbert,<sup>||,2</sup> and Helena Paidassi<sup>\*,†,‡,§,¶,2</sup>

Activation of TGF- $\beta$  by dendritic cells (DCs) expressing  $\alpha$  $\beta$ 8 integrin is essential for the generation of intestinal regulatory T cells (Tregs) that in turn promote tolerance to intestinal Ags. We have recently shown that  $\alpha$  $\beta$ 8 integrin is preferentially expressed by CD103<sup>+</sup> DCs and confers their ability to activate TGF- $\beta$  and generate Tregs. However, how these DCs become specialized for this vital function is unknown. In this study, we show that  $\beta$ 8 expression is controlled by a combination of factors that include DC lineage and signals derived from the tissue microenvironment and microbiota. Specifically, our data demonstrate that TGF- $\beta$  itself, along with retinoic acid and TLR signaling, drives expression of  $\alpha$  $\beta$ 8 in DCs. However, these signals only result in high levels of  $\beta$ 8 expression in cells of the cDC1 lineage, CD8 $\alpha$ <sup>+</sup>, or CD103<sup>+</sup>CD11b<sup>-</sup> DCs, and this is associated with epigenetic changes in the *Itgb8* locus. Together, these data provide a key illustrative example of how microenvironmental factors and cell lineage drive the generation of regulatory  $\alpha$  $\beta$ 8-expressing DCs specialized for activation of TGF- $\beta$  to facilitate Treg generation. *The Journal of Immunology*, 2016, 197: 1968–1978.

Dendritic cells (DCs) serve a unique sentinel role in the body, surveying tissues, integrating peripheral cues, and instructing the adaptive immune system accordingly. DCs can orchestrate powerful pathogen-directed immunity or regulate and suppress immune responses to self-associated or innocuous Ags, and the complexity of these roles is reflected in the diverse populations of DCs found in different tissues and under different conditions

(1, 2). Determining how DCs differentiate to carry out specialized functions is critical for our understanding of immunity in health and disease.

The intestine provides a particular challenge for the immune system, containing a high local concentration of microbes, including commensals and potential pathogens, as well as diverse dietary and environmental Ags (3). To prevent inappropriate inflammatory responses to these mostly innocuous Ags, the mucosal immune system has robust immunoregulatory mechanisms that include regulatory lymphocytes, which reside in the mucosa and associated lymphoid organs and actively suppress immune responses to intestinal Ags (4). The best characterized of these are CD4<sup>+</sup> Foxp3<sup>+</sup> regulatory T cells (Tregs), which either originate in the thymus (thymic or natural Tregs) or are generated in the periphery from naive CD4<sup>+</sup> T cells (peripheral or adaptive Tregs). In the intestine, peripheral Tregs are generated by DCs that constitutively acquire and present self and foreign Ags (5, 6), resulting in Ag-specific tolerance (7). The generation of peripheral Tregs requires TGF- $\beta$ , and we and others have shown that an essential characteristic of DCs that generate Tregs is their ability to activate TGF- $\beta$  from its inactive or latent precursor to an active form that can engage the TGF- $\beta$  receptor (8, 9). This requires the action of a specific cell surface integrin,  $\alpha$  $\beta$ 8, and, underscoring the importance of this process, deletion of either the  $\alpha$  or  $\beta$ 8 subset from DCs results in failure to generate intestinal Tregs and subsequent development of colitis (10, 11).

Recently, we have shown that expression of  $\alpha$  $\beta$ 8 is tightly regulated in DCs. Although the  $\alpha$  subunit, the only known partner of  $\beta$ 8 (12), is ubiquitously expressed,  $\beta$ 8 expression is restricted to specific subsets of cells in the intestine (8). Under homeostatic conditions,  $\alpha$  $\beta$ 8 is expressed predominantly on DCs from mesenteric lymph nodes (MLN) and intestinal lamina propria (LP) that express the mucosal integrin  $\alpha$ E $\beta$ 7 (CD103), conferring on these cells their preferential ability to activate TGF- $\beta$  and generate Tregs (8, 9). CD103<sup>+</sup> DCs have previously been implicated in the generation of intestinal Tregs, which has also been attributed to their

\*CIRI, Centre International de Recherche en Infectiologie, Université de Lyon, 69365 Lyon, France; <sup>†</sup>INSERM, U1111, 69007 Lyon, France; <sup>‡</sup>Ecole Normale Supérieure de Lyon, 69007 Lyon, France; <sup>§</sup>Université Claude Bernard Lyon 1, Centre International de Recherche en Infectiologie, Lyon, France; <sup>¶</sup>CNRS, UMR5308, 69365 Lyon, France; <sup>#</sup>Benaroya Research Institute, Seattle, WA 98101; <sup>||</sup>Centro de Genómica y Bioinformática, Facultad de Ciencias, Universidad Mayor, Santiago 8580745, Chile; <sup>\*\*</sup>Medical Research Council Centre for Inflammation Research, Queen's Medical Research Institute, University of Edinburgh, Edinburgh EH16 4TJ, United Kingdom; and <sup>††</sup>Gastrointestinal Unit, Massachusetts General Hospital, Boston, MA 02114

<sup>1</sup>M.B.-J. and D.K. contributed equally to this work.

<sup>2</sup>A.L.-H. and H.P. contributed equally to this work.

ORCID: 0000-0002-2936-1649 (D.K.); 0000-0002-0861-1565 (M.-L.E.A.); 0000-0002-3927-1399 (J.D.C.); 0000-0002-6488-156X (A.Z.); 0000-0001-8819-0950 (J.R.M.); 0000-0003-2079-1586 (J.S.).

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Address correspondence and reprint requests to Dr. Helena Paidassi, Centre International de Recherche en Infectiologie, 21, Avenue Tony Garnier, 69365 Lyon Cedex 07, France. E-mail address: helena.paidassi@inserm.fr

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Abbreviations used in this article: DC, dendritic cell; FL-DC, bone marrow-derived DC generated in the presence of Flt3-ligand; IRF, IFN regulatory factor; KO, knock-out; LP, lamina propria; *l*-TGF- $\beta$ , latent TGF- $\beta$ ; MLN, mesenteric lymph node; ODN, oligodeoxynucleotide; RA, retinoic acid; Treg, regulatory T cell; TRIF, Toll/IL-1R domain-containing adapter-inducing IFN- $\beta$ ; VAD, vitamin A-deficient.

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ability to synthesize all-trans retinoic acid (RA) that promotes Treg generation in the presence of TGF- $\beta$ . These data therefore support the concept that subsets of intestinal DCs are specialized for generation of Tregs. However, the precise mechanisms by which this population of DCs acquires this specialized ability to activate TGF- $\beta$  and how microenvironmental cues and cell lineage are integrated in this process remain to be fully determined.

In this study, we set out to identify the signals that regulate  $\beta 8$  expression in intestinal DCs. We report that  $\alpha v\beta 8$  is expressed preferentially on the CD103<sup>+</sup>CD11b<sup>-</sup> subset of DCs in the MLNs, and that expression is acquired in the LP. We show that signals from the mucosal microenvironment, specifically TGF- $\beta$ , RA, and TLR agonists, together promote expression of  $\beta 8$  integrin in DCs and that inhibition of signaling through these pathways in mice leads to reduction in  $\alpha v\beta 8$  expression by DCs. Furthermore, we provide evidence that DC lineage is critical in establishing DC subset-specific expression of  $\beta 8$ , demonstrating that DCs derived from the CD103/CD8 $\alpha$  cDC1 lineage respond more robustly to these conditioning factors to upregulate  $\beta 8$ . Together these data show that the combination of cell lineage, immune mediators, and both dietary and microbe-derived environmental factors shape intestinal DCs into critical gatekeepers of TGF- $\beta$ -dependent immune responses through regulation of  $\beta 8$  integrin expression.

## Materials and Methods

### Mice

All animals were housed under specific pathogen-free conditions at Plateau de Biologie Expérimentale de la Souris (Lyon, France), at Benaroya Research Institute (Seattle, WA), or at Massachusetts General Hospital (Boston, MA). Female C57BL/6 mice from Charles River Laboratories (L'Arbresle, France) or The Jackson Laboratory (Bar Harbor, ME) were used between 6 and 15 wk of age. Vitamin A-deficient (VAD) mice were generated at Massachusetts General Hospital. Pregnant C57BL/6 mice were maintained on a vitamin A-sufficient diet (4 IU/g, TestDiet 58M1) through day 10 of gestation and then switched to a VAD diet (TestDiet 5T2P; both diets from Pharmaserv, Framingham, MA) through weaning. Pups were then maintained on either vitamin A-sufficient or VAD diets. Antibiotic-treated mice were generated at Plateau de Biologie Expérimentale de la Souris. Mice were maintained on drinking water containing 1 g/l metronidazole, 1 g/l ampicillin, 1 g/l neomycin (all from Sigma-Aldrich, St. Quentin Fallavier, France), and 500 mg/l vancomycin (Mylan, St. Priest, France) for 4 wk. Transgenic CD11-Cre [B6.Cg-Tg(Igax-cre)1-1Reiz/J] and *Ccr7*<sup>-/-</sup> [B6.129P2(C)-*Ccr7*<sup>tm1Rfor</sup>/J] mice were obtained from The Jackson Laboratory. *Myd88*<sup>-/-</sup> and *Trif*<sup>-/-</sup> mice were provided by R. Mora (Massachusetts

General Hospital). *Tgfb2*<sup>fl/fl</sup> mice (B6.129S6-*Tgfb2*<sup>tm1Hlm</sup>) were obtained from H. Moses (13) (Vanderbilt-Ingram Cancer Center, Nashville, TN). *Foxp3*<sup>IRES-eGFP</sup> reporter mice were obtained from D. Kaiserlian (Centre International de Recherche en Infectiologie). All mice were on C57BL/6 background, except for *Tgfb2*-*flox* mice, which were on a mixed B6.129S6 background. Animal experiments were performed under appropriate licenses within local and national guidelines for animal care.

### DC isolation

DCs from spleen, MLN, and LP were isolated as previously described (8). Bone marrow-derived DCs generated in the presence of Flt3-ligand (FL-DCs) were cultured, as previously described (14). Briefly, bone marrow cells were cultured in the presence of 100 ng/ml recombinant Flt3-ligand (PeproTech, Neuilly-sur-Seine, France). Fresh medium was added to the cultures on day 3 and 6, and cells were harvested at day 9. FL-DC subsets were then sorted by flow cytometry, as indicated.

### DC conditioning in culture

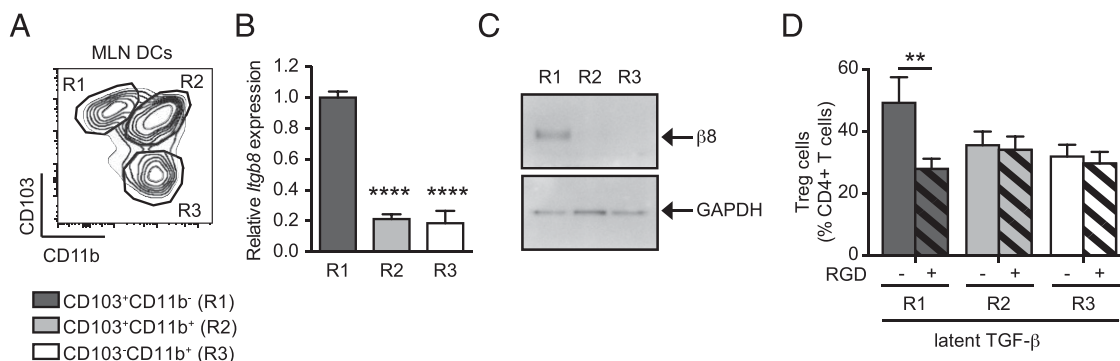
Cells were stimulated in vitro at 37°C in X-vivo 15 medium (Lonza, Levallois-Perret, France) supplemented with MEM nonessential amino acids, 2 mM L-glutamine, 10 mM HEPES, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, and 50  $\mu$ M 2-ME (all from Life Technologies, Saint Aubin, France). Unless stated otherwise, the stimuli were used at the following final concentrations: 0.2 ng/ml TGF- $\beta$  (R&D Biosystems, Lille, France); 0.2  $\mu$ M all-trans RA (Sigma-Aldrich); 5 ng/ml thymic stromal lymphopoietin, 5 ng/ml IL-10, 5 ng/ml IL-1 $\beta$ , and 5 ng/ml IFN- $\gamma$  (all from PeproTech); 2% culture supernatant containing GM-CSF (4 ng/ml final); and 500 ng/ml Pam<sub>3</sub>CSK<sub>4</sub>, 10<sup>8</sup> cells/ml HKLM, 1  $\mu$ g/ml poly(I:C), 100 ng/ml LPS-EK, 100 ng/ml FLA-ST, 100 ng/ml FSL1, 1  $\mu$ g/ml ssRNA40/LyoVec, and 2.5  $\mu$ M oligodeoxynucleotide (ODN)1826 (mouse TLR1-9 agonist kit; InvivoGen, Toulouse, France).

### $\beta 8$ integrin expression analysis

*Igfb8* gene expression by quantitative RT-PCR for MLN and spleen DCs was performed as previously described (8). For LP DCs, *Igfb8* gene expression was quantified by nested RT-PCR using the following pre-amplification primers: forward, 5'-AGTGAACAATAGATGTGGCTC-3' and reverse, 5'-CCGTCATTCGGCACCACCTAT-3'; and quantitative PCR primers: forward, 5'-TGGCCCTTATTCCTGGAC-3' and reverse, 5'-GGGTGGATACTAATGTATGGCGA-3'.  $\beta 8$  integrin protein expression was measured by Western blot, as previously described (8), using an anti- $\beta 8$  antiserum generated in rabbit using the C-terminal tail of the human  $\beta 8$  cytoplasmic region, and provided by Joseph McCarty (15).

### In vitro Treg generation assay

In vitro Treg generation assay was performed, as previously described (8). For RGD blockade experiments, cRGD or control cRAD peptides (both Enzo Life Sciences, Villeurbanne, France) were added at 2  $\mu$ g/ml, as previously described (16).



**FIGURE 1.** CD103<sup>+</sup>CD11b<sup>-</sup> MLN DCs express high levels of  $\alpha v\beta 8$  expression and activate TGF- $\beta$  to generate Tregs. CD11c<sup>+</sup> MHC-II<sup>+</sup> DCs from MLN were sorted by FACS into three populations based on expression of CD103 and CD11b. **(A)** Representative FACS plot gated on CD11c<sup>+</sup>MHC-II<sup>+</sup> DCs from MLN shows gating strategy for isolating indicated populations. **(B)** Quantitative RT-PCR analysis of  $\beta 8$  integrin gene (*Igfb8*) expression relative to  $\beta$ -actin (*Actb*) and presented relative to levels in CD103<sup>+</sup>CD11b<sup>-</sup> DCs (R1) in indicated MLN DC subpopulations. Histogram shows mean  $\pm$  SEM from three individual experiments and at least nine individual mice. **(C)** Western blot analysis of  $\beta 8$  integrin and GAPDH expression in indicated MLN DC subsets. **(D)** FACS-sorted MLN DC subsets were cultured with naive CD4<sup>+</sup>Foxp3<sup>GFP</sup> T cells in serum-free medium with or without addition of latent TGF- $\beta$ , RGD (+, hatched bars), and/or RAD (-, solid bars) peptides, as indicated. After 4 d in culture, Treg generation was assessed by Foxp3<sup>GFP</sup> expression. Data show mean  $\pm$  SEM of individual DC:T cell cultures with six independent pools of MLN DCs from two separate experiments. \*\**p* < 0.005, \*\*\*\**p* < 0.0001, ANOVA with Dunnett's (B) or Sidak's (D) post hoc test.

## Abs

The following Abs were used: anti-CD11c PE-Cy7 (HL3), anti-I-A/I-E FITC (2G9), anti-CD8 $\alpha$  allophycocyanin (53-6.7), anti-CD45RB/B220 PerCP-Cy5.5 (RA3-6B2), anti-CD103 PE (M290), anti-CD172/Sirp $\alpha$ -PE (P84), anti-CD24 FITC (M1/69), and anti-CD11b PE (M1/70; all from BD Biosciences, Pont de Claix, France); anti-I-A/I-E allophycocyanin (M5/114.15.2) and anti-CD103 PerCP-Cy5.5 (2E7; BioLegend, Ozyme, Saint-Quentin-Yvelines, France); and anti-CD86 FITC (B7-2) and anti-CD11b allophycocyanin-eFluor780 (M1/70; eBioscience, Paris, France).

## Computational analysis

Chromatin immunoprecipitation sequencing datasets of the CD24<sup>+</sup> and CD172<sup>+</sup> FL-DCs were retrieved from Gene Expression Omnibus (GSE66899) (17). Raw reads were cleaned using Trimmomatic-0.33 (18) with the following parameters: ILLUMINACLIP:adapters.fa:2:30:10 LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:30. The remaining sequences were aligned to the mouse genome (NCBI37/mm9) by bowtie2 version 2.2.5 with default parameters (19). Density tracks were generated by the *makeUCSCfile* program from the HOMER software suite v4.7 (20). To allow comparison between the two subpopulations, density profiles were normalized based on the library size.

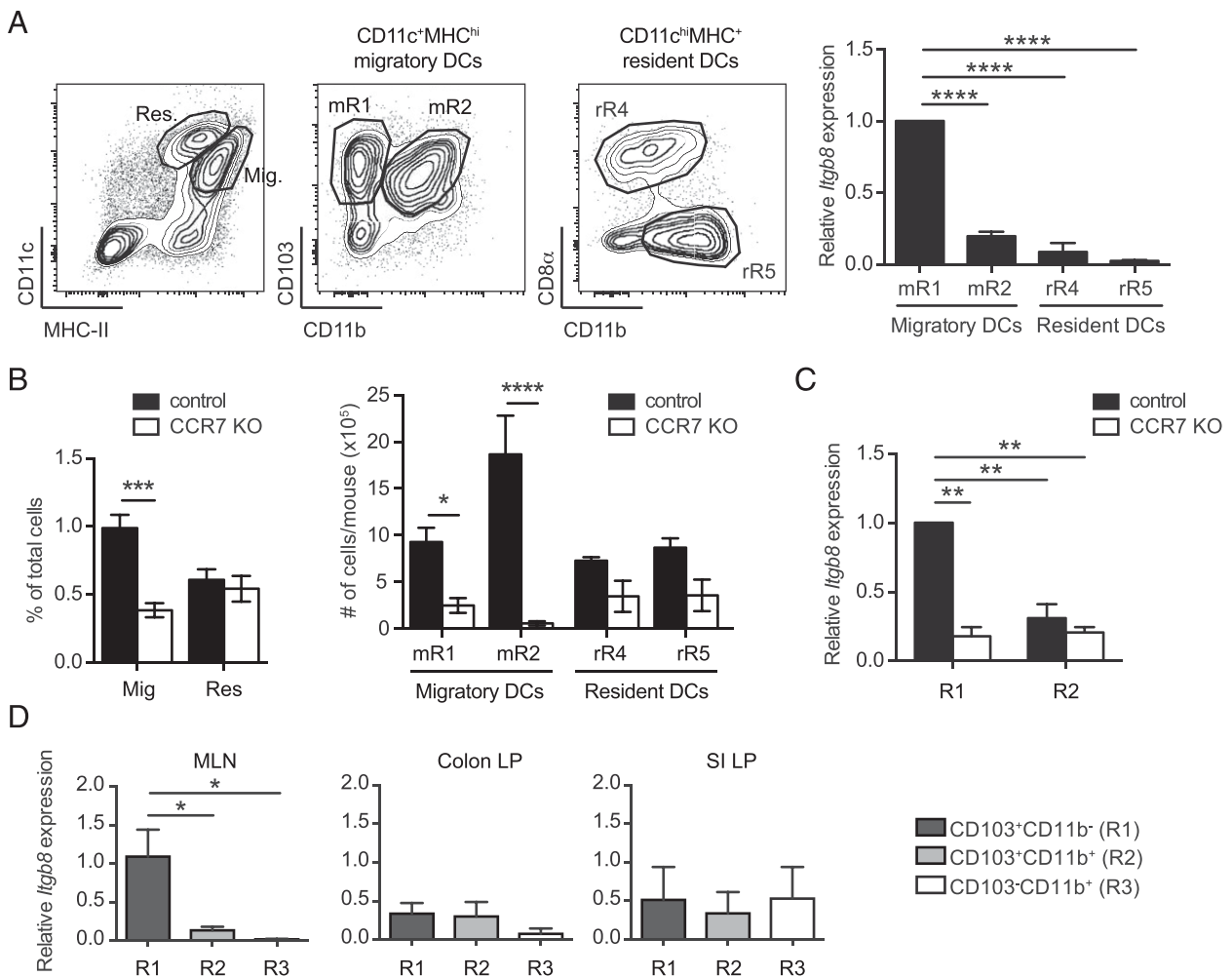
## Statistical analysis

Data were analyzed using GraphPad Prism Software 6.0a. Statistics were calculated using either unpaired *t* test when comparing two groups or one-way ANOVA with Dunnett's post hoc test when comparing more than two groups. For grouped analysis, statistics were calculated using two-way ANOVA with Tukey's or Sidak's post hoc test, where appropriate.

## Results

 $\alpha$ v $\beta$ 8 is preferentially expressed by CD103<sup>+</sup>CD11b<sup>-</sup> DCs

Our previous studies revealed that under homeostatic conditions integrin  $\alpha$ v $\beta$ 8 is expressed preferentially in the CD103<sup>+</sup> subpopulation of MLN DCs and confers on these DCs the ability to generate peripheral Tregs through activation of TGF- $\beta$  (8, 9). Intestinal CD103<sup>+</sup> DCs comprise two distinct populations, distinguished by expression of CD11b, and, in further analysis of MLN DCs, we found that *Itgb8* (the gene encoding  $\beta$ 8) was expressed at much higher levels in CD103<sup>+</sup>CD11b<sup>-</sup> DCs than in the CD103<sup>+</sup>CD11b<sup>+</sup> subset (Fig. 1A, 1B), whereas  $\alpha$ v integrin gene (*Itgav*) did not show



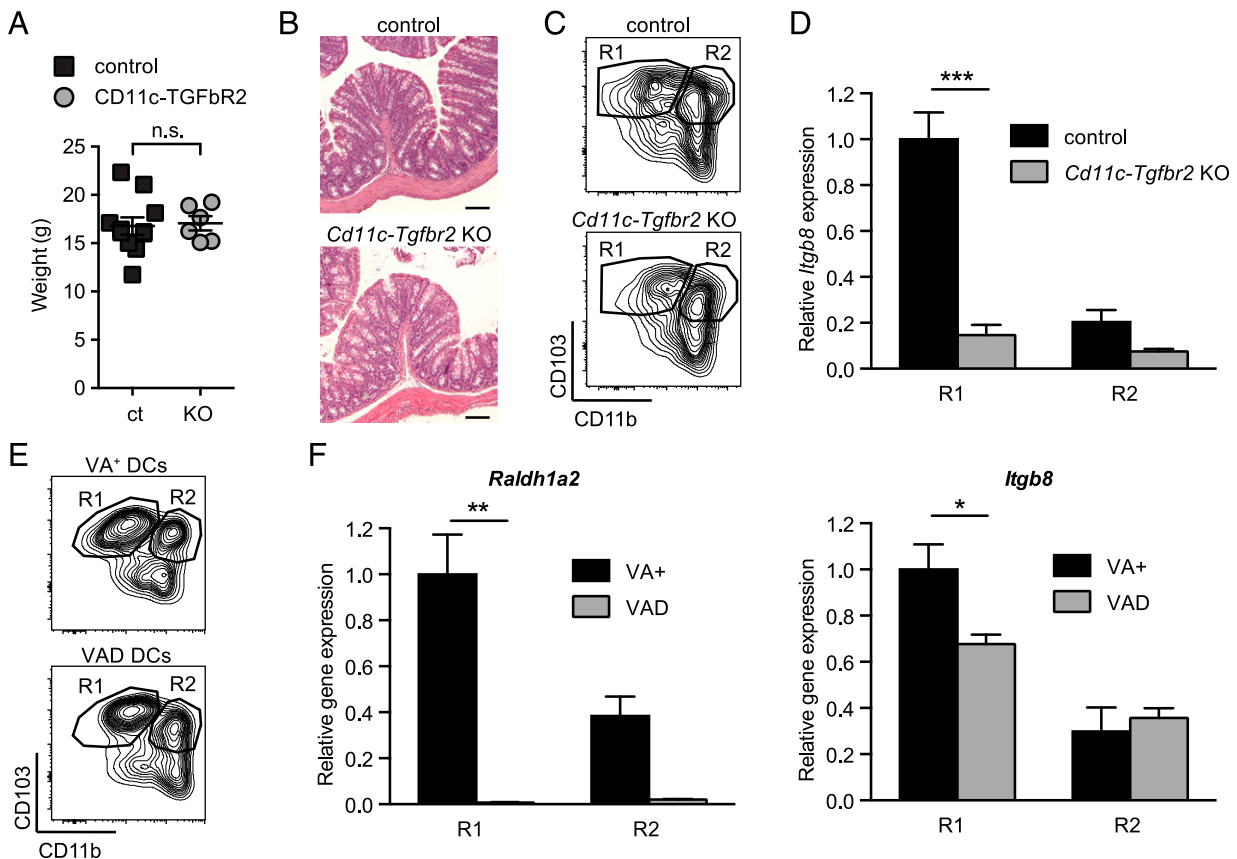
**FIGURE 2.** Migratory CD103<sup>+</sup>CD11b<sup>-</sup> DCs acquire  $\beta$ 8 integrin expression in the LP. (A) Quantitative RT-PCR analysis of  $\beta$ 8 integrin gene (*Itgb8*) expression in migratory (Mig) CD11c<sup>+</sup>MHC-II<sup>hi</sup> and resident (Res) CD11c<sup>hi</sup>MHC-II<sup>+</sup> MLN DC subsets sorted by FACS based on expression of CD103 and CD11b (mR1, mR2) or CD8 $\alpha$  and CD11b (rR4, rR5), respectively, using the gating strategy indicated in the representative FACS plots. Histogram shows mean  $\pm$  SEM of six independent pools of mice from two separate experiments, presented relative to levels in CD103<sup>+</sup>CD11b<sup>-</sup> DCs (mR1). (B) Frequencies and numbers of indicated migratory and resident DC subsets gated as in (A) from MLN of CCR7<sup>-/-</sup> and control mice. (C and D) Quantitative (C) and nested (D) RT-PCR analysis of *Itgb8* expression relative to  $\beta$ -actin (*Actb*) in indicated cell subsets sorted as in Fig. 1 from total CD11c<sup>+</sup>MHC-II<sup>+</sup> DCs from MLN of CCR7<sup>-/-</sup> and control mice (C) or CD11c<sup>hi</sup>MHC-II<sup>hi</sup> cells from MLN, colon, and small intestinal (SI) LP of wild-type mice (D). (B–D) Histogram shows mean  $\pm$  SEM from at least three individual experiments and in (C) are presented relative to levels in CD103<sup>+</sup>CD11b<sup>-</sup> DCs. \**p* < 0.05, \*\**p* < 0.005, \*\*\**p* < 0.0005, \*\*\*\**p* < 0.0001, two-way ANOVA with Tukey's (A and D) or Sidak's (B and C) post hoc test.

any significant difference (Supplemental Fig. 1A). Preferential expression of  $\beta 8$  integrin protein by  $CD103^+CD11b^-$  MLN DCs was confirmed by Western blot analysis (Fig. 1C). The expression of high levels of  $\beta 8$  by these DCs was associated with their preferential ability to activate latent TGF- $\beta$  (*l*-TGF- $\beta$ ) and generate Tregs (Fig. 1D). Furthermore, this difference in TGF- $\beta$  activation activity was dependent on  $\alpha v\beta 8$  ligand-binding integrins, as treatment with an  $\alpha v$  ligand mimetic peptide, cRGD, specifically reduced the effects of *l*-TGF- $\beta$  on induction of Tregs by  $CD103^+CD11b^-$  DCs. The partial reduction of the effects of *l*-TGF- $\beta$  by RGD on Treg generation is most likely due to contaminating active TGF- $\beta$  in the *l*-TGF- $\beta$  preparation or to an additional  $\alpha v$ -independent TGF- $\beta$  activation pathway.  $\beta 8$  only pairs with the  $\alpha v$  integrin subunit, and deletion of either integrin results in identical effects on TGF- $\beta$  activation and Treg generation (8, 9, 11, 16), and we therefore feel confident attributing this activity to  $\alpha v\beta 8$ . As these results closely resemble those we had previously reported with pooled  $CD103^+$  DCs from MLN or from intestinal LP (8), we concluded that  $CD103^+CD11b^-$  DCs were the cells responsible for the high levels of expression of  $\alpha v\beta 8$  and activation of TGF- $\beta$  in the  $CD103^+$  fraction.

*CD103<sup>+</sup> DCs that acquire  $\beta 8$  expression are derived from the intestine*

$CD103^+CD11b^-$  DCs in the MLN are composed of cells that develop in the intestinal LP and then migrate (21), and others that

reside in the MLN, and these populations can be distinguished by relative levels of CD11c and MHC-II expression (22). Previous findings by ourselves and others that  $CD103^+$  DCs derived from the LP can preferentially activate TGF- $\beta$  in an  $\alpha v\beta 8$ -dependent mechanism (8, 9) suggested that DCs may acquire expression of  $\alpha v\beta 8$  in the LP. Supporting this, we found that *Itgb8* expression is restricted to LP-derived migratory  $CD11c^+MHC-II^{hi}CD103^+CD11b^-$  MLN DCs (mR1), whereas neither of the resident DC subsets expressed significant levels of  $\beta 8$  integrin (Fig. 2A). To investigate this further, we analyzed expression of *Itgb8* in DCs from *Ccr7<sup>-/-</sup>* mice. CCR7 is required for migration of DCs from the LP to MLN (6, 23, 24), and MLN of *Ccr7<sup>-/-</sup>* mice contain predominantly blood-derived DCs with reduced numbers of DCs that have migrated from the intestine (22) (Fig. 2B). Notably, the expression of *Itgb8* was much lower in  $CD103^+CD11b^-$  DCs from *Ccr7<sup>-/-</sup>* cells than in cells from control mice, and was not significantly different from expression on  $CD103^+CD11b^+$  DCs (Fig. 2C). These data therefore confirm that *Itgb8* is preferentially expressed on MLN  $CD103^+CD11b^-$  DCs that have migrated from the LP. We also measured expression of *Itgb8* in  $CD11c^{hi}MHC-II^{hi}$  cells purified directly from the LP, but, in contrast to our findings with MLN DCs, we found that none of the major populations of  $CD11c^{hi}MHC-II^{hi}$  cells isolated from the LP ( $CD103^+CD11b^-$  DCs,  $CD103^+CD11b^+$  DCs, or  $CD103^-CD11b^+$  DCs/macrophages) expressed *Itgb8* at levels equivalent to those seen in MLN (Fig. 2D) when freshly isolated from tissue. This was in agreement with data



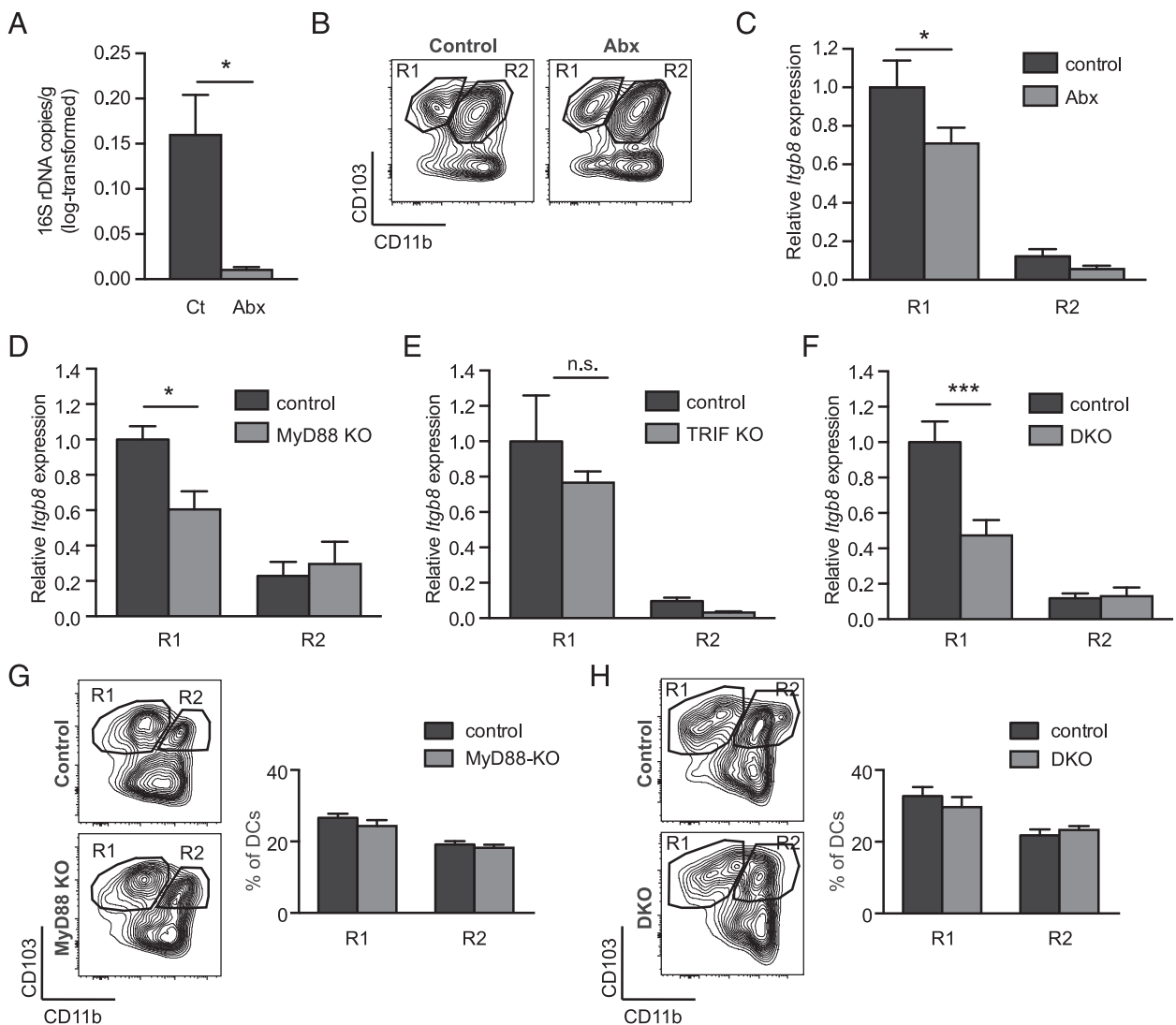
**FIGURE 3.** Reduced expression of  $\beta 8$  integrin expression in RA- and TGF- $\beta$  signaling-deficient mice.  $\beta 8$  integrin (*Itgb8*) expression was assessed in MLN DC subpopulations FACS sorted from control, *CD11c-Tgfb2* KO mice (A–D) and animals fed with a VAD or vitamin A-sufficient (VA<sup>+</sup>) diet (E and F). (A) Weight and (B) representative H&E-stained sections of colons from *CD11c-Tgfb2* KO and control mice. Scale bar, 100  $\mu$ m. (C and E) Representative FACS plot of  $CD11c^+MHC-II^+$  DCs from MLN showing gating strategy for isolating indicated populations. (D and F) Quantitative RT-PCR analysis of *Itgb8* (D and F, right panel) and *Raldh1a2* (F, left panel) expression relative to *Actb* and presented relative to levels in control  $CD103^+CD11b^-$  DCs (R1) in indicated MLN DC subpopulations. All histograms show mean  $\pm$  SEM of at least six individual mice from three separate experiments. \**p* < 0.05, \*\**p* < 0.005, \*\*\**p* < 0.0005, two-way ANOVA with Tukey’s post hoc test.

reported by Atarashi et al. (25), who showed that  $\alpha\beta 8$  was not expressed at significant levels by LP CD11c<sup>+</sup> cells in the steady state.

#### TGF-β and RA contribute to DC β8 expression in vivo

The ability of MLN DCs to generate Tregs and regulate local and systemic immune responses is thought to be due to conditioning events that occur in the intestinal LP, and has been postulated to depend on a number of conditioning factors produced by epithelial cells, including TGF-β and RA (26–30). To determine whether these factors regulate expression of β8 integrin in intestinal DCs, we used genetic and dietary manipulation to inhibit potential conditioning factors in mice in vivo. We first investigated TGF-β signaling in DCs, making use of conditional knockout (KO) mice in which the TGF-β type II receptor is specifically deleted in DCs (*Cd11c-cre; Tgfr2<sup>fl/fl</sup>*, referred to as *Cd11c-Tgfr2* KO mice).

*Cd11c-Tgfr2* KO mice develop normally until 4 wk of age but then gradually develop severe multiorgan autoimmune inflammation associated with spontaneous colitis (31). To exclude the potential contribution of intestinal inflammation on β8 integrin, we used mice <5 wk old with no clinical signs of colitis or wasting disease (Fig. 3A, 3B). CD103<sup>+</sup>CD11b<sup>-</sup> and CD103<sup>+</sup>CD11b<sup>+</sup> DCs were both present in MLNs of *Cd11c-Tgfr2*-KO mice, at frequencies similar to those seen in control mice, suggesting that development of these cells was largely normal in the absence of TGF-β signaling (Fig. 3C). However, CD103<sup>+</sup>CD11b<sup>-</sup> DCs from MLNs of *Cd11c-Tgfr2* KO mice expressed much lower levels of *Itgb8* than equivalent DCs from control mice; indeed, expression was not significantly different from that seen in CD103<sup>+</sup>CD11b<sup>+</sup> DCs from either control or *Cd11c-Tgfr2* KO mice (Fig. 3D). These findings therefore established that TGF-β signaling in DCs is required for preferential expression of β8 in CD103<sup>+</sup>CD11b<sup>-</sup>



**FIGURE 4.** Intestinal microbes and MyD88 signaling promote β8 integrin expression in CD103<sup>+</sup>CD11b<sup>-</sup> MLN DCs. β8 integrin (*Itgb8*) expression was assessed in MLN DC subpopulations FACS sorted from control, antibiotics-treated (A–C), MyD88- (D and G), Toll/IL-1R domain-containing adapter-inducing IFN-β- (TRIF) (E), and MyD88-TRIF-deficient (F and H) mice. (A) Log-transformed 16S rDNA copy numbers per gram of stool in control and antibiotics-treated (Abx) animals, as determined using real-time PCR. (B) Representative FACS plot of CD11c<sup>+</sup>MHC-II<sup>+</sup> DCs from MLN showing gating strategy for isolating indicated populations. (C–F) Quantitative RT-PCR analysis of *Itgb8* expression relative to *Actb* and presented relative to levels in control CD103<sup>+</sup>CD11b<sup>-</sup> DCs (R1) in indicated MLN DC subpopulations in control and antibiotics-treated (C), MyD88-deficient (MyD88 KO) (D), TRIF-deficient (TRIF KO) (E), and MyD88-TRIF-deficient mice (DKO) (F). (G and H) Representative FACS plot and frequencies of CD11c<sup>+</sup>MHC-II<sup>+</sup> DCs from MLN of MyD88-KO (G) and MyD88-TRIF double KO mice (H). Histograms show mean ± SEM of *n* individual mice from one [(E) *n* = 3], two [(D) *n* ≥ 8, (F) *n* ≥ 6], or three [(C) *n* ≥ 5] separate experiments. \**p* < 0.05, \*\*\**p* < 0.0005, unpaired Student *t* test (A) or two-way ANOVA with Tukey's post hoc test (C–H).



DCs in vivo. We next assessed the role of RA in  $\beta 8$  expression. Mice were depleted of RA by maintenance on a VAD diet, as previously described (32, 33). Confirming that we had depleted RA signaling in DCs, expression of *Raldh1a2*, which is induced by RA in immune cells (30, 34), was completely lost in both  $CD103^+CD11b^-$  and  $CD103^+CD11b^+$  DCs from VAD mice (Fig. 3E, 3F). *Itgb8* expression was also significantly reduced in  $CD103^+CD11b^-$  MLN DCs from VAD mice (Fig. 3F, right panel), indicating that RA signaling also contributed to the preferential expression of  $\alpha\beta 8$  in this DC subset in vivo.

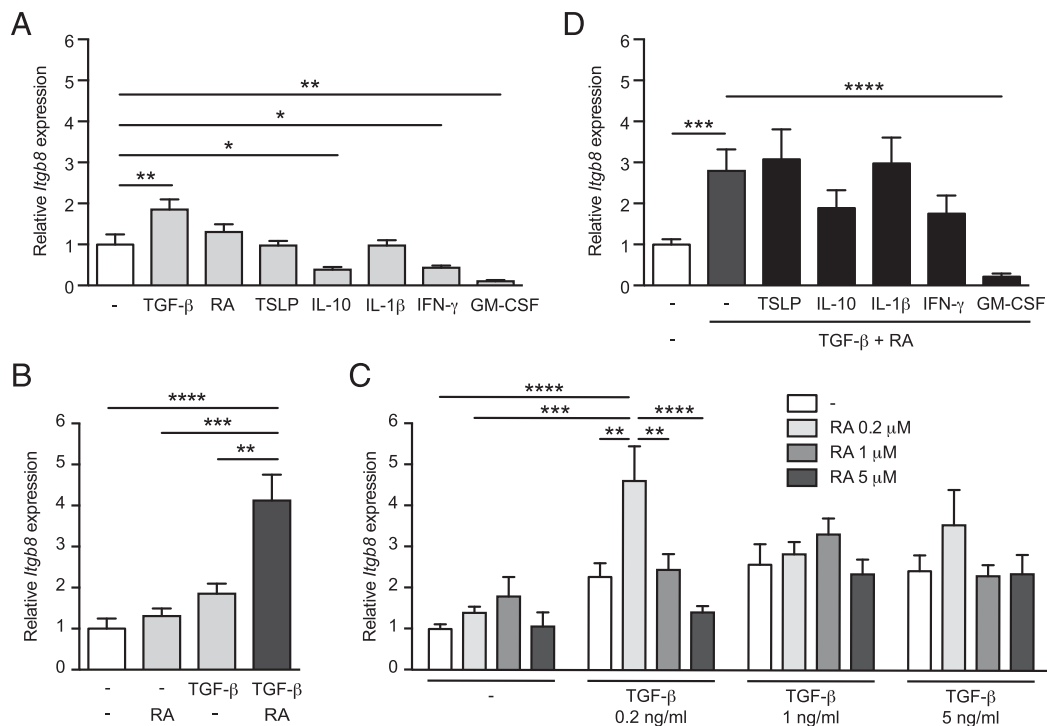
*Intestinal microbes and TLR signaling contribute to expression of Itgb8 in DCs*

The presence of intestinal microbes has been linked to effective development of LP DCs and establishment of normal mucosal immunoregulatory responses (30, 35, 36). To determine whether the intestinal microbiota contributed to induction of  $\alpha\beta 8$  expression in DCs, we treated mice with antibiotics, which reduced the presence of microbes in the intestine by 16-fold, assessed by measurement of 16S rDNA in stool (Fig. 4A). As expected based on previous studies (22), intestinal  $CD103^+CD11b^-$  DCs were present and migrated normally to the MLN of antibiotic-treated animals; however, they expressed significantly lower levels of *Itgb8* than MLN DCs from untreated mice (Fig. 4B, 4C), indicating a role for the intestinal microbiota in conditioning of these regulatory DCs. We next analyzed *Itgb8* expression in intestinal DCs from mice deficient in *Myd88* and *Trif*, components of the TLR signaling pathway by which DCs sense microbes.  $CD103^+CD11b^-$  DCs from *Myd88*<sup>-/-</sup> mice had significantly reduced expression of *Itgb8* compared with DCs from control mice (Fig. 4D). In contrast, deletion of Toll/IL-1R domain-containing

adapter-inducing IFN- $\beta$  (TRIF) did not significantly affect  $\beta 8$  expression and showed no additive effect with MyD88 deficiency (Fig. 4E, 4F). Importantly, in both *Myd88*<sup>-/-</sup> and *Myd88*<sup>-/-</sup>*Trif*<sup>-/-</sup> double-KO mice,  $CD103^+CD11b^-$  and  $CD103^+CD11b^+$  MLN DCs were present in similar proportion than in control mice (Fig. 4G, 4H). Notably, the effects of *Myd88* deficiency on *Itgb8* expression were more pronounced than those of antibiotic treatment, which may be due to the continued presence of microbes in antibiotic-treated mice, endogenous TLR ligands, or additional MyD88-dependent signaling pathways, such as IL-1 $\beta$ .

*Signals encountered in the mucosal environment induce  $\beta 8$  expression by direct effects on DCs*

Together, these data indicated that TGF- $\beta$ , RA, and TLR signaling contributed to the expression of  $\alpha\beta 8$  in intestinal DCs. To determine whether these stimuli could promote  $\alpha\beta 8$  expression in DCs directly, we treated spleen DCs, which do not express  $\beta 8$  when freshly isolated (8), with TGF- $\beta$ , RA, and TLR ligands. Culture of spleen DCs with TGF- $\beta$  induced a small increase in *Itgb8* (Fig. 5A), whereas other cytokines associated with the intestinal microenvironment either had no effect on *Itgb8* expression (thymic stromal lymphopoietin, IL-1 $\beta$ ) or reduced expression (IL-10, IFN- $\gamma$ , GM-CSF) (Fig. 5A). Culture of DCs with RA alone had no effect on *Itgb8* expression but, when combined with TGF- $\beta$ , further promoted expression of *Itgb8* (Fig. 5B, 5C). Hence, RA synergized with TGF- $\beta$  signaling to increase gene expression, as has been previously shown for induction of Foxp3<sup>+</sup> Tregs in the intestine (28, 29). This synergistic effect did not appear to extend to other cytokines, however, as combined treatment with TGF- $\beta$ /RA with other cytokines or factors did not significantly increase *Itgb8* expression (Fig. 5D).

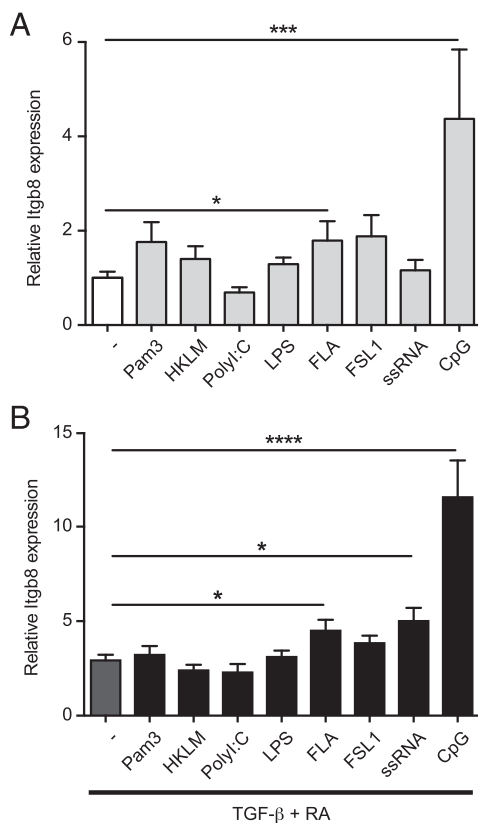


**FIGURE 5.** TGF- $\beta$  and RA directly induce *Itgb8* expression in spleen DCs.  $CD11c^+$  spleen DCs were sorted with magnetic beads and then cultured in serum-free medium for 16 h. Cells were either left untreated (-) or stimulated with TGF- $\beta$ , RA, or both (A-C). In addition, cells were treated with thymic stromal lymphopoietin, IL-10, IL-1 $\beta$ , IFN- $\gamma$ , or GM-CSF, without (B) or with (D) addition of TGF- $\beta$  and RA. A total of 0.2 ng/ml TGF- $\beta$  and 0.2  $\mu$ M RA was used, unless indicated otherwise.  $\beta 8$  integrin (*Itgb8*) gene expression was assessed by quantitative RT-PCR analysis relative to *Actb* and presented relative to levels in unstimulated spleen DCs. Histograms show mean  $\pm$  SEM of cultures from 15 (A), 6 (B and D), or 9 (C) independent pools of mice from at least three separate experiments. \* $p$  < 0.05, \*\* $p$  < 0.005, \*\*\* $p$  < 0.0005, \*\*\*\* $p$  < 0.0001, one-way ANOVA with Dunnett's post hoc test (A, B, and D) or two-way ANOVA with Tukey's post hoc test (C).

We next tested the role of TLR signaling in DC induction of *Itgb8*. Stimulation of spleen DCs with the TLR9 ligand CpG ODN and the TLR5 ligand Flagellin both stimulated expression of *Itgb8* (Fig. 6A). This induction of *Itgb8* was significantly enhanced by addition of RA/TGF- $\beta$ , which in combination with CpG ODN increased expression by >10-fold over unstimulated DCs (Fig. 6B), in line with the preferential expression of *Itgb8* that we have seen in MLN DCs. Together these data therefore show that TGF- $\beta$ , RA, and TLR ligands were sufficient to induce expression of *Itgb8* in DCs, and in combination induced levels similar to those seen in vivo.

#### *$\beta 8$ expression is differentially regulated in distinct DC subsets*

These data therefore support the hypothesis that factors encountered in the intestinal LP induce  $\beta 8$  integrin expression in DCs. However, it is unclear why  $\alpha v\beta 8$  is expressed preferentially by the CD103<sup>+</sup>CD11b<sup>-</sup> subset of intestinal DCs, and not by other DCs exposed to the intestinal microenvironment. One possible explanation is that distinct lineages of DCs show differential responses to *Itgb8*-inducing signals, and we therefore tested the ability of individual subsets of spleen DCs to upregulate *Itgb8* in response to conditioning signals in culture. Spleen DCs consist of two main subsets, CD11b<sup>-</sup>CD8 $\alpha$ <sup>+</sup> DCs and CD11b<sup>+</sup>CD8 $\alpha$ <sup>-</sup> DCs (Fig. 7A; referred to in this study as CD8 $\alpha$ <sup>+</sup> and CD11b<sup>+</sup> DCs, respectively).



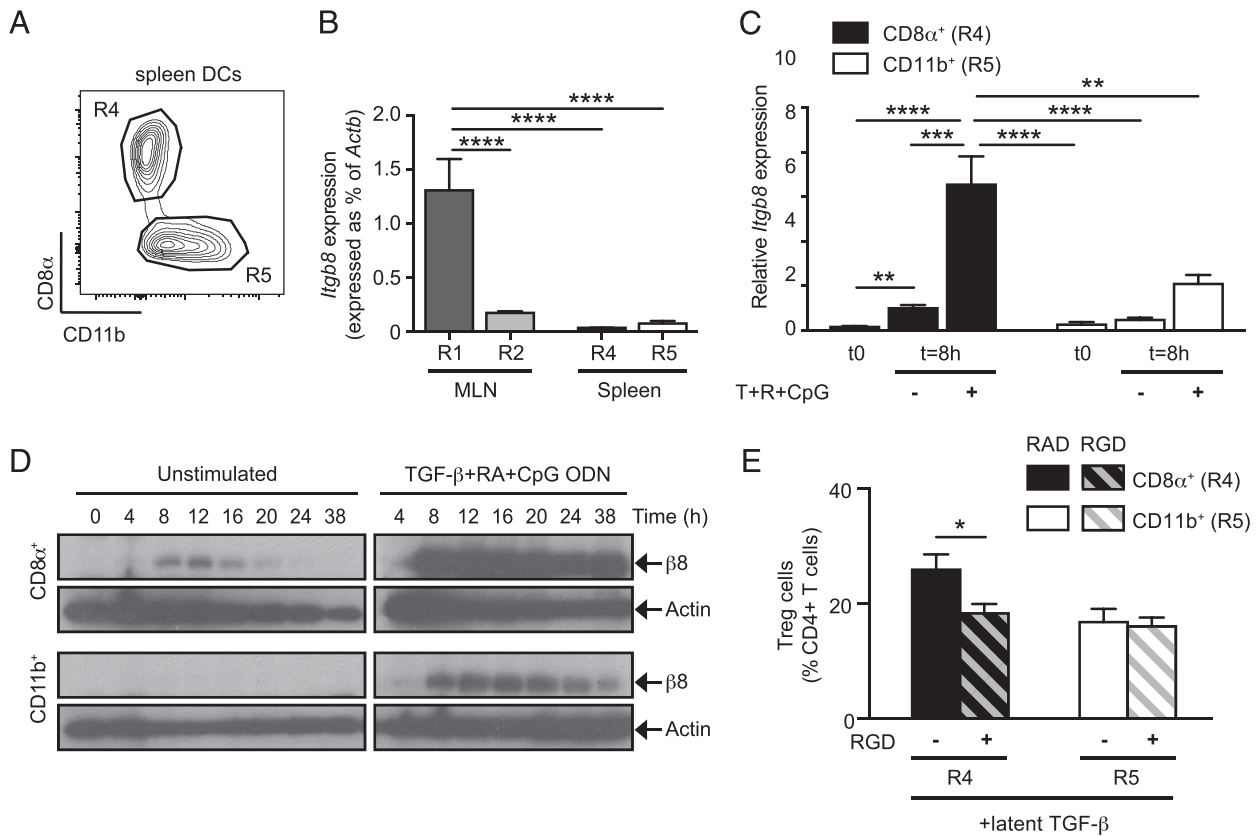
**FIGURE 6.** TLR stimulation induces *Itgb8* expression in spleen DCs. CD11c<sup>+</sup> spleen DCs were sorted with magnetic beads and then cultured in serum-free medium for 16 h. Cells were either left untreated (-) or stimulated with agonists for TLR-1 (Pam<sub>3</sub>), TLR-2 (HKLM), TLR-3 (PolyI:C), TLR-4 (LPS), TLR-5 (FLA), TLR-6 (FSL1), TLR-7 (ssRNA), or TLR-9 (CpG), with (B) or without (A) addition of TGF- $\beta$  and RA.  $\beta 8$  integrin (*Itgb8*) gene expression was assessed by quantitative RT-PCR analysis relative to *Actb* and presented relative to levels in unstimulated spleen DCs. Histograms show mean  $\pm$  SEM of cultures from nine independent pools of mice from three separate experiments. \* $p$  < 0.05, \*\*\* $p$  < 0.0005, \*\*\*\* $p$  < 0.0001, one-way ANOVA with Dunnett's post hoc test.

Sorted spleen CD8 $\alpha$ <sup>+</sup> DCs did not express significant levels of *Itgb8* when freshly isolated (Fig. 7B), but gene expression could be detected after 8 h in culture without additional stimulation (Fig. 7C). Furthermore, treatment of CD8 $\alpha$ <sup>+</sup> DCs with the combination of TGF- $\beta$ , RA, and CpG ODN induced significant further increase in *Itgb8* expression (Fig. 7C), as we had seen for cultures of total spleen DCs. Notably, Western blot analysis showed that combined treatment with TGF- $\beta$ , RA, and CpG ODN increased both levels and duration of  $\beta 8$  protein expression (Fig. 7D). In contrast, spleen CD11b<sup>+</sup> DCs did not express *Itgb8* spontaneously in culture (Fig. 7C, 7D). Furthermore, although these cells did express *Itgb8* mRNA and  $\beta 8$  protein after stimulation with TGF- $\beta$ , RA, and CpG ODN, expression was significantly lower than seen in CD8 $\alpha$ <sup>+</sup> DCs, and was not sustained, diminishing to near background levels by 24–36 h (Fig. 7D). This differential induction of *Itgb8* in DC subsets was not due to a generalized inability of CD11b<sup>+</sup> DCs to respond to the conditioning factors, as these cells expressed similar or higher levels of TLRs and receptors for TGF- $\beta$  and RA than MLN CD103<sup>+</sup>CD11b<sup>-</sup> and spleen CD8 $\alpha$ <sup>+</sup> DCs (Supplemental Figs. 1A, 2), and both spleen subsets showed equivalent levels of TGF- $\beta$  and TLR signaling when stimulated (Supplemental Fig. 3). Furthermore, consistent with their differential ability to express  $\alpha v\beta 8$  in culture, spleen CD8 $\alpha$ <sup>+</sup> DCs were able to generate Tregs in the presence of *l*-TGF- $\beta$  through an  $\alpha v$ -dependent mechanism, whereas CD11b<sup>+</sup> DCs could not (Fig. 7E). To determine whether similar differences in response to inducing stimuli may underlie differential expression of  $\alpha v\beta 8$  in intestinal DCs, we cultured MLN DCs with TGF- $\beta$ , RA, and CpG ODN and followed *Itgb8* expression. As we have shown in previous figures, in freshly isolated cells, *Itgb8* was expressed preferentially in CD103<sup>+</sup>CD11b<sup>-</sup> DCs (R1) compared with CD103<sup>+</sup>CD11b<sup>+</sup> DCs (R2) from MLN, or either population of spleen DCs (Fig. 7B). Culture under *Itgb8*-inducing conditions for 8 h increased expression of *Itgb8* in both CD103<sup>+</sup>CD11b<sup>-</sup> and CD103<sup>+</sup>CD11b<sup>+</sup> MLN DCs. However, the absolute level of *Itgb8* remained significantly lower in CD103<sup>+</sup>CD11b<sup>+</sup> cells than CD103<sup>+</sup>CD11b<sup>-</sup> (Supplemental Fig. 1B). Based on these results, we therefore concluded that the difference in  $\alpha v\beta 8$  expression between CD103<sup>+</sup>CD11b<sup>-</sup> and CD103<sup>+</sup>CD11b<sup>+</sup> DCs could not be explained solely by differential exposure to conditioning factors, but was also determined by the ability of those factors to induce *Itgb8* expression.

Furthermore, recent studies establishing that splenic CD8 $\alpha$ <sup>+</sup> DCs (R4) and intestinal CD103<sup>+</sup>CD11b<sup>-</sup> DCs (R1) share a common lineage (cDC1) (37–39), whereas spleen CD11b<sup>+</sup> DCs (R5) appear to be more closely related to intestinal CD103<sup>+</sup>CD11b<sup>+</sup> DCs (R2) of the cDC2 lineage (40–42), indicate that the CD8 $\alpha$ <sup>+</sup>/CD103<sup>+</sup>CD11b<sup>-</sup> DC lineage may be uniquely specialized for high expression of  $\alpha v\beta 8$  and activation of TGF- $\beta$ .

#### *DC lineage determines the pattern of $\beta 8$ gene expression*

To further investigate whether lineage determines the ability of DCs to express  $\alpha v\beta 8$  in the intestinal microenvironment, DCs were generated from bone marrow precursors in culture with Flt3-ligand, and their ability to express  $\beta 8$  integrin was measured. This method generates two main populations of conventional DCs, CD24<sup>+</sup>CD172<sup>-</sup>CD11b<sup>low</sup> DCs (CD24<sup>+</sup> FL-DCs), which resemble CD8 $\alpha$ <sup>+</sup> or CD103<sup>+</sup>CD11b<sup>-</sup> DCs, and CD24<sup>-</sup>CD172<sup>+</sup>CD11b<sup>+</sup> DCs (CD172<sup>+</sup> FL-DCs), which are more related to CD11b<sup>+</sup> DCs (14, 38, 39) (Fig. 8A). CD24<sup>+</sup> FL-DCs showed consistently higher levels of *Itgb8* expression than CD172<sup>+</sup> DCs from the same culture. Furthermore, this difference was significantly enhanced after stimulation with TGF- $\beta$ , RA, and CpG ODN (Fig. 8B). These findings are consistent with our data from sorted spleen DC subsets (Fig. 7C) and suggest that the preferential ability of CD8 $\alpha$ <sup>+</sup>/CD103<sup>+</sup>CD11b<sup>-</sup>



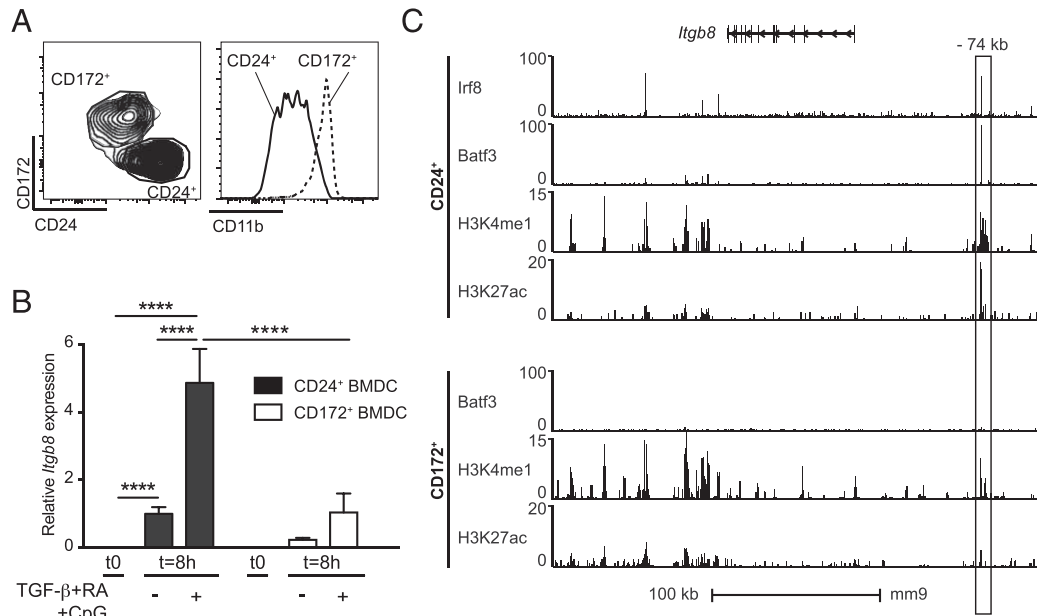
**FIGURE 7.** CD8 $\alpha^+$  spleen DCs preferentially express  $\alpha\beta 8$  and generate Treg in vitro. DC subsets were sorted by FACS. **(A)** Representative FACS plot gated on CD11c $^+$ MHC-II $^+$  DCs from spleen shows gating strategy for isolating indicated populations. MLN DC subsets were sorted as in Fig. 1. **(B)**  $\beta 8$  integrin gene (*Itgb8*) expression was analyzed by quantitative RT-PCR in freshly isolated DC subsets from MLN and spleen and expressed as percentage of *Actb*. **(C and D)** Sorted DC subsets were cultured in serum-free medium for 8 h (C) or indicated times (D). Cells were either left untreated (–) or stimulated with TGF- $\beta$ , RA, and CpG ODN (+) and harvested either in TRIzol (C) or radioimmunoprecipitation assay buffer (D) for analysis of  $\beta 8$  integrin expression. Alternatively for each freshly isolated DC subset, their ability to induce Treg generation was assessed in vitro **(E)**. **(C)** Quantitative RT-PCR analysis of *Itgb8* expression relative to *Actb* and presented relative to levels in unstimulated (–) CD8 $\alpha^+$  DCs (R4). **(D)** Western blot analysis of  $\beta 8$  integrin and  $\beta$ -actin. **(E)** FACS-sorted spleen DC subsets were cultured with naive CD4 $^+$ Foxp3 $^{GFP-}$  T cells in serum-free medium with or without addition of latent TGF- $\beta$ , RGD (+, hatched bars), and/or RAD (–, solid bars) peptides, as indicated. After 4 d in culture, Treg generation was assessed by Foxp3 $^{GFP}$  expression. Data show mean  $\pm$  SEM of individual DC:T cell cultures with eight independent pools of spleen DCs from two separate experiments. Histograms show mean  $\pm$  SEM of *n* independent pools of mice from two [(B) *n* = 5] or four [(C) *n* = 10] separate experiments. \**p* < 0.05, \*\**p* < 0.005, \*\*\**p* < 0.0005, \*\*\*\**p* < 0.0001, one-way ANOVA with Dunnett's post hoc test (B) or two-way ANOVA with Tukey's post hoc test (C and E).

DCs to express *Itgb8* expression is acquired during development. To determine whether this might be due to epigenetic programming, we compared the epigenetic landscape of the *Itgb8* locus in CD24 $^+$  and CD172 $^+$  FL-DCs, using existing chromatin immunoprecipitation followed by sequencing datasets (17). In CD24 $^+$  FL-DCs, we identified an active enhancer marked by both monomethylation of histone 3 lysine 4 (H3K4me1) and acetylation of histone 3 lysine 27 (H3K27ac) 74 kb upstream of the *Itgb8* coding region (Fig. 8C, upper panel). This active enhancer was also associated with binding to the lineage-specific transcription factors Batf3 and IFN regulatory factor (IRF)8. The same site in CD172 $^+$  FL-DCs showed reduced H3K4me1 marks and no H3K27ac (Fig. 8C, lower panel), and no other H3K27ac peak could be detected in the vicinity of *Itgb8*, suggesting that this locus is poised but not active in these cells. The presence of an active enhancer 74 kb upstream of *Itgb8* specifically in CD24 $^+$  FL-DCs is consistent with the preferential ability of these cells to express  $\beta 8$  integrin. Altogether, these data therefore demonstrate that cell lineage plays a critical role in permitting expression of  $\beta 8$  in response to conditioning factors from the microenvironment and support the concept that the ability of DCs to express high levels of  $\beta 8$  integrin is determined during development.

## Discussion

Despite major advances in determining the origin of DC subpopulations, our understanding of the factors that determine their functional diversity and tissue specialization remains limited. In this study, we have identified factors that contribute to differentiation of DCs specialized for generation of Tregs in the intestine. We show that expression of integrin  $\alpha\beta 8$ , which is required for activation of TGF- $\beta$  by intestinal DCs, is controlled by factors that include DC lineage, tissue microenvironment, and the microbiota. Specifically, we show that the combination of TGF- $\beta$  and RA, factors found at high levels in the intestinal microenvironment, together with TLR signaling, promotes expression of  $\beta 8$ . However, these signals only result in high levels of  $\alpha\beta 8$  in the CD8 $\alpha^+$ /CD103 $^+$ CD11b $^-$  lineage of DCs. Together, these data provide a key illustrative example of how microenvironmental factors and cell lineage drive the generation of DCs that perform critical specialized functions, in this case the local activation of TGF- $\beta$  to facilitate Treg generation.

Our data emphasize the critical role of signals derived from the mucosal environment, namely TGF- $\beta$ , RA, and TLR signaling, in promoting expression of  $\alpha\beta 8$  both in vitro and in vivo. Furthermore, we find that MLN DCs expressing high levels of  $\alpha\beta 8$



**FIGURE 8.** CD24<sup>+</sup> FL-DCs preferentially express  $\alpha v\beta 8$  and present an active enhancer in *Itgb8* locus. **(A and B)** FL-DCs. After 9 d in culture, FL-DC subsets were sorted by FACS and cultured in serum-free medium for 8 h. Cells were either left untreated (–) or stimulated with TGF- $\beta$ , RA, and CpG ODN (+).  $\beta 8$  integrin gene (*Itgb8*) expression was measured by quantitative RT-PCR relative to *Actb* and presented relative to levels in unstimulated (–) CD24<sup>+</sup> DCs at 8 h. **(A)** Representative FACS plot of CD11c<sup>+</sup>MHC-II<sup>+</sup> FL-DCs showing gating strategy for isolating indicated populations. **(B)** Histogram shows mean  $\pm$  SEM of individual cultures from four independent experiments. **(C)** Chromatin immunoprecipitation sequencing analysis of Batf3, IRF8, H3K4me1, and H3K27ac in CD24<sup>+</sup> and CD172<sup>+</sup> FL-DCs purified by sorting [chromatin immunoprecipitation sequencing data from (17); Gene Expression Omnibus accession code GSE66899]. Box represents outlined area at –74 kb relative to the *Itgb8* transcription starting site. mm9, NCBI37/mm9 assembly of the mouse genome. \*\*\*\**p* < 0.0001, two-way ANOVA with Tukey's post hoc test.

have emigrated from the intestine, further supporting the importance of intestinal conditioning in generation of  $\alpha v\beta 8$ <sup>+</sup> DCs. However, we find that expression of  $\alpha v\beta 8$  is consistently lower in CD11c<sup>hi</sup> cells freshly isolated from the LP than those from MLNs, suggesting to us that the process of migration and maturation of DCs is also required for high expression of  $\alpha v\beta 8$ . Supporting this, we have previously reported that CD103<sup>+</sup> LP DCs develop the ability to activate TGF- $\beta$  through an  $\alpha v$ -dependent mechanism when cultured with T cells (8), conditions that promote activation or maturation of DCs (43, 44). Whether CD103<sup>+</sup> DCs in the LP can acquire high levels of  $\alpha v\beta 8$ , such as during inflammation, remains to be determined. Although RA and TGF- $\beta$  can potentially be derived from various stromal cells in the intestine (27, 45), work from the Rescigno laboratory strongly supports epithelial cells as a likely source of RA and TGF- $\beta$  for DC conditioning. Their laboratory has previously shown that coculture of spleen DCs with intestinal epithelial cells or epithelial cell-derived culture supernatant promotes the ability of spleen DCs to generate gut-trophic Tregs (26), and that this is dependent on epithelial cell-derived TGF- $\beta$  and RA. Our data indicate that upregulation of expression of  $\alpha v\beta 8$  on DCs is the likely mechanism for this observation. Furthermore, the close association of CD103<sup>+</sup> DCs with the intestinal epithelium (46, 47) suggests this DC subset is ideally placed to receive these signals. Furthermore, there is increasing evidence that DCs associated with the intestinal epithelium interact with intestinal microbes or their products, providing a potential source of TLR signals to promote  $\alpha v\beta 8$  expression (47–49). It is likely, therefore, that intestinal DCs differ in their exposure to some or all of these stimuli due to their localization, and this may contribute to differential expression of  $\alpha v\beta 8$  in DC subsets. However, even when cultured under conditions that strongly promote  $\alpha v\beta 8$  upregulation, expression remained lower in CD103<sup>+</sup>CD11b<sup>+</sup> mucosal DCs than CD103<sup>+</sup>CD11b<sup>–</sup> DCs.

Our observation that CD8 $\alpha$ <sup>+</sup> DCs from spleen and CD24<sup>+</sup> FL-DCs also show high and sustained expression of  $\alpha v\beta 8$  when cultured in TGF- $\beta$ , RA, and TLR ligands led us to conclude that DC lineage also contributes to  $\alpha v\beta 8$  expression. Recent studies indicate that these DCs share a common cDC1 lineage with CD103<sup>+</sup>CD11b<sup>–</sup> DCs, distinct from CD11b<sup>+</sup> DCs found in the intestine and spleen (38, 39, 50). We therefore propose a model in which the cDC1 lineage is programmed to respond to stimulation through TGF- $\beta$ , RA, and TLRs to express high levels of *Itgb8*, and this leads to preferential expression of  $\alpha v\beta 8$  in CD103<sup>+</sup>CD11b<sup>–</sup> DCs in vivo. Similar lineage-specific regulation of the *Itgb8* promoter has been reported by the Nishimura laboratory in mesenchymal cells, where the proinflammatory cytokine IL-1 $\beta$  promotes expression of *Itgb8* in lung fibroblasts and astrocytes, but not dermal fibroblasts. In further parallels with our studies, they show that this requires p38 and NF- $\kappa$ B signaling, which are activated downstream of both IL-1 $\beta$  and TLR signaling. IL-1 $\beta$  activates *Itgb8* expression by chromatin remodeling and exposure of binding sites for additional transcription factors, and the expression of *Itgb8* in different cell types corresponds with the chromatin state of the *Itgb8* core promoter (51, 52). p38 $\alpha$  is reported to be required for *Itgb8* expression by CD103<sup>+</sup> MLN DCs, as well as for Treg generation and induction of oral tolerance in mice (53). Hence, we speculate that TLR stimulation likewise promotes expression of *Itgb8* through regulating accessibility of the core promoter, possibly governing access of TGF- $\beta$  and RA-induced transcription factors, and this is further controlled by lineage-specific factors acting at more distal sites. Our model is supported by the presence of an enhancer 74 kb upstream of *Itgb8*, which is preferentially active in the developmentally related CD24<sup>+</sup> bone marrow-derived DCs. It is currently unclear what transcription factors dictate this lineage specificity, and this is an active ongoing area of investigation. However, likely candidates include Batf3 and IRF8, which both bind this enhancer and are required for effective

generation and maintenance of CD8 $\alpha^+$  and CD103 $^+$ CD11b $^-$  DCs in vivo (Fig. 8) (17, 38, 50). Batf3 and IRF8 might thus contribute to licensing the CD8 $\alpha^+$ /CD103 $^+$ CD11b $^-$  DC lineage to express  $\beta$ 8.

DC expression of  $\alpha$ v $\beta$ 8 is essential for generation of intestinal Tregs (8–11), and our data therefore implicate CD103 $^+$ CD11b $^-$  DCs as the major DC subset responsible for Treg generation in the intestine. CD103 $^+$  DCs have also been implicated in intestinal Treg generation in previous studies (28, 29, 54), although this function has also been attributed to mucosal CD103 $^+$ CD11b $^+$  DCs, CD103 $^-$ CX3CR1 $^{\text{int}}$ F4/80 $^-$  DCs, or CD103 $^-$ CX3CR1 $^+$ F4/80 $^+$  macrophages (55, 56). Deletion of all CD103 $^+$  DC subsets also leads to a significant loss of intestinal Tregs in mice (57), further supporting the key role for this subset in Treg generation. However, although other in vitro studies support the specific involvement of the CD103 $^+$ CD11b $^-$  subset in this process (53), deletion of either the CD103 $^+$ CD11b $^-$  population, through KO of the transcription factors IRF8 or Batf3 (38, 50) or the CD103 $^+$ CD11b $^+$  population (41) alone, does not lead to a detectable loss of intestinal Tregs or intestinal inflammation. Hence, it is likely that both populations make some contribution to Treg generation in vivo, or can compensate for each other in this process. Although we have focused on CD103 $^+$ CD11b $^-$  DCs, low levels of *Itgb8* transcript can be detected in CD103 $^+$ CD11b $^+$  DCs at homeostasis, and these and other DC subsets can upregulate expression during infection or inflammation. Further studies will therefore be needed to determine the exact contribution of different DC subsets to Treg generation and regulation of intestinal inflammation.

In this study, we have focused on the factors that regulate  $\alpha$ v $\beta$ 8 expression by DCs during immune homeostasis, where the principal immunological role is likely to be generation of Tregs. In inflammatory settings, where DC  $\alpha$ v $\beta$ 8 is required for Th17 cell generation (16, 58), different mechanisms may be involved. Atarashi et al. (25) have reported that microbe-derived ATP triggers Th17 responses in vivo, and that exogenous ATP promoted  $\alpha$ v $\beta$ 8 expression in DCs. However, in our experiments, ATP did not induce significant  $\alpha$ v $\beta$ 8 expression in spleen DCs when given alone or in combination with RA and TGF- $\beta$  (data not shown). This discrepancy most likely reflects responses of different DC subsets, and although we focused on CD103 $^+$  DCs, during intestinal infection ATP promotes expression of  $\alpha$ v $\beta$ 8 in CD103 $^-$ CD11b $^+$ CX3CR1 $^+$ CD70 $^{\text{hi}}$  DCs. Hence, Treg and Th17 responses appear to differ in both the conditioning signals that induce  $\alpha$ v $\beta$ 8 expression and the subsets of DCs involved. In this context, it is interesting that TGF- $\beta$ , RA, and TLR stimulation (our studies) or ATP stimulation (25) induces only transient expression of  $\alpha$ v $\beta$ 8 in inflammatory DCs, which is likely to favor Th17 over Treg differentiation (59). Conversely, we find that CD103 $^+$ CD11b $^-$  DCs exhibit high and sustained  $\alpha$ v $\beta$ 8 expression, which would be expected to induce regulatory responses. Despite these differences, our data indicate that regulation of  $\alpha$ v $\beta$ 8 expression is a major factor determining the ability of DCs to generate Tregs and Th17 cells.

TGF- $\beta$  and RA have been previously implicated in promoting Treg generation through direct effects on T cells, inducing expression of Foxp3 and intestinal homing receptors (28, 29, 32, 60–62). Our findings, and those of Rescigno and colleagues (26), that these same factors promote differentiation of Treg-generating DCs indicate that these environmental factors produce coordinated effects on the immune system, and highlight a feed-forward effect, in which TGF- $\beta$  signaling in DCs promotes expression of  $\alpha$ v $\beta$ 8, which then promotes TGF- $\beta$  signaling in T cells. Likewise, RA signaling induces the expression of RA-synthesizing enzymes in DCs (30, 34). In this way, the intestinal microenvironment promotes DCs to recapitulate the same environment in the MLN

during T cell presentation, which we propose allows fine spatio-temporal regulation of TGF- $\beta$  signaling and thus induction of appropriate mucosal responses and homing in T cells. In addition, it has recently been shown that  $\alpha$ v $\beta$ 8 is expressed by Tregs and contributes to ongoing immune regulation (63); whether  $\beta$ 8 expression is also activated by RA and TGF- $\beta$  in T cells remains to be determined.

In conclusion, in this study we show that cell lineage and gut-derived factors together shape DCs into critical gatekeepers of TGF- $\beta$ -dependent intestinal immune responses via regulation of  $\beta$ 8 expression. These results also provide a detailed demonstration of how acquisition of a specialized function comes from the unique ability of DC to integrate signals from the periphery and communicate this contextual information forward for induction of appropriate adaptive immunity.

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## Disclosures

The authors have no financial conflicts of interest.

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# Plasmacytoid dendritic cells control dengue and Chikungunya virus infections via IRF7-regulated interferon responses

Brian Webster<sup>1†</sup>, Scott W Werneke<sup>2,3†</sup>, Biljana Zafirova<sup>2</sup>, Sébastien This<sup>1</sup>, Séverin Coléon<sup>1</sup>, Elodie Décembre<sup>1</sup>, Helena Paidassi<sup>1</sup>, Isabelle Bouvier<sup>2</sup>, Pierre-Emmanuel Joubert<sup>2</sup>, Darragh Duffy<sup>2</sup>, Thierry Walzer<sup>1</sup>, Matthew L Albert<sup>2,3\*</sup>, Marlène Dreux<sup>1\*</sup>

<sup>1</sup>CIRI, Inserm, U1111, Université Claude Bernard Lyon 1, CNRS, UMR5308, École Normale Supérieure de Lyon, Univ Lyon, Lyon, France; <sup>2</sup>Immunobiology of Dendritic Cells, Institut Pasteur, Paris, France; <sup>3</sup>Cancer Immunology Department, Genentech, San Francisco, United States

**Abstract** Type I interferon (IFN-I) responses are critical for the control of RNA virus infections, however, many viruses, including Dengue (DENV) and Chikungunya (CHIKV) virus, do not directly activate plasmacytoid dendritic cells (pDCs), robust IFN-I producing cells. Herein, we demonstrated that DENV and CHIKV infected cells are sensed by pDCs, indirectly, resulting in selective IRF7 activation and IFN-I production, in the absence of other inflammatory cytokine responses. To elucidate pDC immunomodulatory functions, we developed a mouse model in which IRF7 signaling is restricted to pDC. Despite undetectable levels of IFN-I protein, pDC-restricted IRF7 signaling controlled both viruses and was sufficient to protect mice from lethal CHIKV infection. Early pDC IRF7-signaling resulted in amplification of downstream antiviral responses, including an accelerated natural killer (NK) cell-mediated type II IFN response. These studies revealed the dominant, yet indirect role of pDC IRF7-signaling in directing both type I and II IFN responses during arbovirus infections.

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**\*For correspondence:**

albert.matthew@gene.com (MLA);  
marlene.dreux@ens-lyon.fr (MD)

†These authors contributed equally to this work

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## Introduction

Upon sensing invading viruses, host cells produce type I interferons (IFNs), leading to the expression of an array of IFN-stimulated genes (ISGs). This first-line response suppresses viral spread by generating an antiviral state within host cells, and supports the initiation of adaptive immunity (*Hoffmann et al., 2015*). Viral sensing may involve non-hematopoietic or hematopoietic cells that are targets of infection. Specific pathogen-associated motifs, such as viral nucleic acids, are recognized by pattern recognition receptors, which can be cytoplasmic (e.g. retinoic inducible gene-I (RIG-I)-like receptors, and NOD-like Receptors) or endosomal (e.g. Toll-like receptors; TLRs) (*Jensen and Thomsen, 2012*). Due to the potency of these innate responses, most viruses have evolved mechanisms to subvert or evade pathogen-sensing pathways (*GarciaGarcía-Sastre, 2017*).

We and others have recently highlighted the existence of alternative or indirect pathogen-sensing mechanisms that circumvents cell-intrinsic viral evasion mechanisms (*Webster et al., 2016*). Such alternative pathways involve the sensing of infected target cells by plasmacytoid dendritic cells (pDCs), a DC subtype specialized in the production of robust level of type I IFNs (referred herein to as IFN-I) (*Swiecki and Colonna, 2015*). Notably, this was illustrated in the context of dengue virus (DENV), a positive-sense single-stranded RNA virus, which represents global health concerns (*Bhatt et al., 2013*). Recent in vitro work highlighted a newly defined mode of pDC activation, which

**eLife digest** Viruses, like the ones responsible for the tropical diseases dengue and chikungunya, are parasites of living cells. As they cannot multiply on their own, these microbes need to infect a host cell and hijack its machinery to make more of themselves.

When a cell is invaded, it can sense the viral particles, and defend itself by releasing antiviral molecules. Some of these molecules, such as interferons, also help recruit immune cells that can fight the germs. However, viruses often evolve mechanisms to escape being detected by the cell they occupy.

Plasmacytoid dendritic cells are a rare group of immune cells, and they are able to detect when another cell is infected by the dengue virus. When they are in close physical contact with an invaded cell, these sentinels can recognize immature viral particles and release large amounts of antiviral molecules. However, it is unclear how important plasmacytoid dendritic cells are in clearing a viral infection.

Here, Webster, Werneke et al. confirmed that plasmacytoid dendritic cells were able to sense cells infected by dengue, but also by chikungunya. When this happened, the dendritic cells primarily produced interferon, rather than other defense molecules.

In addition, mice were genetically engineered so that the production of interferon was restricted to the plasmacytoid dendritic cells. When infected with dengue or chikungunya, the modified rodents resisted the diseases. These results show that, even though they are only a small percentage of all immune cells, plasmacytoid dendritic cells have an outsize role as first responders and as coordinators of the immune response.

Finally, Webster, Werneke et al. showed that when low doses of interferon are added, the plasmacytoid dendritic cells respond more quickly to cells infected by dengue. Together these findings could potentially be leveraged to create new treatments to fight dengue. These would be of particular interest because interferons are not as damaging to the body compared to other types of defense molecules. The issue is timely since climate change is allowing the mosquitos that transmit dengue and chikungunya to live in new places, exposing more people to these serious infections.

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is mediated primarily by non-infectious immature DENV particles and requires physical cell-cell contact with DENV-infected cells (*Décembre et al., 2014*). Importantly, such cell-cell contact-dependent activation of pDCs has also been reported for several other genetically distant viruses (*Webster et al., 2016*). Consistently, DENV, like some other viruses, does not directly infect pDCs (*Décembre et al., 2014; Webster et al., 2016*). We thus aimed to test how such indirect cell-cell sensing of viral pathogens by pDCs, in the absence of pDC infection, engages host mechanisms for in vivo viral clearance.

pDCs function as sentinels of viral infection, predominantly via recognition of single-stranded RNA and unmethylated CpG containing DNA by endosomal TLR7 and TLR9, respectively. Activation of TLR7 or TLR9 results in copious secretion of IFN-I as well as other proinflammatory cytokines (notably TNF $\alpha$ ), driven by the transcription factor(s) interferon regulatory factor (IRF)-7 and nuclear factor-kappa B (NF- $\kappa$ B), respectively (*Swiecki and Colonna, 2015*). Interestingly, despite the inherent ability of pDCs to produce both IFN-I and NF- $\kappa$ B-induced cytokines, previous studies have suggested a 'bifurcated' pattern of TLR7/9 signaling, where IFN-I production may occur in the absence of NF- $\kappa$ B activation, and vice versa (*Swiecki and Colonna, 2015*). The production of IFN-I or pro-inflammatory cytokines may in part be dependent on the sub-cellular compartment in which these TLRs encounter activating signal, as CpG-A accumulates in early endosomes to induce IFN-I whereas CpG-B aggregates in endolysosomes to activate NF- $\kappa$ B (*Swiecki and Colonna, 2015*). In the context of viral infection, pDCs exposed to cell-associated Hepatitis C virus produced IFN-I but not NF- $\kappa$ B-dependent cytokines (*Dental et al., 2012*). Despite these observations, the consequences of pDC IFN-I production in the absence of NF- $\kappa$ B induction remain unclear, and have not yet been shown to be sufficient for in vivo viral control.



Herein, we show that indirect activation of pDCs by contact with DENV or CHIKV infected cells results in an IRF7-induced IFN response, in the absence of NF- $\kappa$ B inflammatory responses. We developed a novel mouse model where *Irf7* expression is pDC-restricted, that is, *Irf3*<sup>-/-</sup>;*Irf7*<sup>-/-</sup> double knockout mice, with *Irf7* expression driven under the pDC-specific promoter *Sialic acid binding Ig-like lectin H (Siglech)* (Blasius et al., 2006; Takagi et al., 2011; Zhang et al., 2006). We demonstrated that pDC-restricted IRF7-induced signaling is sufficient to achieve in vivo control of DENV and CHIKV infections. We further elucidated that the early pDC IRF7-mediated response accelerates type II IFN (IFN $\gamma$ ) responses via natural killer (NK) cell activation, thus positioning pDCs as a cell type that regulates the interplay between type I and type II IFN responses in the control of these viral infections, an immune axis that is independent of other sources of type I IFN or pDC-derived NF- $\kappa$ B-induced cytokines.

## Results

### Cell-cell sensing of DENV-infected cells does not induce inflammatory NF- $\kappa$ B responses by pDCs

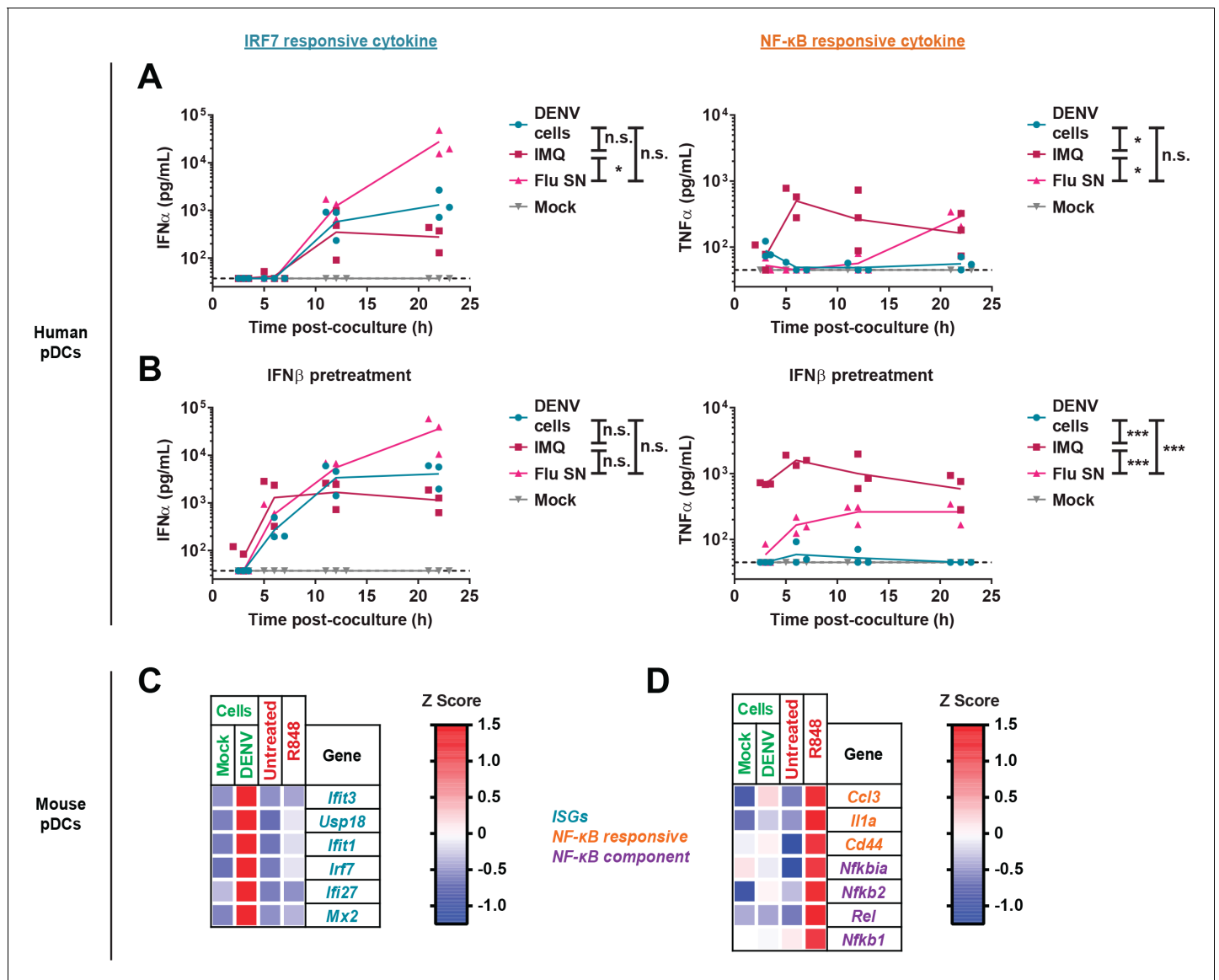
We previously reported that ex vivo activation of an antiviral response by pDCs requires physical contact with DENV-infected cells (Décembre et al., 2014). Prior studies suggested that pDC TLR7/9 signaling differentially induced IRF7 and/or NF- $\kappa$ B, depending on the ligand (Swiecki and Colonna, 2015). Thus, we determined relative pDC secretion of representative NF- $\kappa$ B-induced (TNF $\alpha$ ) and IRF7-induced (IFN $\alpha$ ) cytokines in response to DENV. The sensing of DENV-infected cells by human pDCs failed to induce TNF $\alpha$  secretion (Figure 1A). This was in contrast to the robust TNF $\alpha$  production triggered by direct TLR7 stimuli, including the synthetic ligand imiquimod (IMQ) and influenza A [Flu], which directly activates pDCs as cell-free virus as previously reported (Décembre et al., 2014) (Figure 1A).

pDC activation by DENV, the result of cell-cell transfer of TLR7 ligands, may occur more slowly than direct activation by cell-free stimuli. To assess if differential kinetics of pDC activation underlie the difference in TNF $\alpha$  production, we evaluated pre-treatment of human pDCs with low-dose IFN $\beta$ , prior to coculture with infected cells. Such pre-treatment regimens have been previously shown to accelerate both IFN $\alpha$  and TNF $\alpha$  production in response to influenza virus (Phipps-Yonas et al., 2008). While IFN $\beta$  pretreatment accelerated the kinetics of pDC-derived IFN $\alpha$  (all stimuli) and TNF $\alpha$  production (IMQ/Flu), we observed minimal TNF $\alpha$  production in response to DENV-infected cells (Figure 1B). These observations imply that the defect in DENV-mediated TNF $\alpha$  induction by pDCs might be due to the indirect mechanism of stimulation. Furthermore, we showed that pDC TNF $\alpha$  levels in response to IMQ were not diminished in presence of DENV-infected cells (Figure 1—figure supplement 1A). Together, these results indicated that DENV is neither promoting nor preventing the activation of NF- $\kappa$ B-induced TNF $\alpha$  production by pDCs.

We extended these findings to mouse pDC activation, utilizing cells derived from either bone marrow (BM) or isolated from spleen. We also assessed a broader range of NF- $\kappa$ B-dependent genes. DENV-infected cells induced higher levels of IFN $\alpha$  and interferon-stimulated genes (ISGs) as compared to synthetic TLR7/8 agonists (R848 and IMQ) (Figure 1C, Figure 1—figure supplement 1C–D), results that were consistent with human pDC findings (Figure 1A–B). Conversely, the TLR7/8 agonist R848 induced a strong NF- $\kappa$ B-dependent gene signature in mouse pDCs, whereas limited induction was observed when using DENV-infected cells (Figure 1D and Figure 1—figure supplement 1C). Together, these results demonstrated that pDC-mediated sensing of DENV-infected cells primarily leads to IFN-I production and a strong ISG response in the absence of NF- $\kappa$ B-mediated inflammatory responses.

### An in vivo model for pDC-restricted IFN-I production

To address how this IRF7- and IFN-I-restricted response to DENV may position pDCs as critical in the control of viral infections, we generated a unique mouse model where IRF7 signaling is restricted to pDCs. We established a knock-in strain where *Irf7* expression is driven by the pDC-specific *Siglech* promoter (thus called *Siglech*<sup>Irf7/+</sup>) (Figure 2A). We next backcrossed these mice onto *Irf3*<sup>-/-</sup>;*Irf7*<sup>-/-</sup> double knockout mice to generate hemizygous *Irf7*-expressing animals (*Siglech*<sup>Irf7/+</sup>;*Irf3*<sup>-/-</sup>;*Irf7*<sup>-/-</sup> referred to as 'pDC:*Irf7*<sup>+</sup>' mice). Use of hemizygous mice preserved one copy of the *Siglech* gene



**Figure 1.** Lack of NF- $\kappa$ B response by pDCs in ex vivo coculture with DENV infected cells. (A) Quantification of IFN $\alpha$  and TNF $\alpha$  in supernatants of human pDCs cocultured with DENV-infected (DENV cells) or uninfected BHK-21 cells (Mock), in presence of synthetic TLR7 agonist: Imiquimod; IMQ; 1  $\mu$ g/mL or influenza infectious supernatant; Flu;  $3 \times 10^3$  FFU. (B) pDCs were pretreated for 3 hr with low-dose IFN $\beta$  (50 U/mL) and then cocultured or treated as in (A). Median,  $n = 3$  independent experiments. (C–D) qRT-PCR analysis of gene expression (using mouse-specific primers) by WT murine splenic pDCs cocultured for 22 hr with uninfected- (mock cells) or DENV-infected Huh7.5.1 cells (DENV cells) or treated with TLR7/8 agonist (R848). Expression levels are normalized to housekeeping genes ( $\beta$ -actin) and shown as Z scores (red-blue gradient from maximum to minimum expression).

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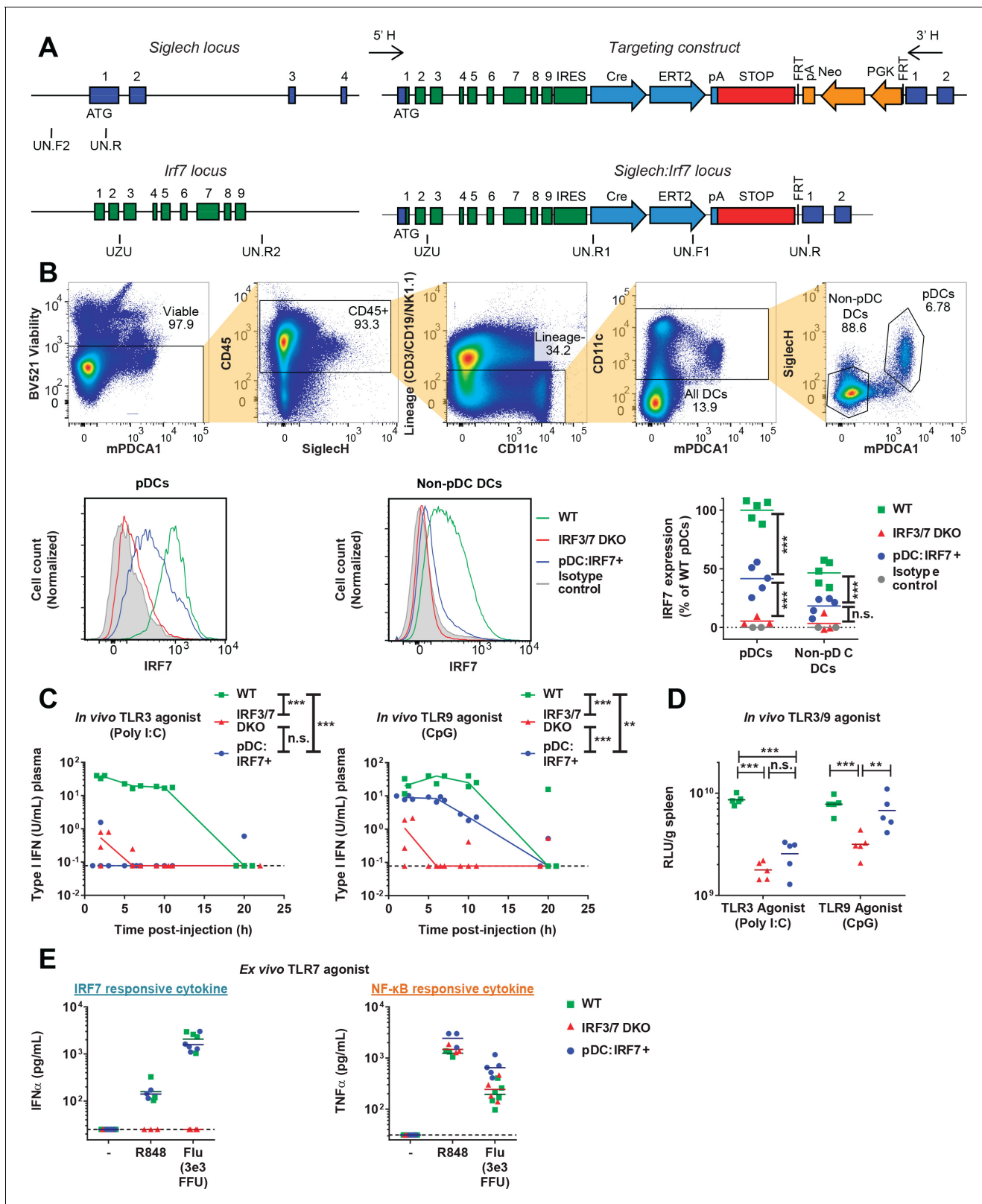
The following figure supplement is available for figure 1:

**Figure supplement 1.** Lack of NF- $\kappa$ B responses to DENV in murine bone marrow and splenic pDCs, and retention of pDC ISG responses in the absence of *Irf3/Irf7*.

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(Figure 1—figure supplement 1B). *Irf3/7* double knockout mice (referred to as *Irf3/7* DKO mice), deficient in IFN-I production (Rudd et al., 2012; Schilte et al., 2012) were used as comparator negative controls in all experiments.

To validate the *Irf7* knock-in in pDC:*Irf7*<sup>+</sup> mice, we analyzed IRF7 protein levels in DC subsets. pDCs were the only cell type to retain significant levels of IRF7 protein expression, seen in both pDC:*Irf7*<sup>+</sup> and WT mice, but not in *Irf3/7* DKO mice (Figure 2B). To functionally validate the pDC:



**Figure 2.** Functional validation of the pDC:Ir7<sup>+</sup> mouse model. (A) Targeting construct for the knock-in of *irf7* under the control of the *siglech* promoter. (B) Expression levels of IRF7 analyzed by FACS in pDCs and non-pDC DCs isolated from spleens of uninfected WT, *Ir3/7* DKO and pDC:Ir7<sup>+</sup> mice. Gating strategy for DCs and pDCs from splenocyte populations (upper panels), IRF7 expression (lower panels); 3–5 mice per condition. (C–D) Quantification of IFN activity by bioassay in plasma (C) and spleen homogenates (D) at various time-points post-injection of mice with agonists of TLR3 (Figure 2 continued on next page

Figure 2 continued

and TLR9, polyinosinic:polycytidylic acid (poly I:C) and CpG-type A oligodeoxynucleotides (CpG), respectively; median,  $n = 3-5$  mice per condition. (E) Quantification of IFN $\alpha$  and TNF $\alpha$  in ex vivo cultures of pDCs (mPDCA1<sup>+</sup> cells) isolated from BM of WT, *Irf3/7* DKO and pDC:*Irf7*<sup>+</sup> mice and treated for 22 hr with TLR7/8 agonist (R848) or influenza virus infectious supernatant (Flu); median,  $n = 3-5$  mice per condition.

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*Irf7*<sup>+</sup> mice, we assessed IFN-I activity induced upon in vivo treatment with agonists of TLR9 and TLR3, which are expressed or not by pDCs, respectively (Swiecki and Colonna, 2015). As expected, we observed IFN-I activity in plasma/spleen of WT mice stimulated by either agonist, whereas little-to-no IFN-I activity was detected in *Irf3/7* DKO mice (Figure 2C–D). Consistent with the TLR expression patterns in pDCs (Swiecki and Colonna, 2015), pDC:*Irf7*<sup>+</sup> mice produced high levels of IFN-I in response to TLR9, but not TLR3 agonists.

Using this model system, we assessed how pDC IRF7-signaling mediates antiviral responses to DENV. First, we purified pDCs from WT, *Irf3/7* DKO and pDC:*Irf7*<sup>+</sup> mice, and treated them with TLR7 agonists (R848/IMQ/cell-free Flu) or DENV-infected cells. pDC:*Irf7*<sup>+</sup> and WT pDCs produced similar amounts of IFN $\alpha$  (Figures 2E and 3A), confirming the functionality of IRF7 signaling in pDC:*Irf7*<sup>+</sup> mice. We also tested NF- $\kappa$ B-signaling in pDCs from *Irf3/7* DKO and pDC:*Irf7*<sup>+</sup> mice induced by the same TLR7 agonists. Confirming independent activation of NF- $\kappa$ B, we observed TNF $\alpha$  secretion levels in both strains to be comparable to WT mice (Figures 2F and 3B). Of note, ISGs previously defined as IRF5-dependent (e.g. *Mx1*, *Ifit1*) (Lazear et al., 2013) were still upregulated in *Irf3/7* DKO pDCs when cocultured with DENV-infected cells or stimulated by other TLR7 agonists (Figure 1—figure supplement 1D).

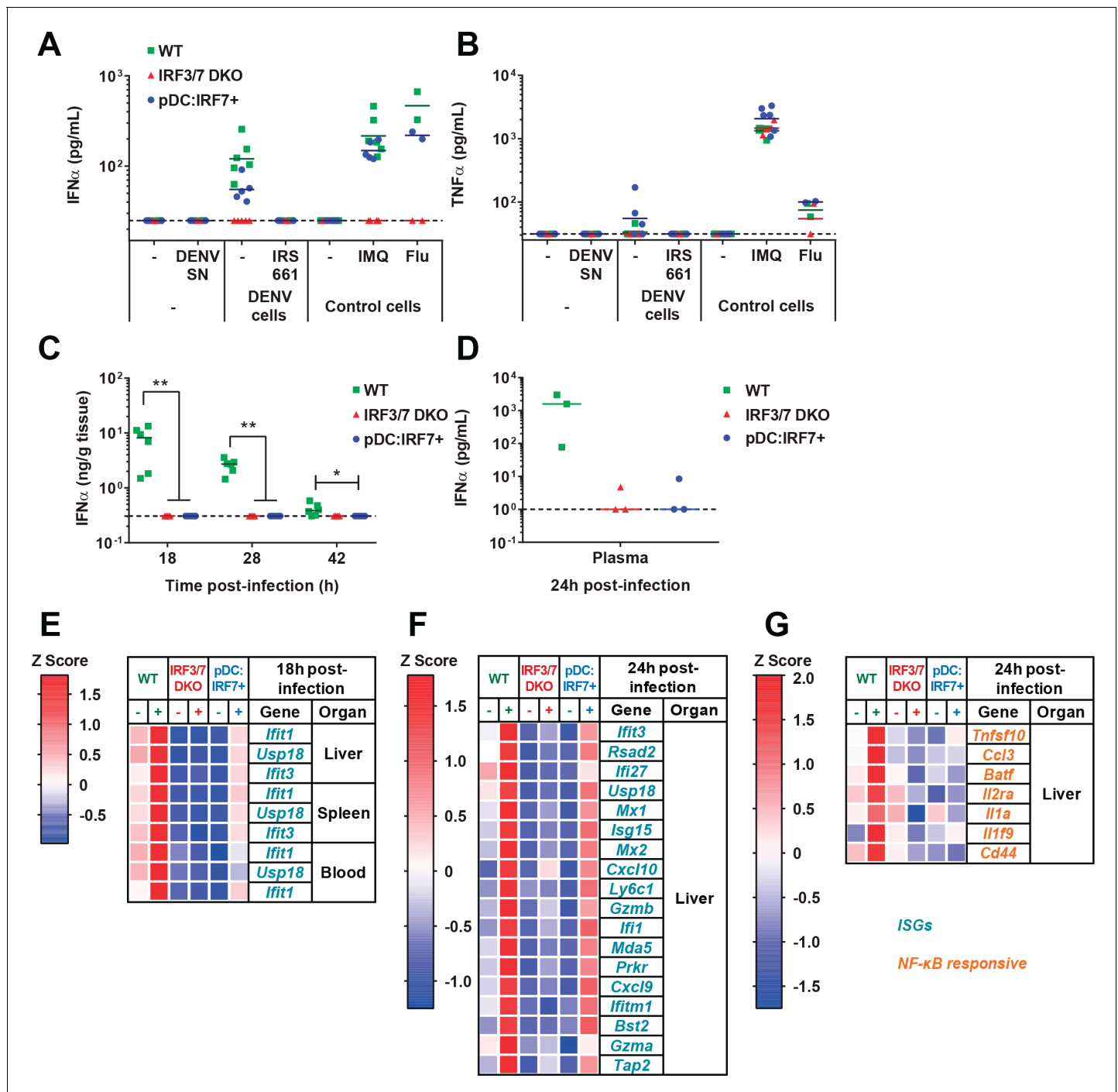
### pDC-IRF7-induced potent downstream ISG responses in absence of detectable IFN-I

To determine whether IFN-I response to viral stimuli was restored in pDC:*Irf7*<sup>+</sup> mice, animals were infected with DENV systemically (intravenously, i.v.), and IFN $\alpha$ / $\beta$  expression was assessed. High levels of IFN $\alpha$  were detected in both the spleen and plasma of infected WT mice (Figure 3C–D), but not *Irf3/7* DKO mice, in agreement with previous results (Chen et al., 2013). IFN $\beta$  levels displayed the same pattern of expression (data not shown). pDC:*Irf7*<sup>+</sup> mice had undetectable IFN $\alpha$ / $\beta$  levels in both the plasma and spleen at all analyzed times post-infection (p.i.) (Figure 3C–D).

We considered the possibility that the pDC response to DENV may occur in a localized manner, with insufficient type I IFN in plasma or spleen to allow its detection. We thus assessed downstream responses of IFN $\alpha$ / $\beta$  receptor (IFNAR) signaling via qRT-PCR analysis of ISGs in different tissues of DENV-infected mice. The ISGs (*Ifit1*, *Usp18*, and *Ifit3*) were selected for their robust expression following by IFN-I stimulation, as compared to type II IFN (Liu et al., 2012). Importantly, despite undetectable IFN $\alpha$ / $\beta$  in DENV-infected pDC:*Irf7*<sup>+</sup> mice, an early induction (i.e. 18 h p.i.) of ISG RNAs was observed in all tissues analyzed. By contrast, this early induction was not observed in *Irf3/7* DKO mice (Figure 3E). Of note, basal ISG expression levels were lower in *Irf3/7* DKO and pDC:*Irf7*<sup>+</sup> mice compared to WT, likely reflecting diminished basal IFNAR signaling in the absence of *Irf3* and *Irf7* (Gough et al., 2012). These results indicate that restricted expression of *Irf7* in pDCs is sufficient to generate a systemic ISG response.

### In vivo activation of pDC IRF7 antiviral signaling is sufficient to control DENV infection

The pDC:*Irf7*<sup>+</sup> model provided a means to distinguish, in vivo, the role of pDCs in regulating antiviral IFN-I-mediated versus NF- $\kappa$ B-induced response(s) to DENV infection. We therefore measured IRF/STAT-dependent ISG expression in multiple organs, and a larger panel of ISGs and NF- $\kappa$ B-dependent transcripts in the liver of DENV-infected animals. DENV infection induced ISGs and NF- $\kappa$ B-dependent transcripts in WT, but not *Irf3/7* DKO mice (Figure 3E–G; expressed as magnitude-independent Z score and Figure 3—figure supplement 1; expressed as magnitude-dependent fold-change), confirming previous findings (Chen et al., 2013). Strikingly, despite a strong upregulation of ISGs in DENV-infected pDC:*Irf7*<sup>+</sup> mice, we observed minimal induction of NF- $\kappa$ B-dependent inflammation-related transcripts (Figure 3F–G and Figure 3—figure supplement 1). Based on these



**Figure 3.** pDCs respond in vivo to DENV in a TLR7/IRF7 but not an NF-κB-dependent manner. (A–B) Quantification of IFNα (A) and TNFα (B) by ELISA in ex vivo culture supernatants of pDCs isolated from BM of WT, Irf3/7 DKO and pDC:IRF7<sup>+</sup> mice and treated for 22 hr with DENV infectious supernatant (DENV SN), TLR7 agonist (IMQ and flu, 3 × 10<sup>2</sup> ffu), or cocultured with DENV-infected Huh7.5.1 cells ± TLR7 inhibitor IRS661; median, n = 3–5 independent experiments. (C–G) Intravenous (i.v.) DENV infection followed by the analysis of IFNα and gene expression in organs collected at the indicated time points p.i. (C–D) Quantification of IFNα in spleen homogenates and plasma by ELISA; median; each data point corresponds to an individual mouse: n = 5–6 and n = 3 mice per condition, for spleen and plasma samples, respectively. IFNα was undetectable in uninfected control mice. (E–G) qRT-PCR analysis of gene expression in the indicated tissues at 18 hr (E) and 24 hr (F–G) p.i., and normalized to housekeeping panel (*hprt1*, *β-actin*, *18S*). Expression levels shown as Z scores, n = 2 (E) or 3 (F–G) infected and n = 1 (E) or 2 (F–G) uninfected mice per genotype. DOI: <https://doi.org/10.7554/eLife.34273.006>

The following figure supplement is available for figure 3:

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Figure 3 continued

**Figure supplement 1.** Strong early ISG but not NF- $\kappa$ B response to DENV occurs in organs of pDC:*Irf7*<sup>+</sup> mice.

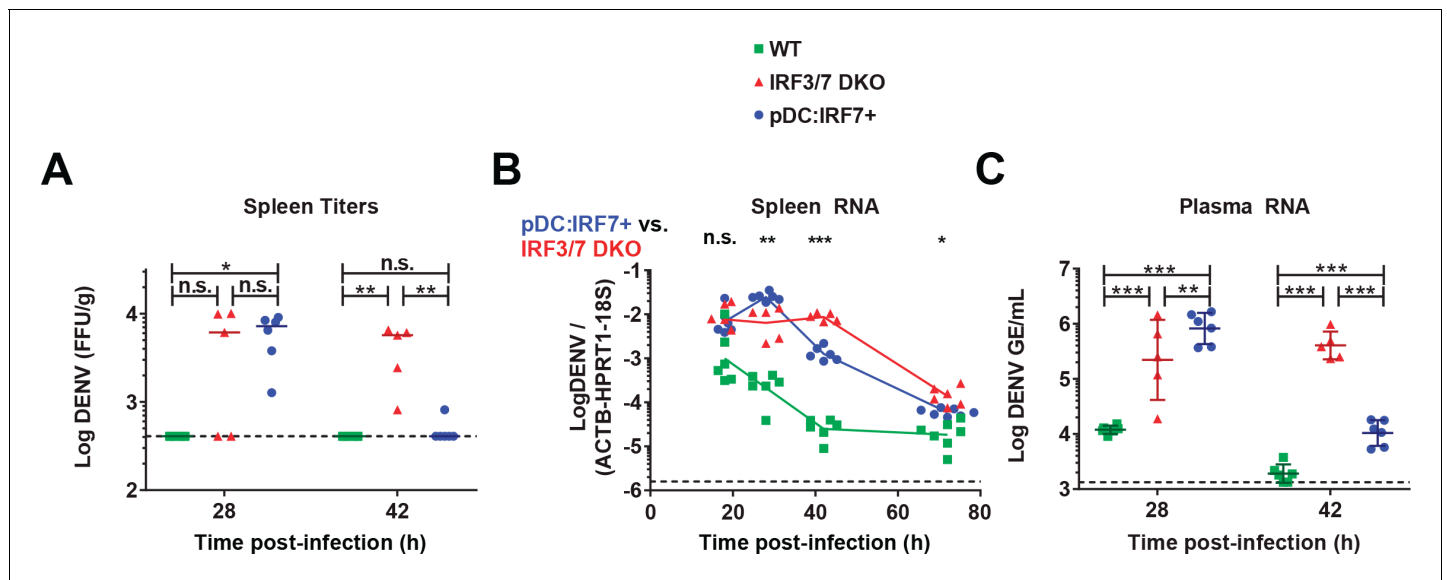
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in vivo and ex vivo observations, we concluded that the principal signaling pathway downstream of DENV-activated pDCs is IRF7-dependent induction of the IFN-I pathway, which could be wholly segregated from the activation of NF- $\kappa$ B-induced cytokines.

We next sought to determine whether pDC IRF7-induced responses are sufficient to protect mice from DENV. Mice were infected i.v. by DENV and viral RNA and titers were assessed in the spleen, the primary site of replication in infected mice. Viral titer and RNA levels were lower in WT mice than either *Irf3/7* DKO or pDC:*Irf7*<sup>+</sup> mice (Figure 4 and Figure 4—figure supplement 1A), likely due to the higher IFN-I response. Notably, at 42–72 hr post-infection, pDC:*Irf7*<sup>+</sup> mice displayed significantly reduced levels of DENV titer and RNA in the spleen and plasma compared to *Irf3/7* DKO mice (Figure 4 and Figure 4—figure supplement 1A). The higher DENV levels in pDC:*Irf7*<sup>+</sup> mice as compared to *Irf3/7* DKO mice at early time points (i.e. 28 h p.i.) may reflect an enhanced recruitment of DENV-susceptible cells in the spleen of pDC:*Irf7*<sup>+</sup> mice due to the localized pDC-induced IFN-I response (Schmid and Harris, 2014; Seo et al., 2011). Our results indicate that IRF7 expression in pDCs is sufficient to control DENV through the local production of IFN-I.

### Type I and type II IFN-induced ISG responses in pDC:*Irf7*<sup>+</sup> mice

To examine the downstream effector mechanisms initiated by pDC-derived IFN-I, we considered the relative contribution of lymphocyte-derived type II IFN (IFN-II) to viral clearance. Notably, an IFN-II response has been observed even in the absence of *Irf3* and *Irf7* expression in response to DENV infections, and was demonstrated to eventually control DENV infection (Chen et al., 2013). To assess IFN-II responses, we measured *Irfng* and *Gbp2* expression, the latter being known to specifically define an IFN-II response (Hall et al., 2012). Interestingly, *Irfng* and *Gbp2* up-regulation

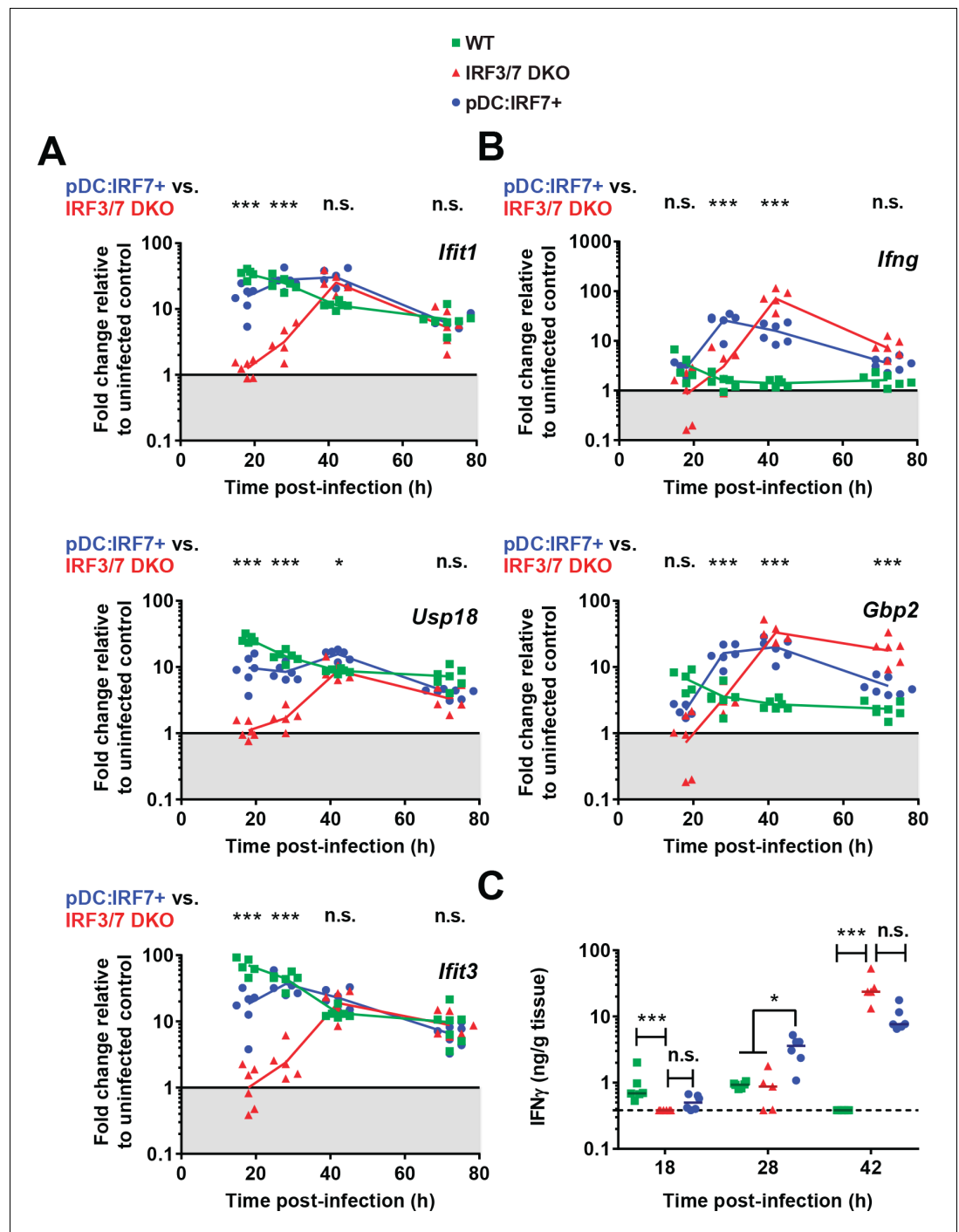


**Figure 4.** pDC:*Irf7*<sup>+</sup> mice control DENV viremia. DENV infection (i.v.) of mice with the indicated genotypes followed by the analysis of DENV titers/RNA in spleens (A–B) and plasma (C) collected at the indicated time points p.i. DENV RNA levels are normalized to a panel of housekeeping genes (*hprt1*,  *$\beta$ -actin*, and *18S* rRNA) and to exogenous RNA (*xef1A*) for spleen and plasma samples, respectively. Results are expressed as normalized Log<sub>10</sub> DENV genome equivalents (GE) or Log<sub>10</sub> foci forming unit (ffu)/g tissue; RNA: mean  $\pm$ SD, titers: median,  $n = 5$ –7 mice (spleen),  $n = 5$ –6 mice (plasma).  
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The following figure supplement is available for figure 4:

**Figure supplement 1.** Regulation of splenic pDC markers and DENV levels is concomitant with IFN responses.

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**Figure 5.** pDC:Irf7<sup>+</sup> mice display elevated IFN-I and accelerated IFN-II responses compared to Irf3/7 DKO mice during DENV infection. Mice were infected as in **Figure 4**. (A–B) Type I IFN-induced ISGs (A) and ISG specifically induced by type II IFN signaling (B). RNA levels were assessed in the spleen and normalized to housekeeping panel (*hprt1*,  *$\beta$ -actin*, *18S*). Transcript levels expressed as the fold change relative to uninfected mice for each genotype; geometric mean,  $n = 5–7$  mice per condition, each data point corresponds to an individual mouse. (C) Quantification of IFN $\gamma$  in spleen homogenates by ELISA; median,  $n = 5–6$  mice per condition. IFN $\gamma$  was undetectable in uninfected control mice.

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The following figure supplement is available for figure 5:

**Figure supplement 1.** Protection in pDC:Irf7<sup>+</sup> mice involves IFNAR1.

Figure 5 continued on next page

Figure 5 continued

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(Figure 5B–C) temporally correlated with the induction of IFN-I-induced ISGs in DENV-infected *Irf3/7* DKO mice (Figure 5A and Figure 4—figure supplement 1B–E). Of note, these ISGs including *Ifit1/Usp18/Ifit3* respond more highly to IFN-I, but will still be induced to some extent by type II IFN [(Chen et al., 2013; Hall et al., 2012; Liu et al., 2012) and data not shown]. Therefore, in agreement with these prior studies, IFN-II signaling likely mediates the late induction of a larger set of ISGs observed in *Irf3<sup>-/-</sup>/Irf7<sup>-/-</sup>* mice.

The kinetic profile of the type II IFN response was markedly different in pDC:*Irf7*<sup>+</sup> mice as compared to *Irf3/7* DKO mice, with *Irfng* and *Gbp2* expression detected as early as 18 h p.i. and peaking at 28 h p.i. (Figure 5B–C). This led to the compelling hypothesis that early pDC IRF7-mediated signaling accelerates the IFN-II response, which is known to be part of the eventual control of DENV in mouse models (Chen et al., 2013; Costa et al., 2017; Shresta et al., 2004).

Given that the ex vivo response of pDCs was biased toward IRF7-mediated IFN-I production (Figures 1 and 3A–B), we hypothesized that the early ISG response in pDC:*Irf7*<sup>+</sup> mice was mediated by this pathway. To formally test the dependence of this early ISG response on IFN-I and thus IFNAR signaling, we employed an IFNAR1 blocking antibody and challenged mice with DENV. IFNAR blockade in pDC:*Irf7*<sup>+</sup> mice abrogated this early ISG response (Figure 5—figure supplement 1A) and correspondingly increased both DENV viremia in these mice, as readily observed in the spleen (Figure 5—figure supplement 1C) and to a lesser extent in the blood (Figure 5—figure supplement 1D; not significant; likely as the consequence of the limited dynamic range of DENV detection at this time point in blood). Of note, consistent with the results of Figure 5, ISG expression was still detected in the *Irf3/7* DKO and pDC:*Irf7*<sup>+</sup> at a later time point (72 h p.i.) (Figure 5—figure supplement 1A–B), further suggesting that the late induction of ISGs is independent of pDC IRF7-mediated IFN-I and likely induced by IFN-II. Along the same line, recent work demonstrates that IRF1-mediated IFN-II can be induced by DENV, independent of *Irf3/Irf7* expression (Carlin et al., 2017). In accordance, we showed that the IFNAR1-dependent response contributes to the control of DENV in *Irf3/7* DKO mice (Figure 5—figure supplement 1C).

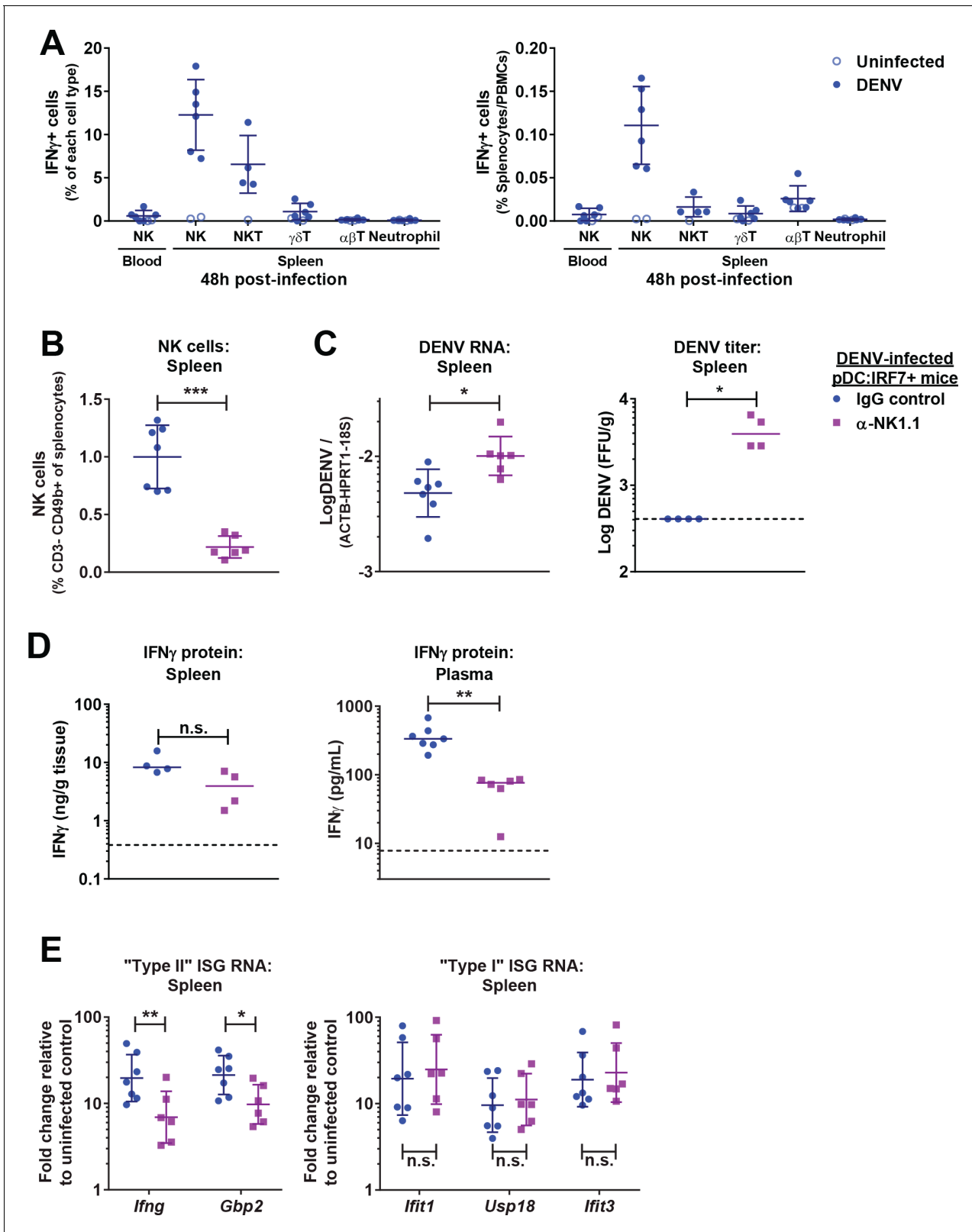
Together, our results demonstrated that, despite undetectable IFN-I at a systemic level, the early ISG induction and control of DENV by the pDC-IRF7-induced response relies on IFN-I/IFNAR1 signaling. Importantly, we also observed an acceleration of IFN-II signaling as a result of IRF7 expression in pDCs, temporally associated with viral control in DENV-infected pDC:*Irf7*<sup>+</sup> mice.

## Mediation of IFN-II production and viral control by NK cells

We next aimed at identifying the cell type(s) responsible for IFN-II production and its subsequent impact on signaling in infected pDC:*Irf7*<sup>+</sup> mice. We observed that an elevated fraction (~12%) of splenic NK cells produced IFN $\gamma$  in DENV-infected pDC:*Irf7*<sup>+</sup> mice (Figure 6A). For other cell types, including NKT cells,  $\gamma\delta$ -T cells,  $\alpha\beta$ -T cells and neutrophils, IFN $\gamma$ <sup>+</sup> cells represented an extremely small fraction of total splenocytes (Figure 6A, right panel and S5A–B). Interestingly, splenic, but not blood-circulating NK cells, produced IFN $\gamma$  protein in response to DENV infection, consistent with the absence of upregulation of blood *Irfng* mRNA (data not shown) and limited induction of the activation marker CD69 on blood NK cells (Figure 6—figure supplement 1C). This implies that NK cell response is localized to the spleen, the primary site of DENV replication. In agreement with a localized host response, we showed that the accelerated IFN-II response in DENV-infected pDC:*Irf7*<sup>+</sup> mice was associated with an early recruitment of neutrophils to the spleen (Figure 6—figure supplement 1D) as well as maturation of monocytes (identified as Ly6C<sup>++</sup>/Lineage<sup>-</sup> cells), as represented by upregulation of MHCII, which did not occur in *Irf3/7* DKO mice (Figure 6—figure supplement 1E). Of note, both cell types have been previously shown to be involved in the modulation of NK cell activation (Costa et al., 2017; Kang et al., 2008).

As NK cells were the primary producers of IFN-II (Figure 6A), we sought to identify their role during DENV infection in pDC:*Irf7*<sup>+</sup> mice in NK cell depletion studies. An efficient depletion of NK cells (i.e. reduction by >80% of splenic and blood NK cells, Figure 6B and Figure 6—figure supplement 2A) lead to a significant increase of both splenic DENV RNA and viral titers in pDC:*Irf7*<sup>+</sup> mice





**Figure 6.** NK cells produce IFN $\gamma$  and control DENV infections in pDC:Irf7<sup>+</sup> mice. (A) Quantification of IFN $\gamma$ <sup>+</sup> cells in spleen/blood samples from DENV infected pDC:Irf7<sup>+</sup> mice at 48 h p.i. by FACS. Cell subsets were identified as shown in **Figure 6—figure supplement 1A**. Results presented as a percentage of each cell type (left panel) or relative to total splenocytes/PBMCs (right panel); mean  $\pm$  SD,  $n = 4-6$  infected,  $n = 1-2$  uninfected mice per cell population. (B–E) pDC:Irf7<sup>+</sup> mice were injected i.p. with anti-NK1.1 depleting or IgG2a control antibody and infected 24 hr later with DENV i.v.; *Figure 6 continued on next page*

Figure 6 continued

$n = 6-7$  mice per condition. Analyses were performed at 48 h p.i. in the indicated tissues. (B) NK cells as a percentage of splenocytes were identified by FACS as in **Figure 6—figure supplement 2A**; mean  $\pm$  SD. (C)  $\text{Log}_{10}$  DENV genome levels; mean  $\pm$  SD and  $\text{Log}_{10}$  foci forming unit (ffu)/g tissue ; median. (D) Quantification of  $\text{IFN}\gamma$  by ELISA; median.  $\text{IFN}\gamma$  was undetectable in uninfected control mice. (E) ISG RNA levels are expressed as fold change relative to uninfected IgG2a-treated control mice; geometric mean  $\pm$  SD.

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The following figure supplements are available for figure 6:

**Figure supplement 1.** Activation of NK cells in DENV-infected mice, neutrophil influx into the spleen and monocyte activation correlate with pDC-dependent responses to DENV.

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**Figure supplement 2.** Depletion of NK cells in DENV infected mice.

DOI: <https://doi.org/10.7554/eLife.34273.014>

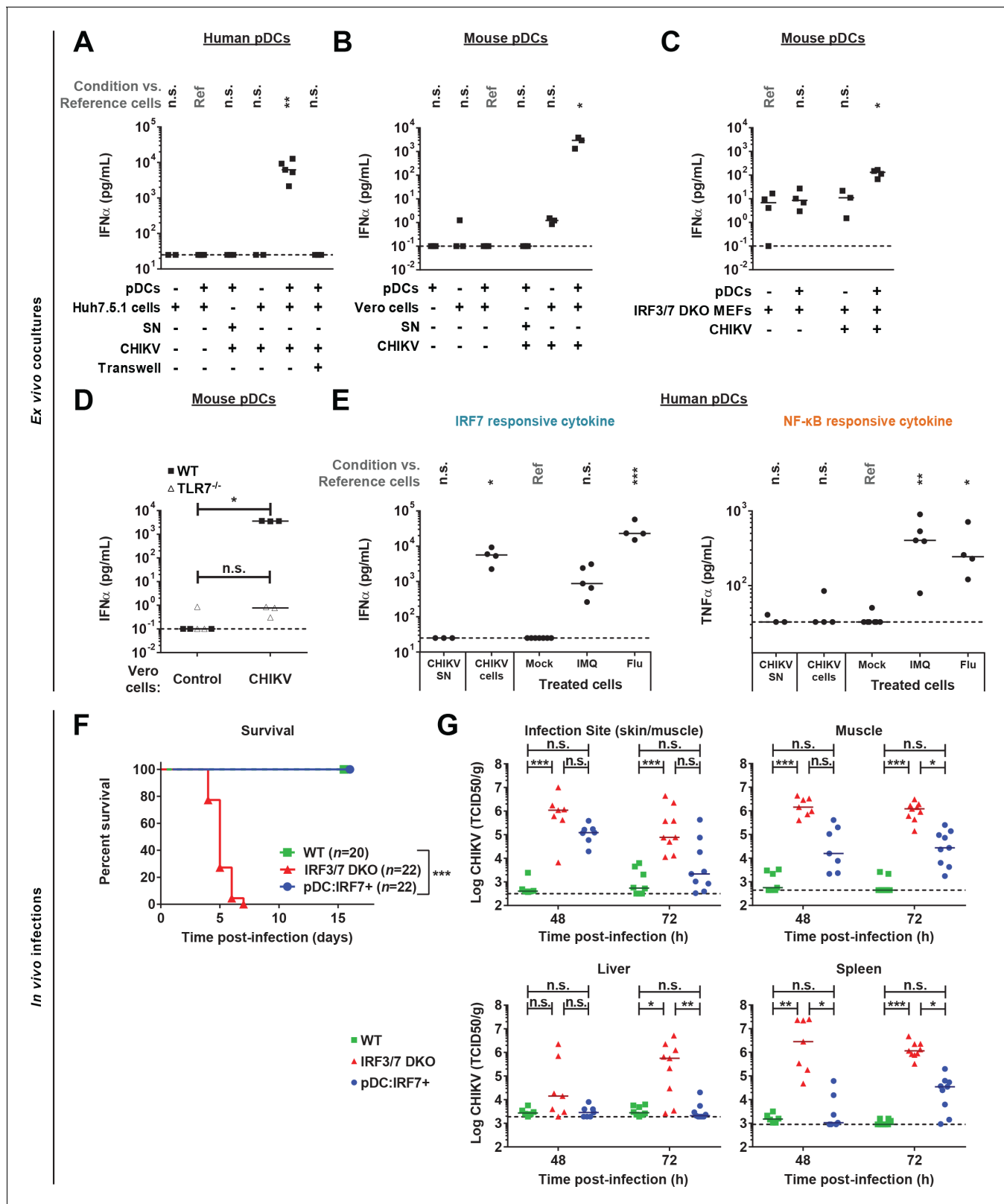
(**Figure 6C**). This was associated with a decrease in  $\text{IFN}\gamma$  mRNA,  $\text{IFN}\gamma$  protein and IFN-II ISG signature in the spleen and plasma (**Figure 6D–E**), along with reduced neutrophil recruitment to the spleen and maturation of monocytes (**Figure 6—figure supplement 2B–C**). Closing the circle and establishing  $\text{IFN}\alpha$  responses as upstream, we observed that IFN-I-induced ISG levels were not impacted by NK cell depletion in pDC:*Irf7*<sup>+</sup> mice at 48 h p.i. (**Figure 6E**).

### CHIKV activates a pDC IRF7-restricted response via cell-cell contact and, which controls in vivo infection

We extended the study to CHIKV, also a positive-sense single-stranded RNA mosquito-borne virus of major concern for human health. We tested whether pDC activation by CHIKV shares the same features as DENV, showing that indeed, human pDCs produced robust  $\text{IFN}\alpha$  in response to CHIKV only when in contact with CHIKV-infected cells (**Figure 7A**). Similarly, robust  $\text{IFN}\alpha$  levels were detected when WT mouse pDCs were co-cultured with CHIKV infected Vero cells or with syngeneic co-cultures of *Irf3/7* DKO mouse embryonic fibroblasts (MEFs) (**Figure 7B–C**). By contrast, cell-free CHIKV and CHIKV-infected cells physically separated from pDCs did not induce  $\text{IFN}\alpha$  secretion (**Figure 7A–B**). CHIKV-infected cells failed to activate pDCs deficient for *Tlr7*, whereas pDCs deficient for *Mavs* (i.e. the downstream signaling adaptor of RLR pathway) produced levels of  $\text{IFN}\alpha$  comparable to WT pDCs (**Figure 7D** and data not shown). Comparable to DENV-induced responses, the sensing of CHIKV-infected cells by pDCs led to an IRF7-mediated cytokine production ( $\text{IFN}\alpha$ ), but not NF- $\kappa$ B-induced inflammatory cytokines ( $\text{TNF}\alpha$ ) (**Figure 7E**). Together, this demonstrated that, like DENV, pDC induction by CHIKV required the cell-to-cell sensing of infected cells by TLR7-induced signaling and led to an IRF7-restricted response.

We thus tested whether pDC IRF7-induced responses were sufficient to protect mice from CHIKV. Mice were infected s.c. with CHIKV, and as previously reported WT mice showed no clinical symptoms, whereas *Irf3/7* DKO mice rapidly succumbed to CHIKV infection (**Figure 7F**) (Schilte et al., 2012). By contrast, pDC:*Irf7*<sup>+</sup> mice experienced no overt clinical symptoms with 100% of mice surviving infection (**Figure 7F**). Early control of viremia was improved in pDC:*Irf7*<sup>+</sup> mice compared to *Irf3/7* DKO mice, although reduced as compared to WT mice (**Figure 7G**). By day 7 p.i., pDC:*Irf7*<sup>+</sup> mice had cleared CHIKV infections (data not shown). Importantly, there was differential control of CHIKV in pDC:*Irf7*<sup>+</sup> mice depending on the tissue. The initial sites of CHIKV replication (i.e. the infection site: skin/muscle and muscle) displayed relatively higher viral titers as compared to more distal sites (i.e. the spleen and liver), where virus was barely detectable at 48 h p.i. in pDC:*Irf7*<sup>+</sup> mice (**Figure 7G**). It is possible that the pDC antiviral response controls infection once virus spreads systemically, as previously proposed in the context of Herpes Simplex virus infection (Swiecki et al., 2013).

We established a mouse  $\text{IFN}\alpha$  Single Molecule Assay (SiMoA) (Rissin et al., 2010), with a quantification limit at 0.1 pg/ml (i.e. >100 fold increased sensitivity compared to current assays, data not shown). Nonetheless plasma  $\text{IFN}\alpha$  remained undetectable in CHIKV-infected pDC:*Irf7*<sup>+</sup> mice at both 24 and 48 h p.i. (data not shown). Therefore, we validated the dependence on IFN-I-induced signaling for the control of CHIKV by pDCs using IFNAR1 blocking antibody. IFNAR blockade in pDC:*Irf7*<sup>+</sup> mice induced lethality and, consistently, greatly increased CHIKV viremia (**Figure 7—figure supplement 1A–B**). By contrast with results for DENV (**Figure 5—figure supplement 1C**), IFNAR1-



**Figure 7.** CHIKV activates a pDC IRF7-restricted response via cell-cell contact and pDC:Ir7<sup>+</sup> mice control CHIKV viremia. (A) Quantification of IFN $\alpha$  by ELISA in supernatants of human pDCs cocultured for 22–24 hr with CHIKV-infected or uninfected Huh7.5.1 cells [seeded together or separated by a 0.4  $\mu$ m transwell membrane] or treated with CHIKV infectious supernatant (SN); median,  $n = 3$ –5 independent experiments. (B–D) Quantification of IFN $\alpha$  levels by single molecule array (SiMoA) assay in supernatants of pDCs isolated from spleens of WT or TLR7<sup>-/-</sup> mice and cocultured with Vero cells (B, D) Figure 7 continued on next page

Figure 7 continued

or *Irf3/7* DKO MEFs (C) for 24 hr. Cultured cells were infected or not with CHIKV-GFP, as indicated; median,  $n = 3-4$  independent experiments. (E) Quantification of IFN $\alpha$  and TNF $\alpha$  by ELISA in supernatants of human pDCs treated for 22–24 hr with CHIKV infectious supernatant (CHIKV SN), TLR7 agonists [IMQ; 1  $\mu$ g/mL and Flu;  $3 \times 10^3$  FFU], or cocultured with CHIKV-infected Huh7.5.1 cells; median,  $n = 4-5$  independent experiments. (F–G) CHIKV infection subcutaneously (s.c.) of mice with the indicated genotypes followed by analysis of the survival rate (F) and CHIKV infectious titers in the indicated tissue homogenates (G); median,  $n = 7-22$  mice per condition.

DOI: <https://doi.org/10.7554/eLife.34273.015>

The following figure supplement is available for figure 7:

**Figure supplement 1.** Protection in pDC:*Irf7*<sup>+</sup> mice involves IFNAR1 and NK response.

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dependent response did contribute to the control of CHIKV in *Irf3/7* DKO mice (**Figure 7—figure supplement 1B**). This discrepancy between DENV and CHIKV infections likely reflects the greater impact of IFN-I signaling in CHIKV versus DENV infection and/or a differential contribution of the alternate IRF-mediated signal transduction. Similar to DENV, NK cell depletion in pDC:*Irf7*<sup>+</sup> mice increased CHIKV titers (**Figure 7—figure supplement 1C**, ~10 fold-increase, although not statistically significant).

Together, these data indicate that IRF7 expression in pDCs is sufficient to control two genetically distant RNA viruses (CHIKV and DENV) via the local production of IFN-I and IFN $\gamma$  secretion by NK cells.

## Discussion

DENV and CHIKV are important mediators of human diseases, causing a significant human and economic burden worldwide. We provide compelling evidence that pDCs are a key cell type in the initiation of antiviral responses to these two distinct RNA viruses. We demonstrate that pDC activation by DENV/CHIKV is characterized by IRF7-prioritized signaling, while passively excluding NF- $\kappa$ B-mediated responses. Despite undetectable levels of systemic IFN-I, pDC IRF7 signaling is critical for the induction of a potent antiviral response. This might reflect a local pDC IFN-I response and is in keeping with the requirement for physical contact with infected cells to activate pDCs, in turn inducing a potent ISG response. Notably, pDC-IRF7 activation accelerates NK-mediated IFN-II induction, which assists in control of viremia.

pDCs have previously been demonstrated to be required for the control of several viral infections, including herpes simplex virus (HSV), murine hepatitis virus (MHV), murine cytomegalovirus (MCMV), vesicular stomatitis virus (VSV) and lymphocytic choriomeningitis virus (LCMV) (**Swiecki and Colonna, 2015**). In contrast, others reported that pDCs are dispensable and/or do not contribute to IFN-I production in the case of certain infections, including influenza virus, respiratory syncytial virus (RSV), Newcastle disease virus (NDV) and MCMV (**Del Prete et al., 2015; GeurtsvanKessel et al., 2008; Jewell et al., 2007; Kumagai et al., 2007; Swiecki et al., 2013**), which may be a reflection of viral replication mechanisms, inoculum size, or location (**Swiecki et al., 2010; Swiecki et al., 2013**). These observations bring the emerging concept that while antiviral response (i.e. IFN-I production) in infected cells acts as a first line defense, pDCs may serve as a failsafe, triggered upon systemic infection and therefore failure of viral control (**GarciaGarcía-Sastre, 2017; Tomasello et al., 2014**). This assumption is in line with the multiple inhibitory mechanisms of antiviral sensing by viral proteins expressed by infected cells (**GarciaGarcía-Sastre, 2017**). Nonetheless, previous investigations have primarily demonstrated the importance of pDCs in viral control by depletion of pDCs (**Swiecki and Colonna, 2015**). Here, we validated a model consisting of a pDC-restricted IFN response, which offers a unique opportunity to define pDC function. This new model also permitted the study of the possible cryptic pDC-mediated control of certain infections. For instance, pDC antiviral function might be masked due to the homeostasis of the innate immunity, including an enhanced response by other cell types when pDCs are depleted.

We demonstrated that pDCs are sufficient to initiate early control of viral infections, via induction of IRF7-prone signaling. In this mouse model, the restoration of *Irf7* expression in pDCs does not reflect control under the endogenous promoter, as the *Irf7* gene is under the control of the pDC-specific *Siglech* promoter. However, *Irf7* overexpression artifacts are avoided in the pDC:IRF7+

model as *Irf7* steady-state expression is reduced under the control of the *Siglech* promoter relative to the *Irf7* promoter (Figure 2B). Furthermore, *Siglech* expression by pDCs is inversely correlated to ISG upregulation in vivo (Figure 4—figure supplement 1E), in agreement with prior study (Puttur et al., 2013). Importantly, while systemic IFN-I was not detectable in response to DENV/CHIKV infection, the pDC-IRF7 response rapidly and potently induces a broad array of ISGs in multiple organs, dependent on IFN-I signaling. Therefore, in line with our ex vivo results in this and previous studies (Décembre et al., 2014), we propose that pDCs respond in a contact-dependent manner to infected cells, and thus induce highly localized IFN-dependent responses. Importantly, this response is sufficient to impart a large-scale antiviral response, which protects from viral infections.

The engagement of TLR7/9 leading to IFN-I production and NF- $\kappa$ B-dependent pro-inflammatory cytokines by pDCs may affect downstream immunological parameters, such as for example, B cell differentiation induced by pDC response to influenza virus (Jego et al., 2003). We observed that pDCs fail to respond via NF- $\kappa$ B-mediated inflammatory responses to DENV/CHIKV-infected cells, indicating that pDC TLR7 signaling induced by these viruses primarily leads to IRF7 induction. Consistently, on the whole-organism level, while ectopic IRF7 expression in pDCs fully restores ISG responses, NF- $\kappa$ B responses to DENV were absent in both pDC:*Irf7*<sup>+</sup> and *Irf3/7* DKO mice, thus underlining the importance of the IRF7 and dispensability of the NF- $\kappa$ B responses in pDC-mediated control of DENV and CHIKV infections. Our results also imply that NF- $\kappa$ B induction by these viruses in other cell types likely requires cross-talk between IRF3 and NF- $\kappa$ B (Freaney et al., 2013; Iwanaszko and Kimmel, 2015).

While pDC IFN-I responses were important in early control of both DENV and CHIKV infections in vivo, substantial differences were observed in the overall outcome of infections. CHIKV infections were invariably lethal in the *Irf3/7* DKO genetic background, and pDC IFN-I responses provided 100% protection to this lethal phenotype. By contrast, DENV infections were well-controlled even in *Irf3/7* DKO mice by 72 h p.i. Notably, pDC IFN-I responses lead to substantial control of DENV viremia at time points intermediate between WT and *Irf3/7* DKO mice (~42–48 hr). In our study, the lethal phenotype in CHIKV infection in *Irf3/7* DKO mice correlated with substantially higher viral titers, as compared to DENV levels. Interestingly, blockade of IFN-I signaling in *Irf3/7* DKO mice only increased viremia in situations where lower basal viral titers were observed: for example, DENV infections in all organs, or CHIKV viral titers in the liver. We thus suggest that the persistence of IFN-I-mediated viral control under these conditions depends on IRF5 or IRF1 signaling (Carlin et al., 2017; Chen et al., 2013; Lazear et al., 2013; Rudd et al., 2012), as control of flaviviral infections is still contingent on IFNAR signaling in the absence of *Irf3/Irf7* expression. The contribution of these alternate IRF proteins to the antiviral response may only be revealed in case of relatively low viremia.

Eventual control of DENV in the *Irf3/7* DKO background depended on the induced IFN $\gamma$  response (Chen et al., 2013). In agreement, we observe an IFN-II response in DENV-infected *Irf3/7* DKO mice peaking at ~42 h p.i., which is largely absent in WT mice. Importantly, the antiviral IFN-II response is accelerated in pDC:*Irf7*<sup>+</sup> mice relative to *Irf3/7* DKO mice. These pDC-regulated IFN-II responses are consistent with prior work showing a defect in type II IFN activation in a pDC-deficient mouse model (Guillerey et al., 2012). We demonstrated that IFN $\gamma$  production in DENV-infected pDC:*Irf7*<sup>+</sup> mice is largely mediated by NK cells in the spleen, the primary site of DENV replication. These activated NK cells mediate downstream IFN-II responses that contribute to control of DENV viremia, in accordance with a recent report (Costa et al., 2017). A similar decrease in viremia was observed in CHIKV-infected pDC:*Irf7*<sup>+</sup> mice depleted of NK cells, although these results did not reach statistical significance. DENV may be more sensitive to type II IFN signaling (relative to type I IFN) compared to CHIKV, as DENV infections cause higher lethality in *Ifngr*<sup>-/-</sup> compared to *Ifnar*<sup>-/-</sup> mice, a situation that is reversed in the context of CHIKV infection (Gardner et al., 2012; Shresta et al., 2004).

Importantly, IFN $\gamma$  production by NK cells was not observed in the blood, a site of low DENV viremia, likely indicating a necessity for NK cells to be closely apposed to DENV-infected cells and/or DENV-activated cells to achieve NK cell activation of IFN-II responses. We also observed an association with other immune cell parameters, including a pDC IRF7-mediated influx of neutrophils and monocyte activation in the spleens of DENV-infected mice. While some reports suggested that type I IFN directly contributes to NK cell response, NF- $\kappa$ B-dependent cytokines are well-known to regulate IFN $\gamma$  production by NK cells (Schulthess et al., 2012). Along the same lines, in vitro studies suggested that pDCs act in concert with monocytes to potentiate NK-cell-mediated IFN $\gamma$  production in

the context of HCV and DENV infection (*Costa et al., 2017; Zhang et al., 2013*). Future studies are required to determine how DENV/CHIKV-activated pDCs can directly augment NK cell IFN $\gamma$  responses, or whether pDC-produced type I IFN acts through other cell types and attendant NF- $\kappa$ B-dependent cytokine responses for this amplification of type II induction.

The ability to segregate NF- $\kappa$ B and IRF7 responses may have potential use in developing novel therapies. Notably, NF- $\kappa$ B-dependent cytokines have been associated with disease severity in both DENV and CHIKV infection (*Chen et al., 2007; Dupuis-Maguiraga et al., 2012*). This is juxtaposed with pDC-derived IFN responses supporting viral clearance while avoiding possible inflammatory responses. As arboviral infections tend to be acute, rather than chronic diseases, this study provides an important indication of how early viral control may be mediated by one of the key drivers of the IFN response, the plasmacytoid dendritic cell.

## Materials and methods

### Cells and reagents

Huh7.5.1 cells (*Zhong et al., 2005*) (RRID:CVCL\_E049), Vero cells (CLS Cat# 605372/p622\_VERO, RRID:CVCL\_0059), and BHK-21 cells (ATCC Cat# CCL-10, RRID:CVCL\_1915) were maintained in DMEM (Dulbecco's modified Eagle medium; Life Technologies) supplemented with 10% FBS, 100 units (U)/ml penicillin, 100 mg/ml streptomycin, 2 mM L-glutamine (Life Technologies). Cells were maintained at 37°C in 5% CO<sub>2</sub>. MEFs were maintained in supplemented RPMI growth media. The work involved cell line, with routinely verify status for contamination (e.g. Mycoplasma).

Cell culture reagents included the TLR7 agonist (IMQ; imiquimod) and TLR7/8 agonist (R848) (Invivogen, San Diego, CA); TLR7 antagonist, IRS661 (5'-TGCTTGCAAGCTTGCAAGCA-3') synthesized on a phosphorothioate backbone (MWG Biotech, Ebersberg, Germany); TLR3 agonist (poly-I:C) (Invivogen); TLR9 agonist (CpG) (Invivogen); recombinant IFN $\beta$  (PBL Interferon Source, Piscataway, NJ). General reagents included: paraformaldehyde (PFA) and brefeldin A (BFA) (Sigma-Aldrich, St Louis, MO); Ficoll-Hypaque (GE Healthcare Life Sciences, Piscataway, NJ).

### Viral stocks

Viral stocks of the prototypic DENV-2 strain New Guinea C (NGC) (AF038403) were produced using in vitro RNA transcripts prepared from DENV-2 infectious plasmid clone pDVWS601 plasmid (*Pryor et al., 2001*). Briefly, plasmid was linearized with XbaI (New England Biolabs, Ipswich, MA) and RNA transcripts were produced and purified using mMACHINE T7 Kit (Ambion/Thermo Fisher Scientific, Waltham, MA). RNA transcripts were introduced into BHK-21 cells by electroporation (using 100  $\mu$ g RNA to transfect 8  $\times$  10<sup>7</sup> cells, with 4  $\times$  10<sup>6</sup> cells per electroporation). Electroporation was performed in 0.4 cm cuvettes (Bio-Rad, Hercules, CA) with a Gene Pulser system (Bio-rad), using a square-wave pulse (280 V, 25 ms). Virus-containing supernatants were collected at 3–5 days post-electroporation and clarified through a 0.2  $\mu$ m filter (Corning Inc, Corning, NY). Viral supernatants were concentrated by ultracentrifugation at 75000  $\times$  g for 2 hr at 4°C, resuspended in DMEM growth media, and frozen at –80°C until titration and/or use. Viral stocks were titered on Huh7.5.1 cells, as described below, and adjusted to 2  $\times$  10<sup>7</sup> focus-forming units (ffu)/mL.

CHIKV-21 viral stocks, used for all in vivo infections, were prepared from clinical samples (*Schilte et al., 2010*). Briefly, CHIKV-21 was propagated in C6/36 mosquito cells, and infectious supernatants were harvested, clarified through a 0.2  $\mu$ m filter, and frozen at –80°C until titration and/or use. Recombinant CHIKV-GFP, which expresses GFP under a subgenomic promoter (CHIKV-GFP 5'), was generated from a full-length infectious cDNA clone provided by S. Higgs (*Vanlandingham et al., 2005*) and was used for all CHIKV in vitro mouse studies. Briefly, CHIKV-GFP 5' plasmid was linearized with *NotI* (New England Biolabs) and RNA transcripts were produced and purified using mMACHINE SP6 Kit (Ambion). RNA transcripts were introduced into BHK-21 cells by electroporation (using 1  $\mu$ g RNA to transfect 5  $\times$  10<sup>6</sup> cells). Virus-containing supernatants were collected when the viral cytopathic effect reached 75% of transfected cells, clarified through a 0.2  $\mu$ m filter (Corning Inc), and frozen at –80°C until titration and/or use. Viral stocks were titered on Vero cells, as described below.

DENV infectivity titers in concentrated culture supernatants or mouse organ homogenates were determined by end-point dilution using Huh 7.5.1 cells (*Décembre et al., 2014*). Foci forming units

(ffu) were detected 72 hr after infection. Briefly, Huh7.5.1 cells were fixed with 4% PFA and permeabilized by incubation for 7 min in PBS containing 0.1% Triton. Cells were then blocked in PBS containing 3% BSA for 15 min and incubated for 1 hr with mouse anti-E glycoprotein (clone 3H5) hybridoma supernatant diluted in PBS containing 1% BSA. After three washes with PBS, cells were incubated 1 hr with secondary Alexa 555-conjugated anti-mouse antibody. Virally-infected foci of E-positive cells were determined using a Zeiss Axiovert 135 microscope.

CHIKV infectivity titers in culture supernatants or mouse organ homogenates were titrated as TCID<sub>50</sub> endpoint on Vero cells using a standard procedure. Serial 10-fold dilutions of supernatants were added in six replicates in 96-well plates seeded with 10<sup>4</sup> Vero cells. The cytopathic effect was scored 5 days after infection and the titers were calculated by determining the last dilution giving 50% of wells with cells displaying a cytopathic effect. Results were expressed as TCID<sub>50</sub>/mL.

Viral stocks of Influenza A Virus (FluAV, H1N1/New Caledonia/2006) were produced as previously described (*de Chasse et al., 2013*) and kindly provided by Dr V. Lotteau (CIRI, Lyon, France).

## Mouse models, infection and treatment

All mice, previously backcrossed on a C57BL/6 background, were bred and maintained at the Institut Pasteur (Paris, France) and the Plateau de Biologie Expérimentale de la Souris (PBES, Lyon, France) with Authorization Agreement C 69 123 0303. All animal studies were performed in accordance with the European Union guidelines for approval of the protocols by the Institutional Committees on Animal Welfare of the Institut Pasteur (Paris, France, OLAW assurance #A5476-01) and by the local ethics committee Rhône-Alpes d'Éthique pour l'Expérimentation Animale (Lyon, France, Authorization Agreement C2EA-15).

Housing conditions for mice in the PBES were as follows. Mice were housed under group conditions in individually ventilated cages, from 1 to 8 single-sex mice per cage. Specific-pathogen free (SPF) housing was maintained, with regular sanitary status testing of sentinel mice and bedding for standard mouse pathogens. Mice were fed LASQCDiet Rod16-R (Lasvendi), and temperatures were maintained at 22 ± 2°C. All mice were treatment- and drug-naive at the time of experimental manipulation. Manipulations including and following viral infections were carried out under animal BSL-3 (A3) conditions; mice were anesthetized with xylazine/ketamine (20 mg/kg and 100 mg/kg by body weight) for all A3 manipulations. Similar housing conditions were maintained at the Institut Pasteur: SPF housing, 1–5 single-sex mice per cage, and manipulations carried out under animal BSL3. All in vivo CHIKV experiments were performed at the Institut Pasteur.

*Irf3*<sup>-/-</sup>*Irf7*<sup>-/-</sup> double knockout (referred to as *Irf3/7* DKO) mice were generated and kindly provided by Dr T. Taniguchi (University of Tokyo, Tokyo, Japan) (*Honda et al., 2005*). *cardif*<sup>-/-</sup> mice were generated by Dr J. Tschopp, and *Tlr7*<sup>-/-</sup> mice were provided by Dr S. Akira (Osaka University, Osaka, Japan). Knock-in *Siglech*<sup>Irf7+</sup> mice were generated by Ozgene Pty Ltd company. Next, knock-in *Siglech*<sup>Irf7+</sup> mice were backcrossed onto the *Irf3/7* DKO background strain for approximately 10 generations to generate *Siglech*<sup>Irf7+</sup>*Irf3/7* DKO breeding stocks, referred to as 'Union *Irf3/7* DKO'. As the *Siglech*<sup>Irf7+</sup> transgene replaced the endogenous *Siglech* gene, to preserve *Siglech* expression and functionality in experimental mice, 'Union *Irf3/7* DKO' mice were bred with *Irf3/7* DKO mice to generate *Siglech*<sup>Irf7/+</sup> *Irf3/7* DKO ('pDC:*Irf7*<sup>+</sup>') F1 mice.

For in vivo synthetic agonist experiments, 100 µg of CpG 2216 (Invivogen, tlr-hodna)+DOTAP (Roche) or 10 µg of poly-I:C (Invivogen, tlr-pic) were injected intravenously (i.v.) into 8- to 12-week-old mice. For DENV in vivo experiments, 8- to 16-week-old mice were infected systemically with DENV. 1–3 litters of mice of each genotype were used per experiment; each litter was aged within 3 weeks of all others in the same experiment. Mice were randomly assorted into treatment groups by the experimenter. 2 × 10<sup>6</sup> ffu of DENV was injected i.v. in the retro-orbital sinus using a 31-gauge 0.3 mL insulin syringe (Becton-Dickinson, Franklin Lakes, NJ) for all experiments. In **Figure 3—figure supplement 1**, an additional group of mice were i.v. infected with 2 × 10<sup>5</sup> ffu DENV, as indicated. DMEM growth media was used as a negative injection control. For CHIKV experiments, 8- to 12-week-old adult mice were infected with 10<sup>6</sup> pfu (plaque-forming units) of CHIKV-21 sub-cutaneously in the right flank.

For IFNAR blocking studies, a single dose (800 µg/mouse) of anti-IFNAR1 antibody (Leinco Technologies, Fenton, MO; clone MAR1-5A3) or IgG<sub>1</sub> isotype control was administered i.p. 24 hr (CHIKV) or 3 hr (DENV) before infection.

For NK cell depletion, a single dose (800  $\mu$ g and 200  $\mu$ g for CHIKV and DENV experiments, respectively) of  $\alpha$ -NK1.1 antibody (BioXCell, West Lebanon, NH; clone PK136) or IgG<sub>2A</sub> isotype control was injected i.p. 24 hr before infection. Mice cohorts, representing individual experiments, are provided in **Supplementary file 1**, with mice age and sex.

### Processing and analysis of mouse tissues

Solid tissues were collected from mice and cell suspensions were prepared by pressing tissues through 40  $\mu$ m cell strainers (Becton-Dickinson) or homogenates were prepared with the TissueLyser II (Qiagen, Hilden, Germany) or GentleMACs (Miltenyi, Bergisch-Gladbach, Germany) systems. Blood was collected by cardiac puncture (end-point) or retro-orbital bleeds and anticoagulated with ethylenediamine tetra-acetic acid (EDTA) prior to centrifugation to collect formed elements ('blood') or plasma. For viral titers and protein (ELISA) analysis, tissues were homogenized in PBS, and for qRT-PCR analysis, tissues were homogenized in guanidinium thiocyanate citrate buffer (GTC) (**Dreux et al., 2012**). Virus from tissue homogenates was titrated on Vero cells (CHIKV) or Huh7.5.1 cells (DENV). Results are expressed as median culture infective dose TCID<sub>50</sub>/g (CHIKV) or ffu/g (DENV).

### Cytokine measurements

IFN $\alpha$ / $\beta$  activity from mouse tissue homogenates or plasma was quantified using Luciferase assay (Promega, Madison, WI). Briefly, type I IFN levels were determined by incubating the reporter cell line LL171 with diluted plasma or tissue homogenates (in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 50 mM  $\beta$ -mercaptoethanol) for 8 hr. Type I IFN activity was calculated using serial dilutions of a recombinant standard (IFN $\alpha$ 4; PBL Interferon Source, Piscataway, NJ). Cytokine protein measurements from tissue culture supernatants, plasma, and tissue homogenates were performed by ELISA using commercially available kits: human IFN $\alpha$  and mouse IFN $\alpha$ /IFN $\beta$  (PBL Interferon Source), human TNF $\alpha$  and mouse TNF $\alpha$ /IL6/IFN $\gamma$  (eBioscience/Thermo Fisher Scientific, Waltham, MA). When indicated, mouse IFN $\alpha$  protein levels were determined using a newly developed mouse IFN $\alpha$  assay on the Quanterix SiMoA platform using capture and detection antibodies obtained from eBioscience (capture: Mouse IFN $\alpha$  Platinum ELISA capture antibody; detection: Mouse IFN $\alpha$  Platinum ELISA detection antibody). Recombinant mouse IFN $\alpha$ 2 (eBioscience) was used as a standard.

### Quantitative reverse transcription-PCR (qRT-PCR) analysis

RNAs were isolated from plasma or tissue homogenates prepared in guanidinium thiocyanate citrate buffer (GTC; Sigma-Aldrich) by phenol/chloroform extraction procedure as previously (**Dreux et al., 2012**). Reverse transcription was performed using the random hexamer-primed High-Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA) and quantitative PCR was carried out using the Powerup Sybr Green Master Mix (Applied Biosystems). For DENV and ISG analysis in mouse spleen, liver, and blood, absolute numbers of transcripts were generally normalized to the geometric mean of  $\beta$ -actin, *hprt1*, and *18S* housekeeping gene transcript numbers. For plasma samples lacking housekeeping transcripts, qRT-PCR was controlled by the addition of xenogeneic carrier RNAs encoding *xef1 $\alpha$*  (xenopus transcription factor 1 $\alpha$ ) in vitro transcripts in plasma diluted in GTC buffer. The sequences of the primers used in analysis are described in **Supplementary file 2**.

### Isolation of pDCs and ex vivo coculture experiments

Mouse pDCs were isolated from male and female mice from 8 to 22 weeks of age, of the indicated genotypes. pDCs were isolated from splenocytes/bone marrow via negative selection using the pDC isolation kit II (Miltenyi) or from bone marrow via positive selection using PDCA1-biotin antibody (Miltenyi) followed by anti-biotin microbead (Miltenyi) selection. Human pDCs were isolated from PBMCs derived from leukapheresis of healthy adult human volunteers, obtained according to procedures approved by the 'Etablissement Français du sang' (EFS) Committee. PBMCs were isolated using Ficoll-Hypaque density centrifugation. Human pDCs were positively selected from PBMCs using BDCA-4-magnetic beads (Miltenyi), as we previously reported (**Décembre et al., 2014**).

After isolation, pDCs were cultured with Vero, Huh7.5.1, BHK-21, or Irf3/7 DKO primary MEF cells in RPMI cell growth media (10% FBS, 100 U/ml penicillin, 100 mg/ml streptomycin, 2 mM



L-glutamine, non-essential amino acids, 1 mM sodium pyruvate and 0.05 mM  $\beta$ -mercaptoethanol) in 96-well round bottom plates at 37°C, as previously described (D cembre *et al.*, 2014), using  $8 \times 10^4$  mouse pDCs when cocultured or  $1 \times 10^4$  human pDCs. Cocultured cells were either naive, previously infected or infected 24 hr post-coculture, using a multiplicity of infection of 5 and 2 for CHIKV and DENV, respectively. Cell culture supernatants were collected at 22–24 hr after the beginning of coculture with the infected cells.

### Antibodies, flow cytometry and cell sorting

For flow cytometry, FACs sorting, and magnetic cell isolation, biotin, BV421, Pacific Blue, FITC, PE, PE-Cy7, APC, AlexaFluor-647, AlexaFluor-700, APC-eFluor780 or PerCP-Cy5.5 conjugates of the following anti-mouse antibodies were used (clone in parentheses): Siglec-H (440 c), B220 (RA3-6B2), CD11c (N418), CD11b (M1/70), CD8a (53–6.7), CD2 (RM2-5), CD19 (1D3), CD4 (GK1.5), CD3 (17A2, 500A2), NK1.1 (PK136), CD69 (H1.2F3), Ly6C (HK1.4), Ly6G (1A8), PDCA-1 (JF05-1C2.4.1), CD25 (3C7), CD49b (DX5, HM $\alpha$ 2), TER-119 (TER-119), TCR- $\gamma\delta$  (UC7-13D5), TCR- $\beta$  (H57-597), IFN $\gamma$  (XMG1.2), IRF7 (MNGPKL) (Miltenyi; eBioscience; Becton-Dickinson; Biolegend, San Diego, CA). Dylight-405 and Dylight-680 streptavidin conjugates (Life Technologies) were used to detect biotinylated antibodies.

Intracellular and extracellular staining was performed as follows. Isolated splenocytes and blood cells were depleted for erythrocytes by incubation at room temperature in red blood cell lysis buffer (1.5 M NH $_4$ Cl, 100 mM NaHCO $_3$ , 10 mM Na $_2$ EDTA, pH 7.4) and cells were washed in FACS buffer (PBS + 2 mM EDTA + 5% FCS). For extracellular staining, cell surface marker staining was performed with antibodies diluted in FACS buffer, followed by fixation using 4% PFA. For intracellular staining, cells were incubated for 3 hr at 37°C in RPMI cell growth media + 10  $\mu$ g/mL BFA, and cell surface marker staining was performed in FACS Buffer + 10  $\mu$ g/mL BFA. Fixation, permeabilization, and intracellular staining (IFN $\gamma$ ) was performed using the Cytotfix/Cytoperm kit (Becton-Dickinson).

### Statistical analysis

Statistical analysis was performed using PRISM v7.03 software (Graphpad, La Jolla, CA); only biological replicates representing separate mice and/or human donors are presented as data herein. Technical replicates, representing repeated measurements or treatments of the same cellular populations, were averaged prior to analysis. Mouse experiments were designed to detect a two-fold difference in means of parametric data at 95% confidence ( $n \geq 4$  per condition, assumed CV = 0.3); however, larger sample sizes were used as litter numbers permitted. Viral titers and cytokine levels were considered non-parametric due to the presence of many data points at the assay detection limits; therefore, non-parametric Kruskal-Wallis tests with Dunn’s multiple comparison corrections were performed on this data. Viral RNA levels and transcript fold changes, as log-normal parametric data, were log-transformed prior to analysis using parametric tests. Kinetic analyses of ex vivo pDC cytokines (Figure 1A–B) were analyzed by one-way ANOVA parametric tests of AUC (area under the curve). Parametric tests were: unpaired Student’s t test for comparisons of two conditions, one- or two-way ANOVA with a Holm-Sidak multiple comparison correction for comparison of multiple conditions. Statistical analysis of survival curves was performed using log-rank (Mantel-Cox) tests.

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### Author contributions

Brian Webster, Conceptualization, Formal analysis, Validation, Investigation, Visualization, Methodology, Writing—original draft, Writing—review and editing; Scott W Werneke, Conceptualization, Formal analysis, Validation, Investigation, Visualization, Methodology, Writing—review and editing; Biljana Zafirova, Conceptualization, Investigation, Methodology; Sébastien This, Séverin Coléon, Elodie Décembre, Investigation, Methodology; Helena Paidassi, Formal analysis, Supervision, Writing—review and editing; Isabelle Bouvier, Darragh Duffy, Formal analysis, Supervision; Pierre-Emmanuel Joubert, Formal analysis, Methodology; Thierry Walzer, Resources, Supervision, Writing—review and editing; Matthew L Albert, Conceptualization, Supervision, Funding acquisition, Writing—review and editing; Marlène Dreux, Conceptualization, Supervision, Funding acquisition, Visualization, Writing—original draft, Writing—review and editing

### Author ORCIDs

Brian Webster [id http://orcid.org/0000-0002-6806-0363](http://orcid.org/0000-0002-6806-0363)  
Helena Paidassi [id http://orcid.org/0000-0001-9915-4365](http://orcid.org/0000-0001-9915-4365)  
Thierry Walzer [id http://orcid.org/0000-0002-0857-8179](http://orcid.org/0000-0002-0857-8179)  
Marlène Dreux [id https://orcid.org/0000-0002-6607-4796](https://orcid.org/0000-0002-6607-4796)

### Ethics

Animal experimentation: All mice were bred and maintained at the Institut Pasteur (Paris, France) and the Plateau de Biologie Expérimentale de la Souris (PBES, Lyon, France) with Authorization Agreement C 69 123 0303. All animal studies were performed in accordance with the European Union guidelines for approval of the protocols by the Institutional Committees on Animal Welfare of the Institut Pasteur (Paris, France, OLAW assurance #A5476-01) and by the local ethics committee Rhône-Alpes d'Éthique pour l'Expérimentation Animale (Lyon, France, Authorization Agreement C2EA-15). Housing conditions for mice in the PBES were as follows. Mice were housed under group conditions in individually-ventilated cages, from 1-8 single-sex mice per cage. Specific-pathogen free (SPF) housing was maintained, with regular sanitary status testing of sentinel mice and bedding for standard mouse pathogens. Mice were fed LASQCDiet® Rod16-R (Lasvendi). All mice were treatment- and drug-naïve at the time of experimental manipulation. Manipulations including and following viral infections were carried out under animal BSL-3 (A3) conditions; mice were anesthetized with xylazine/ketamine (20 mg/kg and 100 mg/kg by body weight) for all A3 manipulations. Similar housing conditions were maintained at the Institut Pasteur: SPF housing, 1-5 single-sex mice per cage,

and manipulations carried out under animal BSL3. All in vivo CHIKV experiments were performed at the Institut Pasteur.

### Decision letter and Author response

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## Additional files

### Supplementary files

• Supplementary file 1. Mouse cohorts used for *in vivo* studies. Mouse genotype, age, sex, and number are provided for the *in vivo* experiments performed in **Figures 3–7** and supplements.

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• Supplementary file 2. Primer sets used in quantitative RT-PCR. The primer sets used to amplify genes from cDNA for expression analysis are provided.

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### Data availability

All data generated or analysed during this study are included in the manuscript and supporting files.

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