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# Impact of IL-1 $\beta$ on Tfh and Tfr lymphocytes during physiological and pathological germinal center reaction

Romain Vaineau

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# Sorbonne Université

Ecole doctorale 394 Physiologie, Physiopathologie et Thérapeutique

*Inserm UMR-S 959 Immunologie Immunopathologie Immunothérapie (i3)*

## **Impact of IL-1 $\beta$ on Tfh and Tfr lymphocytes during physiological and pathological germinal center reaction**

Par Romain Vaineau

Thèse de doctorat d'Immunologie

Dirigée par Stéphanie Graff-Dubois  
et co-encadrée par Nicolas Tchitchek

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Devant un jury composé de :

Dr. Helena Paidassi	Rapporteuse
Dr. Lars Rogge	Rapporteur
Dr. Sébastien Lacroix-Desmazes	Président du jury
Dr. Sabine Le Gouvello	Examinatrice
Dr. Nicolas Tchitchek	Co-encadrant de thèse
Pr. Stéphanie Graff-Dubois	Directrice de thèse



**“ L’éducation est l’arme la plus puissante que l’on puisse utiliser pour changer  
le monde ”**

*Nelson Mandela, activiste et homme politique sud-africain*

**“ Le bonheur est un état d'esprit. Il s'agit de la façon dont vous regardez les  
choses ”**

*Walt Disney, réalisateur, scénariste, producteur et animateur américain*

**“ Trouve toujours une raison de rire; cela n'ajoutera peut-être pas des années  
à ta vie, mais ajoutera de la vie à tes années ”**

*Proverbe*

**“ If you can't love yourself, how in the hell you gonna love somebody else? ”**

*RuPaul Charles, drag queen, chanteur et producteur américain*



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## Abstract

---

T follicular helper (Tfh) and T follicular regulatory (Tfr) cells orchestrate the germinal center (GC) reaction by providing balance to B cell maturation. The harmonious interplay between Tfh, Tfr and B cells fosters high affinity antibody production, while its dysregulation can yield to autoimmunity, highlighting the importance of the underlying regulatory mechanisms.

In this context, the role of Interleukin-1 $\beta$  (IL-1 $\beta$ ) emerges as a focal interest. This proinflammatory cytokine is well-documented in modulating innate immune responses through the agonist receptor IL-1R1 while IL-1R2 exerts a decoy function by sequestering IL-1 $\beta$ . However, its specific interaction with Tfh and Tfr is not fully elucidated. This study embarks on a critical exploration of IL-1 $\beta$ 's influence on the dynamic equilibrium of Tfh and Tfr, transcending the limitations of previous studies predominantly confined to murine models.

Here, we carry an original systems immunology approach encompassing the use of varied human secondary lymphoid organs that embody distinct stages of Tfh and Tfr maturation. We reveal differential patterns of IL-1 receptors that govern Tfr heterogeneity, and trajectory inference predicts a bifurcation branching out from GC-Tfh. IL-1R1 expression is associated to Tfh maturation, and its excess over IL-1R2 precipitates the transition to a unique Tfr phenotype contrasting with the one described in mice. The application of single-cell RNA sequencing together with immunophenotyping confirmed Tfh and Tfr responsiveness to IL-1 $\beta$ , which promotes their functionality. A patient cohort suggested a tangible link between dysregulated IL-1 $\beta$  signaling and pronounced aberrancies in Tfh and Tfr responses during autoimmune pathogenesis.

Collectively, our findings underscore IL-1 $\beta$ 's pivotal role in Tfh and Tfr cell dynamics through the interplay of IL-1 receptors, unveiling its influence on GC-mediated antibody responses. This study offers new insights into autoimmune disease mechanisms and potential interventions.



## Résumé

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Les lymphocytes T folliculaires auxiliaires (Tfh) et régulateurs (Tfr) orchestrent la réaction de centre germinatif (GC) en contrôlant la maturation des lymphocytes B. L'équilibre entre ces cellules est crucial pour la production d'anticorps de haute affinité; sa perturbation peut induire l'auto-immunité, mettant en exergue l'importance des mécanismes régulateurs essentiels.

L'interleukine-1 $\beta$  (IL-1 $\beta$ ), une cytokine pro-inflammatoire connue, attire l'attention. Son rôle précis est bien documenté dans le cadre de l'immunité innée grâce au récepteur agoniste IL-1R1 et au récepteur leurre IL-1R2 qui séquestre l'IL-1 $\beta$ . Cependant, son interaction spécifique avec les Tfh et Tfr reste à approfondir. Cette étude, en dépassant les limites des travaux jusqu'alors confinés aux modèles murins, vise à éclairer l'interaction dynamique entre l'IL-1 $\beta$  et ces lymphocytes.

Notre approche novatrice, empruntée à l'immunologie des systèmes, implique l'étude de divers organes lymphoïdes secondaires humains représentant des phases distinctes de maturation des Tfh et Tfr. Nous avons identifié des profils différentiels liés aux récepteurs à l'IL-1 $\beta$  démontrant l'hétérogénéité des Tfr, et l'inférence de trajectoire prédit une bifurcation des lymphocytes GC-Tfh vers certains Tfr. L'expression de l'IL-1R1 est associée à la maturation des Tfh, et son excès par rapport à l'IL-1R2 précipite leur transition vers un phénotype Tfr singulier en contraste avec celui décrit chez la souris. Le séquençage d'ARN en cellule unique et l'immunophénotypage confirment la sensibilité des Tfh et Tfr à l'IL-1 $\beta$ , qui optimise leur fonction cellulaire. Une analyse de cohorte révèle une corrélation entre la signalisation altérée de l'IL-1 $\beta$  et les anomalies des réponses Tfh et Tfr dans l'auto-immunité.

En somme, nous proposons un rôle pivot de l'IL-1 $\beta$  dans la dynamique des lymphocytes Tfh et Tfr à travers le jeu d'expression des récepteurs à l'IL-1, illustrant son impact sur les réponses anticorps induites par le GC. Cette étude ouvre des pistes innovantes pour mieux comprendre et traiter les maladies auto-immunes.



## List of abbreviations

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AICD	Activation Induced Cell Death
AID	activation induced cytidine deaminase
AIRE	AutoImmune Regulator
ANA	antinuclear antibodies
APC	Antigen Presenting Cells
APECED	Autoimmune PolyEndocrinopathy Candidiasis Ectodermal Dystrophy
BALT	Bronchus associated lymphoid tissue
Bcl-2	B cell lymphoma 2
BCR	B cell receptor
C	Constant
CAPS	Cryopyrin-Associated Periodic Syndromes
CCP	Cyclic-citrullinated peptide
CD	Cluster of Differentiation
CDR3	Complementary-determining region 3
COX-2	Cyclooxygenase 2
CRP	C-reactive Protein
CSF	Cerebrospinal fluid
CSR	Class Switch Recombination
cTFh	circulating memory Tfh
CTLA	Cytotoxic T-Lymphocyte Associated protein
CTLs	cytotoxic CD8+ T lymphocytes
D	diversity
DAMP	Damaged-associated Molecular patterns
DC	Dendritic Cell
DISC	Death Induced Signaling Complex
DZ	Dark zone
EAE	autoimmune encephalomyelitis
EBV	Epstein-Barr Virus
ELS	Ectopic lymphoid structures
Eomes	Eomesodermin

ER	Endoplasmic Reticulum
FDC	Follicular dendritic cells
Foxp3	Forkhead box P3
FRC	Follicular reticular cells
GALT	Gut associated Lymphoid Tissue
GC	Germinal Center
GPA	granulomatosis with polyangiitis
HEV	High endothelial Venules
HIV	Human immunodeficiency virus
HLA	Human Leucocyte Antigen
ICAM-1	Inter-cellular adhesion molecule 1
IFN	IntereFroN
IL	InterLeukin
IL-1R1/2	IL-1 Receptor 1/2
IL-1Ra	IL-1 Receptor antagonist
IL-1RAcP	IL-1 Receptor accessory protein
IL-1 $\beta$	Interleukin-1 $\beta$
iNOS	nitric oxide synthase
IPEX	Immunodysregulation Polyendocrinopathy Enteropathy X-linked
IRAKs	Interleukin-1 Receptor Associated Kinases
iTfr	induced Tfr
J	Joining
Lck	lymphocyte-specific protein tyrosine kinase
LZ	Light zone
MALT	Mucosa-associated Lymphoid Tissue
MAPK	Mitogen Activated Protein Kinases
MHC	Major Histocompatibility Complex
MR1	MHC-I related
MS	Multiple sclerosis
MyD88	Myeloid differentiation primary response 88
NF-kB	Nuclear Factor kappa B
NK	Natural killer
NLR	NOD-like Receptors
nTfr	natural Tfr

PAMP	Pathogen-associated molecular patterns
PCA	Principal component analysis
PD	Programmed cell Death
PD-L	PD-Ligand
pMHC	Peptide MHC complex
PRR	Pattern Recognition Receptors
RA	rheumatoid arthritis
SAA	serum amyloid a
SAP	SLAM associated protein
SE	Staphylococcal enterotoxin
SHM	Somatic HyperMutation
sIL-1R1/2	soluble IL-1R1/2
SjS	Sjögren's syndrome
SLE	Systemic Lupus Erythematosus
SLO	Secondary Lymphoid Organs
TAK1	Transforming growth factor-beta-activated kinase 1
T1D	Type 1 diabetes
T2D	Type 2 Diabetes
TCR	T cell receptor
TECs	Thymic Epithelial Cells
Tfh	T follicular helper
Tfr	T follicular regulatory
TGF	Transforming Growth Factor
Th	helper T cells
TLR	Toll-Like Receptor
Treg	regulatory T cell
V	Variable
VCAM-1	Vascular cell adhesion molecule 1
VLP	Virus-like particles
SARS-CoV2	Severe acute respiratory syndrome coronavirus 2
CMV	cytomegalovirus
CCL	chemokine ligand
CCR	chemokine receptor
CXCL	C-X-C chemokine ligand





# Introduction

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## PART I. INTRODUCTION TO NON-SELF-RECOGNITION, IMMUNE TOLERANCE AND PATHOLOGICAL BREAKDOWN

### A. Self and non-self recognition in the immune system

#### 1. Importance of distinguishing self from non-self

Immunity relies on the immune system's ability to distinguish between what is "self" and what is "non-self", meaning the distinction between patterns found in the organism and those of other origin. This fundamental distinction is critical for the immune system to mount appropriate responses and protect the body from harm.

The immune system — consisting of all the organs, tissues, cells and molecules interacting together to ensure the body's integrity — plays a major role in recognizing non-self-antigens that may lead to infections caused by viruses, fungi, bacteria and parasites. This process is the basis of protective immunity against pathogens.

However, non-self-antigens can also come from other organisms, both within the same species (intraspecies) or different species (interspecies). These non-self-antigens, although not derived from microbes, can potentially threaten and trigger immune responses when introduced into the body. This process is the foundation of allogenic rejection, which occurs when transplanted tissues or organs from another individual are recognized as non-self and targeted by the immune system.

Similarly, proteins that undergo somatic mutations can be regarded as either "modified-self" or non-self as they present novel antigenic patterns that have not been previously encountered by the immune system, potentially leading to an immune response. This phenomenon is commonly observed in the field of cancer immunology.

Moreover, the concept of non-self extends beyond infectious agents and allogenic rejection. It encompasses non-self-antigens that are not inherently harmful, such as environmental or dietary antigens. While these antigens are derived from external sources, they are not pathogenic and should be tolerated by the immune system. This

concept of immunological tolerance was first theorized by 1960 Nobel Prize winners Frank Macfarlane Burnet and Peter Brian Medawar (Billingham et al., 1953).

Therefore, immunity against non-self requires a dual process of recognition and tolerance to both self-antigens and harmless non-self-antigens. Immune tolerance is crucial to avoid unnecessary immune reactions against self and instead focus responses towards threatening components. Failure in non-self recognition may result in defective protection against pathogens, whereas aberrant immune tolerance can lead to autoimmunity or allergy.

## 2. Major Histocompatibility Complex (MHC) and antigen presentation

Self and non-self recognition require the presentation of antigens on the surface of cells expressing fundamental molecules named *Major Histocompatibility Complex* (MHC). MHC is a group of genes that encode proteins involved in the recognition of antigens by the immune system and subsequent activation of immune responses. MHC molecules are cell surface proteins that can be present on most nucleated cells in vertebrates, playing an essential role in antigen presentation to T cells (Flajnik and Kasahara, 2010).

There are two main classes of MHC molecules whose genes are found in the *Human Leucocyte Antigen* (HLA) locus. These molecules are MHC class I and MHC class II, and enable the specific antigen presentation to *Cluster of Differentiation* (CD) 4- and CD8-expressing T cells (**Fig.1**) :

- MHC I molecules present antigens to CD8<sup>+</sup> T cells. MHC I heterodimers consist of a heavy chain encoded by 3 highly polymorphic genes — HLA-A, HLA-B and HLA-C in humans — as well as a small invariant  $\beta$ 2-microglobulin protein. Heavy chains are composed of 3  $\alpha$  domains involved either in peptide binding ( $\alpha_1$ ,  $\alpha_2$ ) or in interaction with CD8 T cells ( $\alpha_3$ ).

Presented peptides primarily derive from intracellular pathogens or the endogenous proteome of the cell, thus representing a safeguard to prevent abnormal or infected cells to thrive. Those peptides are generally 8 to 10 amino

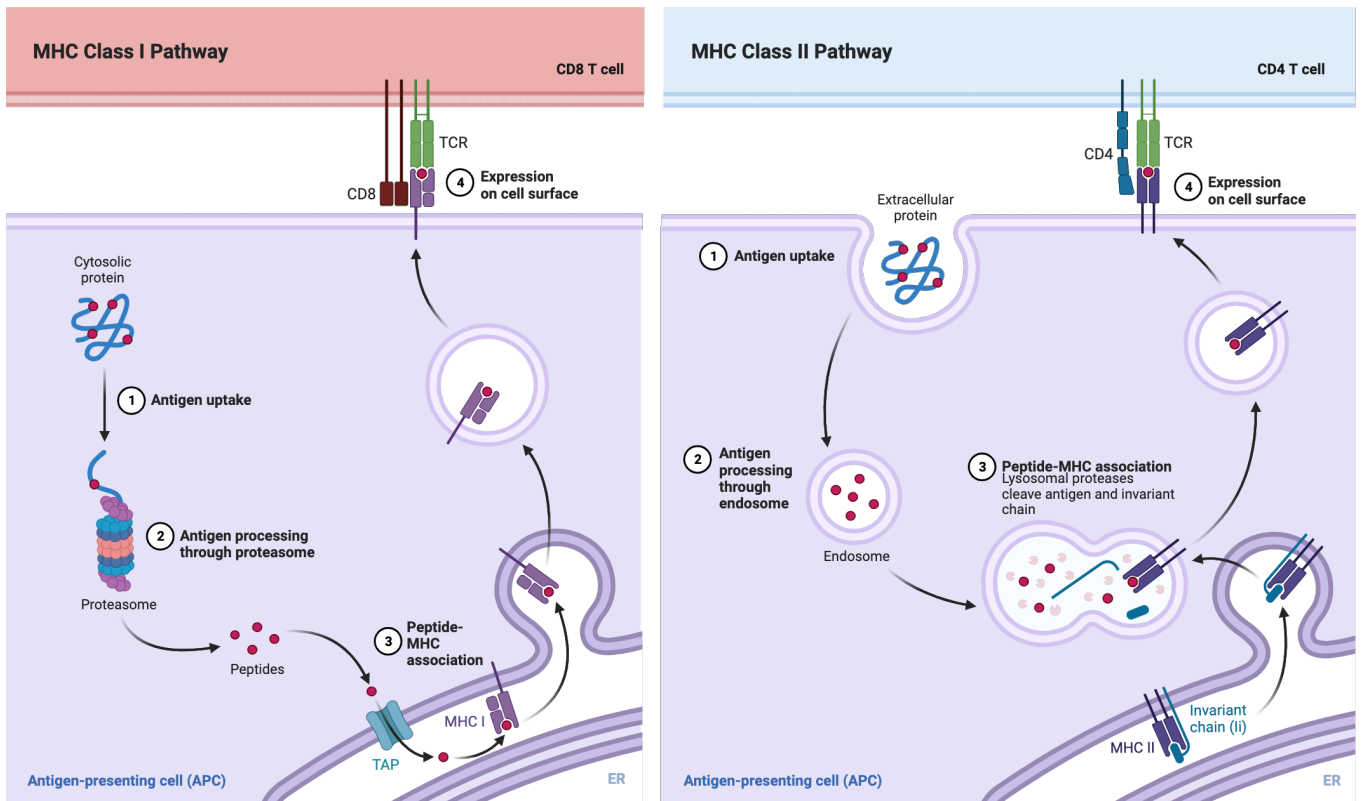
acids in length, loaded onto a closed groove via anchor residues allowing each MHC I molecule to bind millions of different peptides ((Falk et al., 1991);(Jardetzky et al., 1991);(Brusic et al., 2004)).

Mechanistically, proteins inside cells are degraded in peptides by the proteasome, then transported to the endoplasmic reticulum (ER) to be load onto the MHC I molecule, which reaches the cell membrane to present the peptide to CD8<sup>+</sup> T cells. A high turnover of MHC I molecules is observed in each cell, enabling a constant presentation of thousands of peptides (Rock et al., 2016).

- MHC II molecules present antigens to CD4<sup>+</sup> T cells. MHC II are heterodimers as well, composed of two chains,  $\alpha$  and  $\beta$ , each divided in 2 domains —  $\alpha_1$  and  $\beta_1$  forming an open groove that can bind trillions of 13 to 25 amino acid-long peptides (R. M. Chicz et al., 1992). In humans, these chains are encoded by HLA-DP, HLA-DQ and HLA-DR polymorphic and co-dominant genes.

Peptides presented by MHC II molecules principally derive from extracellular proteins, including proteins from pathogens that have been internalized by *Antigen-Presenting Cells* (APCs). Antigens taken up by APCs are digested into peptides within specialized compartments called endosomes or lysosomes. MHC class II molecules are synthesized in the ER and transported to these compartments, where they associated with peptides. The MHC II-peptide complex is then expressed on the cell surface, where it can be recognized by CD4<sup>+</sup> T cells. Like MHC I, MHC II molecules face an important turnover to renew presented antigens (Rock et al., 2016).

An alternative mechanism called cross-presentation, by which APCs can present extracellular antigens on MHC I molecules, exists. This enables the activation of CD8<sup>+</sup> cytotoxic T cells, which are typically responsible for targeting intracellular pathogens or tumor cells. While cross-presentation can occur in various cell types, certain APCs are particularly efficient at this process like *Dendritic Cells* (DCs) (Bevan, 1976). Conversely, endogenous antigens can be presented on MHC II via the cytosolic pathway, notably in APCs, thus activating CD4<sup>+</sup> T cells to promote an extracellular response impeding pathogen spreading (Tewari et al., 2005).



**Figure 1. Antigen presentation pathways of MHC Class I and II molecules in APCs.**

On the left, the MHC Class I pathway is shown: (1) Cytosolic proteins are taken up as antigens. (2) These antigens undergo processing through proteasomes where they are broken down into peptides. (3) The peptides are then transported by the transporter associated with antigen processing (TAP) into the endoplasmic reticulum, where they associate with MHC I molecules. (4) The peptide-MHC I complexes are expressed on the cell surface, ready for recognition by CD8 T cells.

On the right, the MHC Class II pathway is detailed: (1) Extracellular proteins are internalized into the APC. (2) Antigens are processed in endosomes. (3) Lysosomal proteases cleave the antigen, and the resulting peptides associate with MHC II molecules, having invariant chains. (4) Peptide-MHC II complexes are then presented on the cell surface for recognition by CD4 T cells. This figure illustrates the sequential steps and cellular components involved in antigen processing and presentation by MHC Class I and II molecules, crucial for adaptive immune responses.

In addition to classical MHC I and II molecules, there are also non-classical MHC molecules. These non-classical MHC molecules have distinct features and functions compared to the classical ones, since they are encoded by genes that are structurally different. They are less polymorphic and expressed in limited cell types. As an example, CD1 molecules present lipid antigens on the surface of APCs (Blumberg et al., 1995), and MHC-I-related (MR1) presents vitamin and microbial antigens to mucosal-associated T cells (Franciszkiewicz et al., 2016).

### 3. T Cell Receptor (TCR) recognition of antigen-MHC complexes

#### a. *TCR structure and diversity*

We have seen that the process of antigen presentation to T cells relies on the recognition of the antigen-MHC complex displayed on the surface of APCs. For this purpose, T cells express a specialized receptor known as the *T Cell Receptor* (TCR), which exhibits specificity towards a — theoretically — unique antigen epitope bound to a specific MHC molecule.

The TCR is a heterodimer composed of two chains, either  $\alpha$  and  $\beta$  chains in TCR $\alpha\beta$  T cells (found in majority) or  $\gamma$  and  $\delta$  chains in TCR $\gamma\delta$  T cells.

Since T cells can theoretically recognize millions of antigens, the primary theory claimed that millions of different genes each encode a TCR expressed on a T cell. However, in 1976 Susumu Tonegawa pioneered the principle of genetic rearrangement and diversity of antibodies within B cells, close to TCR rearrangement, and for which he was awarded the Nobel Prize in 1987 (Hozumi and Tonegawa, 1976).

During T cell development in the thymus, TCR genes undergo the aforementioned gene rearrangement process. This process is responsible for generating a diverse range of TCRs. TCR genes comprise various segments, including variable (V), diversity (D) — for the  $\beta$  chain only —, joining (J), and constant (C) segments (Kraegel, 2009) (**Fig. 2**). Through the rearrangement process, these gene segments are selected and combined to form a distinctive TCR sequence in each T cell. This mechanism allows for the creation of a vast repertoire of TCRs, enabling T cells to recognize a wide array of antigens including peptides derived from pathogens, cancer cells, and self-antigens.

In detail, for the establishment of T cell repertoire, the TCR undergoes the following process :

- V(D)J recombination: in the early stages of T cell development, the DNA locus encoding the V, D, and J gene segments rearrange to form a functional TCR gene. During V(D)J recombination, one V segment is joined with one D

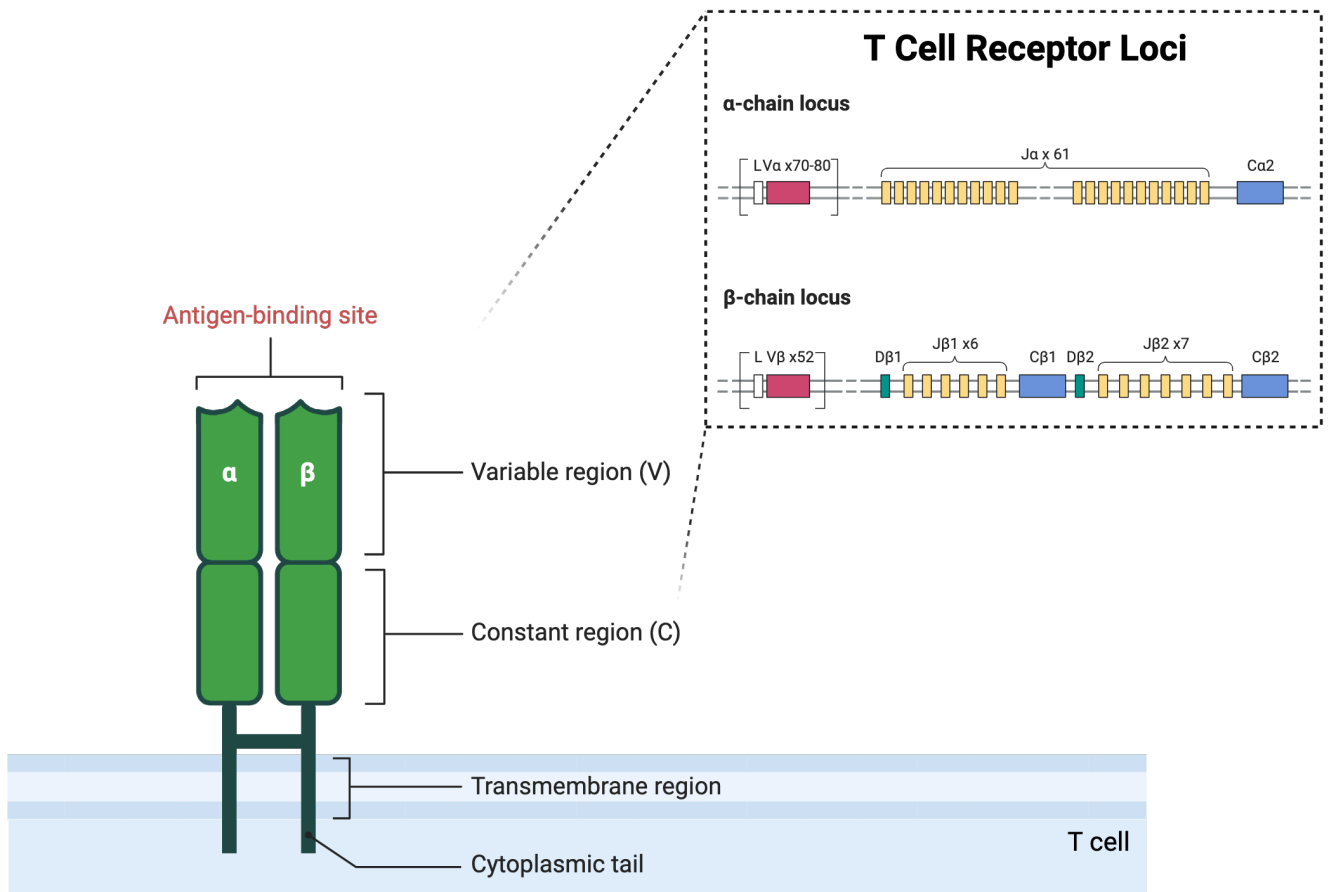


segment (if present) and one J segment, creating a variable region for the TCR chain (Bassing et al., 2002).

- junctional diversity: the rearrangement process involves the addition or deletion of nucleotides at the junctions where the gene segments are joined together. This process, known as junctional diversity, introduces additional variability to the TCR sequence (Hata et al., 1988).

- combinatorial diversity: the multiple V, D, and J gene segments provide a wide range of options for recombination. Different combinations of V, D, and J gene segments can be selected during the rearrangement process, resulting in a large number of potential TCR sequences (Qi et al., 2014).

- allelic exclusion: each T cell expresses only one TCR $\alpha$  chain and one TCR beta chain (Brady et al., 2010). This is achieved through a process called allelic exclusion, which ensures that a T cell has only one functional TCR. Once a productive rearrangement occurs on one allele, the rearrangement process is halted on the other allele, preventing the expression of two different TCRs on the same T cell — although this step is not infallible and can lead to multiple TCRs expressed by a cell.



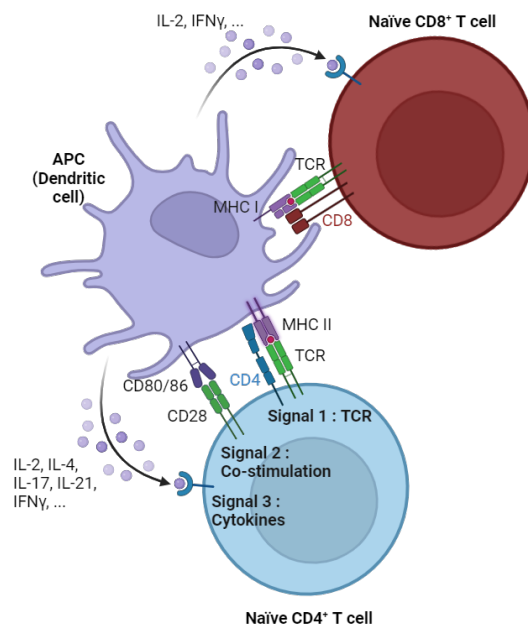
**Figure 2. The structure of the TCR and its gene arrangement.**

On the left, the TCR is depicted as a heterodimer consisting of an alpha (α) and beta (β) chain, each with variable (V) and constant (C) regions. The antigen-binding site is formed at the V region intersection. The TCR traverses the T cell membrane, displaying a cytoplasmic tail and a transmembrane region. On the right, the genetic loci of the TCR alpha and beta chains are shown, detailing the segments involved in V(D)J recombination, which contributes to the diversity of the TCR.

### *b. MHC restriction and TCR interactions*

TCR recognition of antigens is dependent on their presentation by specific MHC molecules; this phenomenon is termed MHC restriction. Indeed, the TCR is designed to interact with both the peptide antigen presented by the MHC molecule and the MHC molecule itself (Shah et al., 2021). As previously mentioned, the TCR from CD4 T cells can only recognize peptide antigens presented by MHC II molecules, while the one from CD8 T cells is designed to recognize MHC I-loaded peptide antigens (**Fig. 3**).

This high specificity binding of the TCR is enabled and stabilized by various molecular contacts. In particular, TCR binding to the antigen relies on the interaction between the exposed aminoacid sequence of the antigen and the complementarity-determining region 3 (CDR3) of TCR  $\alpha\beta$  chains (Springer et al., 2021). CDR3 is a highly polymorphic region of the TCR formed by the variable segments of each TCR chain, allowing antigen binding via hydrogen bonds, Van der Waals forces, electrostatic and hydrophobic interactions. TCR recognition also requires co-receptor binding to ensure MHC restriction : CD4 molecules bind to the invariant  $\beta_2$  region of MHC II whereas CD8 molecules bind to  $\alpha_3$  domain of MHC I, providing additional stability to the TCR-*peptide-MHC complex* (pMHC).



**Figure 3. Antigen presentation : 3 fundamental signals involved in T cell activation.**

An APC interacts with a T cell, providing signals necessary for its activation. Signal 1 involves the TCR recognizing and binding to a specific antigen presented by the MHC on the APC. This interaction requires MHC I when the TCR is from a CD8<sup>+</sup> T cell while MHC II is required when the TCR is from a CD4<sup>+</sup> T cell. Signal 2 is co-stimulation, where additional receptors enhance the activation signal, and Signal 3 involves cytokines, which further modulate the T cell's response. These collective signals ensure the T cell is properly activated to respond to the encountered antigen.

All T cells are characterized by the expression of CD3, a protein complex made of 2 transmembrane heterodimeric subunits ( $\gamma\epsilon$  and  $\delta\epsilon$ ) which associate with the TCR to form the "TCR complex", together with homodimeric CD3 $\zeta\zeta$  (Wegener et al., 1992). When the TCR binds a pMHC, conformational changes bring CD3-associated

phosphorylation sites closer to lymphocyte-specific protein tyrosine kinase (Lck), thus enabling the recruitment of other enzymes of the downstream signaling pathways (Mariuzza et al., 2020). In the end, those pathways trigger the transcription of genes involved in survival, T cell differentiation and proliferation.

## B. Maintenance of immune tolerance to self-antigens

### 1. Central tolerance: negative selection in the thymus

The immense diversity and random generation of TCR repertoire make it possible to recognize any peptide motif and thus mount an immune response against a wide range of threats. However, the way TCR repertoire is generated also enables the production of TCRs that can recognize self-antigens, leading to an autoimmune response.

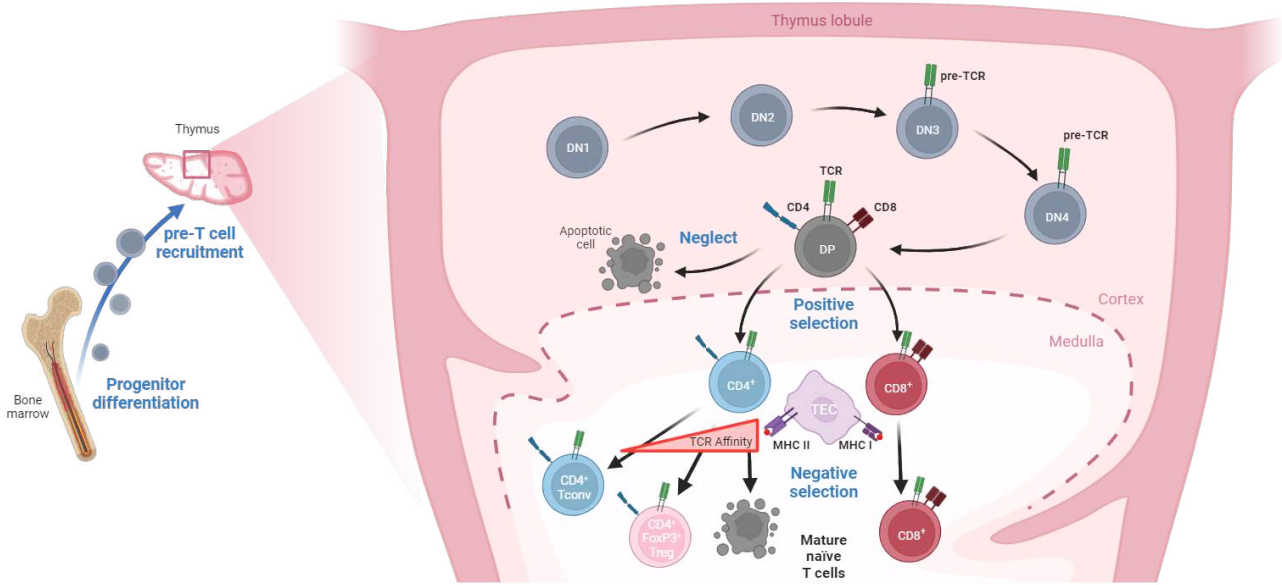
In order to prevent self-reactive T cell clones from being released in the organism, F.M. Burnet and P. Medawar proposed and demonstrated that the immune system has to be educated during its development. This process takes place within primary lymphoid organs (bone marrow and thymus) : following their development in the bone marrow, progenitor T cells enter the thymus — they are called thymocytes — and rearrange their TCR  $\beta$  and  $\alpha$  chains successively. Then, they undergo a dual-step process of positive and negative selection based on TCR affinity to complete their maturation, only completed by up to 5% of all thymocytes (Merkenschlager et al., 1997; Sitnicka, 2009) (**Fig. 4**). Indeed, cortical thymocytes, initially double negative, start acquiring both CD4 and CD8 expression to become double positive, and interacting with *Thymic Epithelial Cells* (TECs). TECs are specialized cells that express MHC class I or II molecules on which a multitude of private self-antigens are presented to thymocytes. If a thymocyte does not engage in any TCR-self-pMHC interaction it will die by neglect, that is by the absence of survival signals, and will not produce positive selection (Klein et al., 2014).

Positive selection occurs when the TCR-pMHC interaction is weak, which ensures limited self-affinity. If the interaction involves MHC class I, then the thymocyte will

become single positive for CD8 by discarding CD4 expression, whereas it will become single positive for CD4 if the TCR interacts with MHC class II molecules.

Single positive thymocytes pursue their maturation in the medullary region of the thymus, where they encounter TECs specialized in the ectopic presentation of tissue-restricted antigens. This self-peptide presentation — elicited by the expression of the transcription factor *AutoImmune REgulator* (AIRE) — leads to the clonal deletion of thymocytes engaging strong TCR-pMHC interactions. Thus, by inducing apoptosis of highly self-reactive T cell clones, TECs guarantee the establishment of central tolerance. This mechanism is exemplified by the onset of *Autoimmune PolyEndocrinopathy Candidiasis Ectodermal Dystrophy* (APECED) syndrome as a result of AIRE mutations, causing lack of tolerance and multiple self-reactive disorders (Ahonen et al., 1990). Of note, in the medulla, TECs can also indirectly mediate negative selection through the help of APCs (mostly DCs) that can carry self-antigens produced by TECs.

Lastly, another mechanism named clonal deviation importantly contributes to the establishment of central tolerance. It consists in the partial maintenance of self-reactive CD4<sup>+</sup> T cells to provide them with a regulatory and suppressive phenotype (Hsieh et al., 2012). Indeed, intermediate TCR-pMHC interactions can lead to the skewing of T cell ontogeny towards a distinct differentiation program characterized by the expression of transcription factor *Forkhead box P3* (Foxp3) and CD25 (Sakaguchi et al., 1995). These thymic CD4<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> T cells, first described in 1995, were referred to as "natural" regulatory T cells (Treg) in contrast with "induced" Tregs which will be discussed later. Natural Treg deficiency, caused by a mutation in FOXP3 gene, leads to a multiorgan autoimmune syndrome termed *Immunodysregulation, Polyendocrinopathy, Enteropathy, X-linked* (IPEX) (Wildin et al., 2001); (M Asano et al., 1996).



**Figure 4. Central tolerance of T cells is established in the thymus.**

Progenitor cells, originating from the bone marrow, enter the thymus where they proceed through various differentiation stages, marked as DN1 to DN4. The thymocytes undergo a series of tests for their TCRs, starting with the formation of the pre-TCR, ensuring functionality and self-tolerance. A pivotal part of this maturation process is the positive and negative selection that occurs primarily in the cortex and medulla, respectively. Positive selection ensures a correct pMHC binding and orientates DP precursors to CD4 or CD8 lineage. Then, thymocytes with TCRs that have a low-moderate affinity for self-antigens presented by MHC molecules are positively selected, while those with TCRs showing high affinity for self-antigens undergo apoptosis, termed negative selection, to prevent autoimmunity. Some of these cells differentiate into regulatory T cells (Tregs) which play a role in maintaining immune tolerance. The mature naïve T cells, now equipped with unique TCRs, exit the thymus to circulate and participate in immune responses.

In sum, T cell ontogeny through positive and negative selection in the thymus allows the formation of a tolerant repertoire of conventional T cells, as well as a self-reactive Treg repertoire via clonal deviation. Mature conventional and regulatory T cells that leave the thymus can then reach peripheral effector sites to ensure the integrity of the organism and promote tolerance.

## 2. Peripheral tolerance mechanisms

The process of central tolerance prevents the organism from mounting a T cell response against self. However, the TCR repertoire could theoretically exceed  $10^{20}$  clones (Zarnitsyna et al., 2013) of which only  $10^6$  to  $10^8$  are actually generated per individual (Arstila et al., 1999);(Qi et al., 2014). Thus, the likelihood of a non-self-peptide-MHC ligand perfectly matching its corresponding TCR is low. This is why each

TCR requires cross-reactivity to many pMHC complexes to cover the estimated  $20^9$  different antigens that may be encountered (Mason, 1998). In addition, there is low probability of presenting all self-antigens for negative selection, and the presentation of food and aero-antigens is impossible in the thymus. All these features highlight that tolerance establishment in the thymus is not sufficient to completely avoid reactivity to self and unharmed non-self. As a consequence, multiple tolerance mechanisms have emerged in the periphery (**Fig. 5**).

a. *Tregs and immune suppression*

The main mechanism ensuring tolerance in the periphery relies on the suppressive function of Tregs. As mentioned earlier, Tregs are a specialized subset of  $CD4^+$  T cells characterized by the expression of Foxp3. Natural Tregs developed in the thymus can be found in the blood as well as in their homing sites which they reach via the expression of different integrins and chemokine receptors. These sites include secondary lymphoid organs, where Tregs remain close to naive T cells to prevent the initiation of self-reactive responses, along with mucosal tissues where Tregs promote tolerance of aero-digestive antigens and commensal microbiota-derived antigens (Sakaguchi et al., 2020).

In situ, Tregs suppress autoreactive T cells by recognizing a common self-antigen presented by APCs and thus inhibiting their activation (**Fig. 5**). Also, Tregs contribute to immune tolerance through bystander suppression, which dampens responses against neighboring antigens unrelated to their cognate antigen. This mechanism prevents unnecessary activation by ensuring that immune responses are focused on relevant targets, while minimizing collateral damage to healthy neighboring tissues.

Importantly, peripheral tolerance is maintained by the trans-differentiation of conventional  $CD4^+$  T cells into Tregs, called "induced" or "peripheral" Tregs (**Fig. 5**). Under certain microenvironment conditions, epigenetic reprogramming allows the expression of Foxp3 and the acquisition of regulatory functions. However these induced Tregs have a less stable phenotype than natural Tregs (Kanamori et al., 2016). It has been shown that exposure to *InterLeukin* (IL)-2 and *Transforming Growth Factor* (TGF)- $\beta$  induce Tregs both in vivo and in vitro (Chen et al., 2003), although

other factors can be involved such as commensal bacteria-derived metabolites (Arpaia et al., 2013).

Treg effector mechanisms strongly rely on the expression of CD25, the high-affinity chain of IL-2 receptor. By constitutively displaying a high expression of CD25 in contrast with conventional T cells, Tregs compete for and capture at first-hand IL-2, thus depriving T cells of this cytokine essential for their activation (Chinen et al., 2016). As a consequence, high doses of IL-2 are required to reach the activation threshold of T cells and counterbalance the IL-2-induced suppressiveness of Tregs.

Tregs employ various molecular and cellular mechanisms to exert their suppressive activity (Xing and Hogquist, 2012). Among those, Tregs can directly inhibit the activation and proliferation of effector T cells through the expression of immune checkpoint molecules like *Cytotoxic T-Lymphocyte-Associated protein (CTLA)-4* and *Programmed cell Death (PD)-1*. These juxtacrine interactions both prevent T cells from engaging TCR-pMHC interactions and provide inhibitory signals. Also, Tregs can secrete immunosuppressive cytokines such as IL-10 and TGF- $\beta$ , which act in an autocrine and paracrine manner (Schmidt et al., 2012). Otherwise, Tregs can modulate extracellular ATP derivatives via CD39 and CD73 ectoenzymes, thus blocking inflammatory signals and leading to the production of immunosuppressive adenosine (Deaglio et al., 2007).

*b. Anergy and functional inactivation of self-reactive T cells*

Anergy corresponds to the functional inactivation of T cells following antigen encounter without proper co-stimulation. In normal immune responses, T cells require 3 signals for full activation : 1) antigen recognition via the TCR-pMHC complex, 2) co-stimulation through interactions with APCs expressing co-stimulatory molecules like CD28, and 3) cytokine signaling like IL-2. When co-stimulation is absent, T cells can become anergic, rendering them unable to carry out their effector functions. This prevents the activation of self-reactive T cells that recognize self-antigens without inflammation, treating the encounter with antigen as a signal of tolerance rather than activation (Xing and Hogquist, 2012) (**Fig. 5**).



Anergy induction involves signaling pathways that reduce TCR signaling such as the *Mitogen-Activated Protein Kinases* (MAPK) pathway downstream of CD28, in which lack of phosphorylation impairs IL-2 production (Fathman and Lineberry, 2007). Accordingly, anergic T cells show reduced expression of activation markers and impaired proliferation.

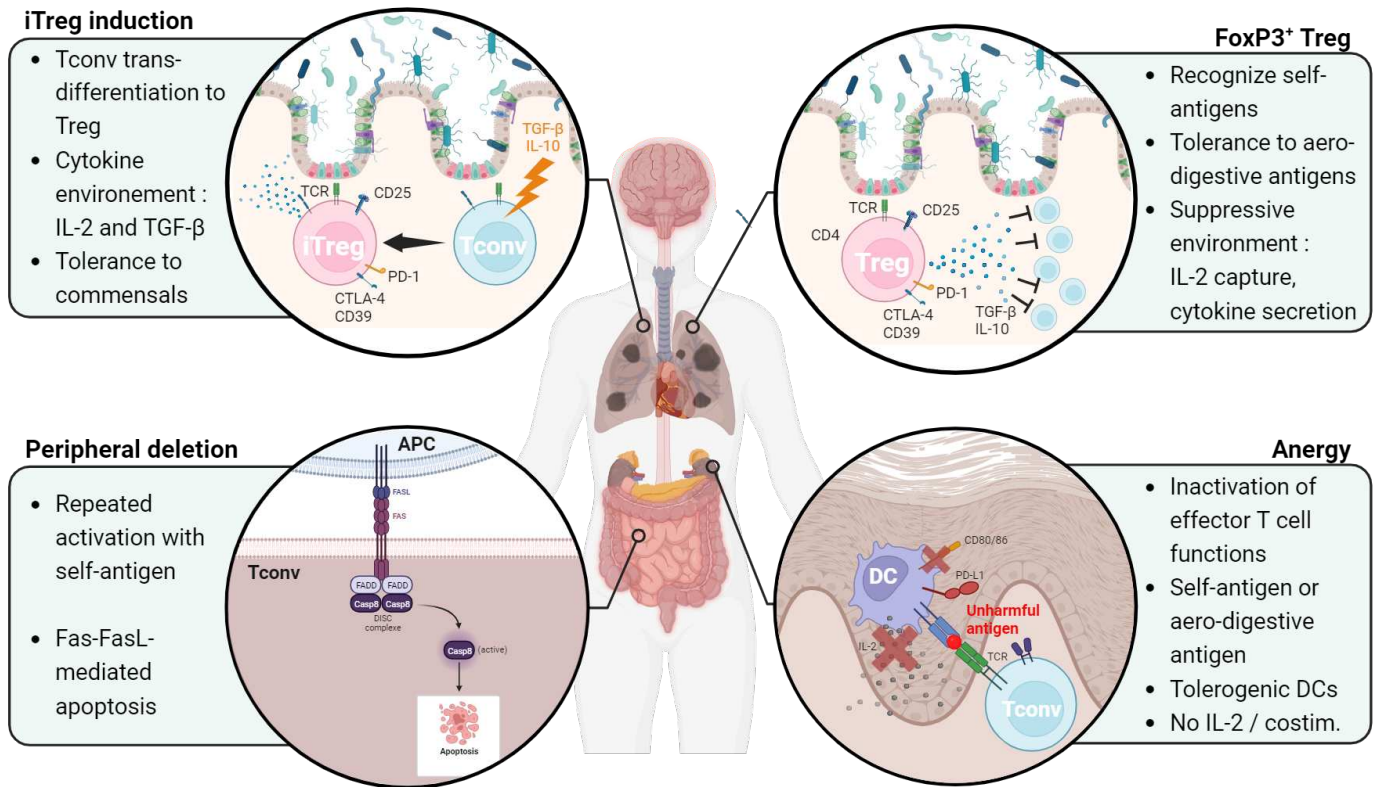
As an important promoter of anergy and tolerance, so-called "tolerogenic" DCs play a crucial role by presenting antigens to T cells without providing sufficient co-stimulatory signals for their full activation (Xing and Hogquist, 2012). These DCs fail to trigger T-cell proliferation and instead deliver coinhibitory signals like *PD-Ligand* (PD-L) 1 and PD-L2 that modulate the orientation towards T-cell activation or tolerance. Tolerogenic DCs arise from unfinished maturation, which can be influenced by factors such as apoptotic cell detection without *Toll-Like Receptor* (TLR) agonists, exposure to anti-inflammatory molecules like IL-10, TGF- $\beta$  and corticosteroids (Morelli and Thomson, 2007).

### c. *Peripheral deletion of autoreactive T cells*

Apoptotic cell death plays a crucial role in eliminating self-reactive lymphocytes and maintaining peripheral tolerance. Among the various mechanisms involved, the Fas-mediated apoptosis pathway stands out as a key player in deleting self-reactive lymphocytes outside of the thymus (**Fig. 5**). Fas, also known as CD95, is a receptor containing a death domain that can be activated by its ligand, FasL (Arakaki et al., 2014).

In the periphery, T cells express Fas on their surface, while the expression of FasL is induced upon T cell activation by antigens and interleukin-2 (IL-2) (Refaeli et al., 1998). When self-reactive T cells are repeatedly stimulated by their cognate or foreign antigen, Fas becomes crucial for their deletion. Upon Fas activation, an intracellular signaling complex called the "death-inducing signaling complex" (DISC) is formed. This process involves the recruitment and activation of Caspase-8, which subsequently activates effector caspases. The activation of these caspases ultimately leads to the induction of apoptosis, a programmed cell death process.

This specific pathway of apoptosis triggered by antigen activation is known as activation-induced cell death (AICD). AICD via the Fas-mediated apoptosis pathway plays a significant role in eliminating self-reactive lymphocytes and preventing the development of autoimmunity or excessive immune responses.



**Figure 5. Mechanisms involved in peripheral tolerance.**

There are four main mechanisms established in the periphery to ensure the adaptive system is tolerant. First, in an ideal cytokine environment rich in IL-2 and TGF- $\beta$ , conventional T cells (Tconv) undergo trans-differentiation into Tregs, which fosters tolerance to commensal microorganisms for instance. Then, Tregs play a pivotal role in recognizing self-antigens, thereby orchestrating a suppressive environment. This milieu is characterized by IL-2 capture and the secretion of various cytokines, reinforcing a state of immune tolerance. Additionally, the repeated activation of conventional T cells with self-antigens leads to peripheral deletion. Presented by APCs, these self-antigens activate mechanisms such as Fas-FasL mediated apoptosis in T cells, promoting a state of tolerance. Lastly, DCs, through presenting unharmed (like allergens) or self-antigens to T cells, contribute to peripheral tolerance by inactivating effector T cell functions in the absence of co-stimulation and IL-2 : this its called anergy.

### C. Breakdown of Immune Tolerance and Development of Autoimmune Pathologies

We previously saw that the immune system is meticulously formed and structured to distinguish self and non-self, ensuring a specific targeting of foreign intruders and the termination of cells that could threaten the body's integrity. This delicate balance is

maintained through immune tolerance to prevent autoimmunity. Yet this mechanism can be compromised and yield to uncontrolled immune responses that can give rise to a variety of clinical manifestations called autoimmune pathologies.

1. Loss of tolerance: mechanisms and triggers

- a. *Genetic predisposition and HLA associations*

Gene variations and HLA combination play significant roles in the loss of immune tolerance and the development of autoimmune diseases. Certain genetic variants can favor loss of tolerance by altering the repertoire of presented self-antigens or the regulatory mechanisms, leading to the activation of autoreactive T cells and autoimmunity.

One key factor is the involvement of HLA genes. Genetic variations in HLA genes can influence the ability to present self-antigens effectively, leading to potential breakdowns in immune tolerance. Specific HLA alleles have been associated with an increased risk of developing autoimmune diseases. For example, certain HLA-DRB1 alleles are strongly associated with the development of multiple sclerosis (MS) (Cook, 1997), as well as HLA-DR3 and HLA-DR4 which are associated to an increased risk of systemic lupus erythematosus (SLE).

In addition, several non-HLA genes play a significant role in genetic predisposition to autoimmune diseases. These genes are involved in crucial immune regulatory mechanisms that help maintain immune tolerance. For example, *CTLA4* gene mutation has been associated with autoimmune disorders such as Graves' disease and type 1 diabetes. Another example is the *PTPN22* gene, which regulates immune cell signaling and has been linked to rheumatoid arthritis and systemic lupus erythematosus. Also, as mentioned above, mutations of *AIRE* and *FOXP3* genes lead to APECED and IPEX syndromes, respectively (Ahonen et al., 1990); (Wildin et al., 2001); (M. Asano et al., 1996). In sum, variations in these non-HLA genes can disrupt immune cell function, cytokine production, and other essential immune processes, increasing the susceptibility to autoimmune disorders.

- b. *Environmental and non-genetic factors*

Environmental factors play a significant role in triggering the loss of immune tolerance and the development of autoimmune diseases. Infections caused by certain pathogens, such as viruses and bacteria, can activate immune cells that mistakenly target self-antigens. Mechanisms can involve molecular mimicry (described later) and bystander activation, which refers to a robust immune cell activation not specific of a given pathogen (Pacheco et al., 2019). Indeed, some microbe-derived antigens, called superantigens, are able to bind both MHC II and TCR, thus stimulating polyreactive CD4<sup>+</sup> T cells and antibody responses. Among those can be cited toxic shock proteins, staphylococcal enterotoxins (SE) like SEB, streptococcal pyrogenic exotoxins, as well as proteins produced by mouse mammary tumor virus (Acha-Orbea, 1993).

Hormonal changes have also been implicated in the disruption of immune tolerance. Fluctuations in hormone levels, such as those that occur during puberty (Ucciferri and Dunn, 2022), pregnancy, or menopause (Desai and Brinton, 2019), can influence the immune response and increase the risk of autoimmunity onset. The influence of hormones is exemplified by the sex-bias of numerous autoimmune diseases. For instance, the hormone estrogen has been linked to increased autoimmunity in diseases like SLE (Billi et al., 2019). Indeed, Treg suppressiveness and profile were modified in transgender individuals receiving gender-affirming hormone therapy, and resembled more to the ones of SLE patients when undergoing estrogen therapy (G. A. Robinson et al., 2022).

Exposure to certain environmental factors, such as chemicals (silica, iodine), pollutants (dioxin), drugs (procainamide) and metals (mercury) can also contribute to immune dysregulation (Pollard et al., 2010). These factors can trigger inflammation, oxidative stress, and alter the balance of immune cells and cytokines, leading to the breakdown of immune tolerance. Additionally, lifestyle factors like diet (gluten consumption, (Briani et al., 2008)) and smoking (Chang et al., 2014) can further exacerbate immune dysregulation and contribute to the loss of immune tolerance.

### *c. Cross-Reactive immunity and Molecular Mimicry*

Cross-reactive immunity takes place when the immune system, triggered by a specific antigen, also recognizes and attacks structurally similar antigens (Petrova et al., 2012). This phenomenon carries a physiological function, since it enables T cells to

recognize a number of antigens theoretically superior to the TCR repertoire. However, as mentioned above, infection can elicit molecular mimicry when a foreign antigen shares structural similarities with self-antigens present in the body. When the immune system encounters the foreign antigen, it may generate an immune response that not only targets the invader but also mistakenly attacks self-tissues expressing similar antigens. As a result, the immune response initially directed against the pathogen can extend to self-tissues, leading to autoimmune reactions.

The consequences of cross-reactive immunity and molecular mimicry can be significant. They can initiate or exacerbate autoimmune diseases by promoting chronic inflammation and tissue damage. For example, in rheumatic heart disease, certain strains of *Streptococcus* bacteria contain antigens that resemble cardiac tissue components (Marijon et al., 2012). Antibodies generated against the bacterial antigens cross-react with cardiac antigens, leading to inflammation and damage to the heart valves. As well, Epstein-Barr virus (EBV) chronic infection can lead to T cell targeting of myelin proteins of the central nervous system in MS due to mimicry with viral protein EBNA1 (Marijon et al., 2012; Bjornevik et al., 2022).

## 2. Pathogenesis of Autoimmune Diseases

As mentioned above, loss of tolerance can emerge from both internal or external factors, all of which yielding to the activation of self-reactive cells (**Fig. 6**). But multiple mechanisms exist and lead to the targeting of the body's tissues, sustained by inflammation and giving rise to distinct pathological conditions.

### *a. Different effector mechanisms lead to autoimmunity*

Several primary effector mechanisms are pivotal in the erroneous attack of self, and they can be separated in 2 closely intertwined categories: autoreactive T cell activation and autoantibody production.

Autoreactive T cells are activated when APCs present self-antigens — or self-antigen alike antigens — in the context of MHC molecules. This interaction, typically occurring in lymphoid organs, is facilitated and strengthened by co-stimulatory signals. In an autoimmune setting, these self-antigens are often post-translationally modified or aberrantly expressed, leading to the activation of T cells that have escaped thymic

deletion (Raposo et al., 2018). Upon activation, naïve autoreactive T cells undergo clonal expansion and differentiation into various effector subsets, including cytotoxic CD8<sup>+</sup> T lymphocytes (CTLs) or helper T cells (Th) like Th1, Th2, and Th17 — in the case of CD4<sup>+</sup> T cells, each characterized by distinct cytokine profiles and mechanisms of action.

In target tissues, CTLs express perforin and granzymes, molecules that induce apoptosis in target cells. In type 1 diabetes, for example, CTLs are instrumental in the destruction of insulin-producing beta cells in the pancreas, leading to insulin deficiency and hyperglycemia (Liblau et al., 2002). Similarly, CTLs have also been associated with the destruction of myelin patches in MS.

In effector sites, Th1 cells produce interferon-gamma (IFN $\gamma$ ), a potent pro-inflammatory cytokine that activates CTLs, as well as macrophages and enhances their ability to phagocytose and destroy targeted cells. In autoimmune diseases like rheumatoid arthritis (RA), the activation of macrophages by Th1 cells amplifies the inflammatory response, leading to joint inflammation and erosion (Raphael et al., 2020). In addition, Th17 cells secrete IL-17, a cytokine that promotes the recruitment of neutrophils and macrophages to the site of inflammation. In psoriasis, Th17 cells contribute to skin inflammation and the hyperproliferation of keratinocytes, leading to the characteristic plaques observed in affected individuals.

On the other hand, the activation of autoreactive B cells can result from encounters with self-antigens, facilitated by a failure in tolerance or exposure to modified self-antigens too. Interactions with T cells, in particular Tfh, can further potentiate this activation, leading to B cell proliferation and differentiation into antibody-secreting plasma cells and memory B cells. This process is often exacerbated by molecular mimicry, where foreign antigens resembling self-antigens induce cross-reactive immune responses.

One of the most damaging consequences of autoreactive B cell activation is the production of autoantibodies (Giltiay et al., 2012). These antibodies target self-antigens, resulting in various forms of tissue damage, depending on the specificity of

the autoantibodies and their abundance. For example, in SLE, autoantibodies target nuclear antigens, forming immune complexes that precipitate in tissues, particularly the kidneys, causing inflammation and damage termed lupus nephritis. Other consequences also emerge from autoreactive B cell activation: B cells are APCs able to activate T cells and promote self-reactive cellular responses and tissue destruction (Shlomchik, 2008). The release of cytokines such as interferons and IL-12 can also be damaging by amplifying T cell activation.

However, categorizing autoimmune diseases in T cell-mediated versus antibody-mediated mechanisms is reductive, since a strong overlap exists. Indeed, the activation of some autoreactive CD4<sup>+</sup> T cells, notably Tfh, can lead to the stimulation of autoreactive B cells and thus the production of autoantibodies, as observed in celiac disease (Tye-Din et al., 2018), highlighting how interconnected these mechanisms are. Plus, there are diseases displaying both features at different stages, like type 1 diabetes (T1D) with autoantibody production at early stages while T cell-mediated destruction of pancreatic islets occurs years later.

#### *b. Autoimmunity and inflammation*

Primary effector mechanisms involved in autoreactive cell activation underline the importance of adaptive immunity in this context. However, the innate immune system also plays a significant role in the awakening and maintenance of autoimmunity. A strong dichotomy has long been established between strictly inflammatory diseases and autoimmune diseases. The former were primarily characterized by the activation of the innate immune system through the pathway of IL-1 $\beta$ , a pro-inflammatory cytokine with diverse and systemic effects (Dinarello, 2007). On the other side, autoimmunity was described according to the presence of self-reactive adaptive cells and antibodies. A different approach emerged and defined a continuum between those two categories, given the shared features between inflammation and autoimmunity (McGonagle and McDermott, 2006).

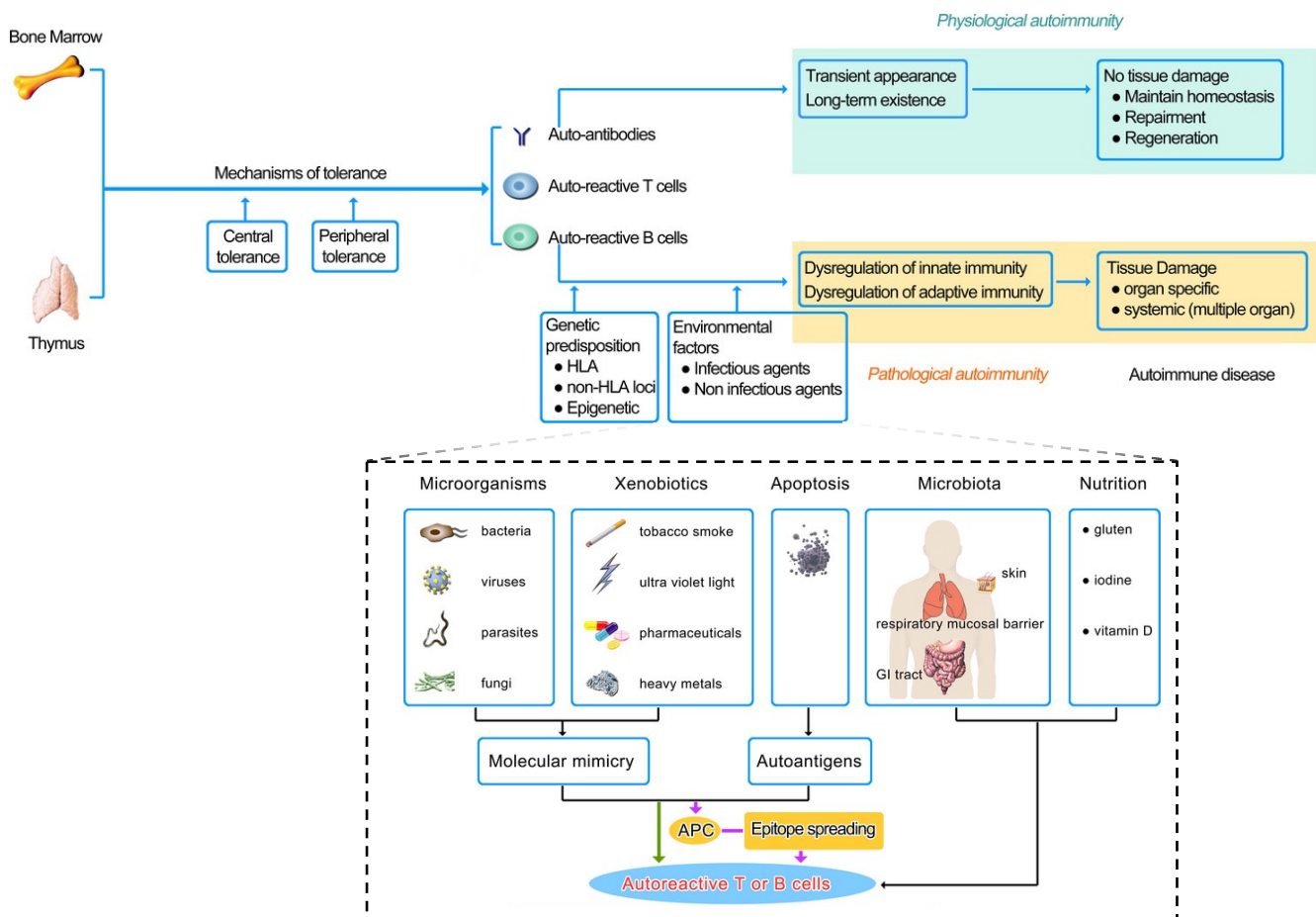
The innate immune system indeed plays a significant role in autoimmunity. Components like natural killer (NK) cells, macrophages, and the complement system, often act in concert with adaptive immune responses. NK cells are proven to destroy enterocytes in celiac disease following T cell production of IFN $\gamma$ , while macrophages

can erode the joints in RA (Saferding and Blüml, 2020). The complement system plays a key role in SLE by forming immune complexes with autoantibodies in the kidney, allowing the recruitment of inflammatory cells and tissue injury. Cytokines and chemokines, produced during both adaptive and innate immune responses, appear to exacerbate autoimmunity by promoting inflammation and recruiting additional immune cells to the targeted sites.

The recruitment of innate cells provokes tissue damage and so-called antigen spreading, which corresponds to the release of both the targeted epitope and other uncommon epitopes, thus exacerbating inflammation and activation of autoreactive cells via APCs (Wang et al., 2015) (**Fig. 6**).

Finally, the destruction of immune privilege sites as a result of inflammation enables the exposition of usually sequestered antigens to the immune system (**Fig. 6**). In MS, the breach of the blood-brain barrier allows autoreactive T cells to infiltrate the central nervous system, attacking myelin and leading to neurodegeneration (Goverman, 2009).





**Figure 6. From tolerance to autoimmunity : contributing factors and consequences.**

Initially, the mechanisms of tolerance, both central, occurring in the thymus, and peripheral, are illustrated, originating from progenitor cells in the bone marrow. The diagram then bifurcates to portray the differentiation between physiological autoimmunity, which entails transient auto-reactivity with no lasting tissue damage, and pathological autoimmunity.

In the pathway toward pathological autoimmunity, contributing factors such as genetic predispositions, including HLA and non-HLA loci, and environmental factors, like infectious and non-infectious agents, are illustrated. In the lower section, a spectrum of environmental triggers, including microorganisms, xenobiotics, apoptosis, microbiota, and nutrition, are meticulously mapped, each contributing to the molecular mimicry, epitope spreading, and ultimately, the activation of autoreactive T or B cells. These factors, combined with genetic predispositions and immunoregulatory disruptions, culminate in a multifactorial landscape that nurtures the development and perpetuation of autoimmune disorders. Overall, this leads to the dysregulation of innate and adaptive immunity, culminating in tissue damage, which could be organ-specific or systemic, thus leading to autoimmune disease. Adapted from Wang et al., 2019.

### c. Clinical manifestations of autoimmunity

Autoimmune diseases present a spectrum of clinical manifestations that are as diverse as the mechanisms involved in their pathogenesis. They can broadly and porously be

categorized into tissue-specific and systemic autoimmune diseases, each with distinct clinical presentations attributed to the respective targets of the immune response.

Tissue-specific autoimmune diseases are characterized by the immune system's selective attack on a particular tissue or organ. The clinical manifestations are often directly related to the impaired function of the targeted tissue. In type 1 diabetes, autoantibodies and autoreactive T cells target and destroy pancreatic  $\beta$ -cells, leading to a deficiency in insulin production which clinically translates into hyperglycemia. In Hashimoto's Thyroiditis, the thyroid gland is attacked, leading to hypothyroidism.

Interestingly, in many tissue-specific autoimmune diseases, the formation of ectopic lymphoid structures (ELS) is observed (Pitzalis et al., 2014). These structures resemble secondary lymphoid organs, with T cells, B cells and dendritic cells, and are implicated in perpetuating chronic inflammation and autoantibody production. For instance, in Sjögren's syndrome, their presence in the salivary and lacrimal glands correlates with severe glandular dysfunction and heightened autoantibody production.

Systemic autoimmune diseases are characterized by immune responses against multiple tissues and organs (Krainer et al., 2020). They often present with a broad array of symptoms, making diagnosis challenging. SLE is notorious for its varied clinical presentation, which can include malar rash, joint pain, renal dysfunction, and neurological abnormalities. The presence of autoantibodies, such as antinuclear antibodies (ANA), is a hallmark of the disease. RA is characterized by chronic inflammation of the joints, leading to pain, swelling, and eventual joint deformity. Systemic manifestations can also include lung, heart, and skin involvement. In RA and some other systemic autoimmune diseases, the formation of ELS within tissues like the synovium is associated with severe disease and joint damage. These structures support the local expansion and differentiation of autoreactive B and T cells, contributing to chronic inflammation and tissue destruction.

The diverse clinical manifestations of autoimmune diseases are defined by the specificity of the immune response and the affected tissues or organs. The presence of ELS can exacerbate disease severity, underscoring the importance of autoreactive T and B cells in association with inflammation for autoimmune pathophysiology. A

thorough understanding of these varied presentations is vital for accurate diagnosis and tailored treatment, aiming to address both the underlying autoimmune response and the resulting clinical manifestations.

One current challenge in the field of autoimmunity is the integration of complex and diversion features across diseases to provide a patient-specific care, in the era of precision medicine. In fact, the traditional classification of diseases in either inflammatory or autoimmune is now over, given the large spectrum of overlapping manifestations. Clinical symptoms and classic biological markers do not suffice to classify and find the ideal treatment for patients. New approaches are now emerging, by taking into account multiple parameters such as blood markers, cell phenotype, genetic susceptibility (Vaitinadin et al., 2023) and epigenetic abnormalities (Surace and Hedrich, 2019). An increasing number of studies even highlights the interplay between the immune system and the commensal flora : multiple autoimmune diseases have been associated to dysbiosis (De Luca and Shoenfeld, 2019).

All these approaches require new technologies that provide huge amounts of data: for instance genome-wide association studies for genetic susceptibility, 16s RNA sequencing for microbiome studies, multiparameter flow cytometry for cell phenotyping, Luminex for blood markers, single-cell bisulfite sequencing for epigenomics (Davis et al., 2017). However, new challenges arise when it comes to analyzing these data, both in terms of bioinformatics knowledge and in informatic infrastructure. The latter is a critical step, since storage of the data and their manipulation requires computers and servers with terabytes of capacities. The former is essential to visualize and interpret the data. Multiple software exist, such as R, Python, Matlab, and the basic Linux system, all contributing to data analysis. Integrating so many different and complex data is enabled by dimensionality reduction methods to simplify what the human brain cannot capture, like uniform manifold approximation and projection (UMAP), principal component analysis (PCA), multidimensional scaling (MDS) or diffusion maps (Becht et al., 2018). In addition, the classification of heterogeneous patterns can be achieved through clustering methods, which are based on individual proximity of biological objects (Weber and Robinson, 2016). To illustrate and quantify such findings, recent visualization techniques have emerged, such as volcano plots to compare differential expression or abundance, and heatmaps to summarize similar patterns. In the end,

the whole process allows the integration of multifaceted data, thereby providing new insight into patient heterogeneity and identify novel biomarkers or therapeutical targets.

To summarize this first part, we have seen that the repertoire of T and B cells is educated within the primary lymphoid organs to limit self-reactivity. Alongside these so-called "central" mechanisms, other regulatory mechanisms contribute to maintaining a controlled adaptive response in the periphery, in particular those provided by regulatory T cells. In the next chapter, we will focus on the balance between conventional and regulatory CD4<sup>+</sup> T cells within lymphoid structures, i.e. between T<sub>fh</sub> and T<sub>fr</sub>.

## PART II. TFH/TFR BIOLOGY AND THEIR INVOLVEMENT IN AUTOIMMUNITY

The first part underscored that discerning self from non-self is the foundation of immunological tolerance. Yet, this equilibrium is not always maintained. Such breakdowns set the stage for the complex dynamics of Tfh and Tfr cell biology. These distinct T cell subsets play pivotal roles in guiding the germinal center reaction, a process critical for the maturation of B cells and the production of antibodies. As we dig deeper into the realms of Tfh and Tfr, their significant role in the initiation and advancement of autoimmunity becomes increasingly evident.

### A. T cell polarization following antigen presentation

A bridge exists between the surveillance of the body's tissues and the establishment of an appropriate adaptive response. This bridge is antigen presentation. It dictates the destiny of a recognized antigen: either to be ignored or targeted by the adaptive immune system. Various paths come out from this bridge. One leads to a cytotoxic response implemented by CD8<sup>+</sup> T cells, another to an antibody response mediated by B cells, while yet another instructs innate cells to destruction under the guidance of CD4<sup>+</sup> helper T cells (Seder and Ahmed, 2003). An adequate orchestration of these processes is critical to mount an effective and controlled immune response.

#### 1. T cell subsets and their functions

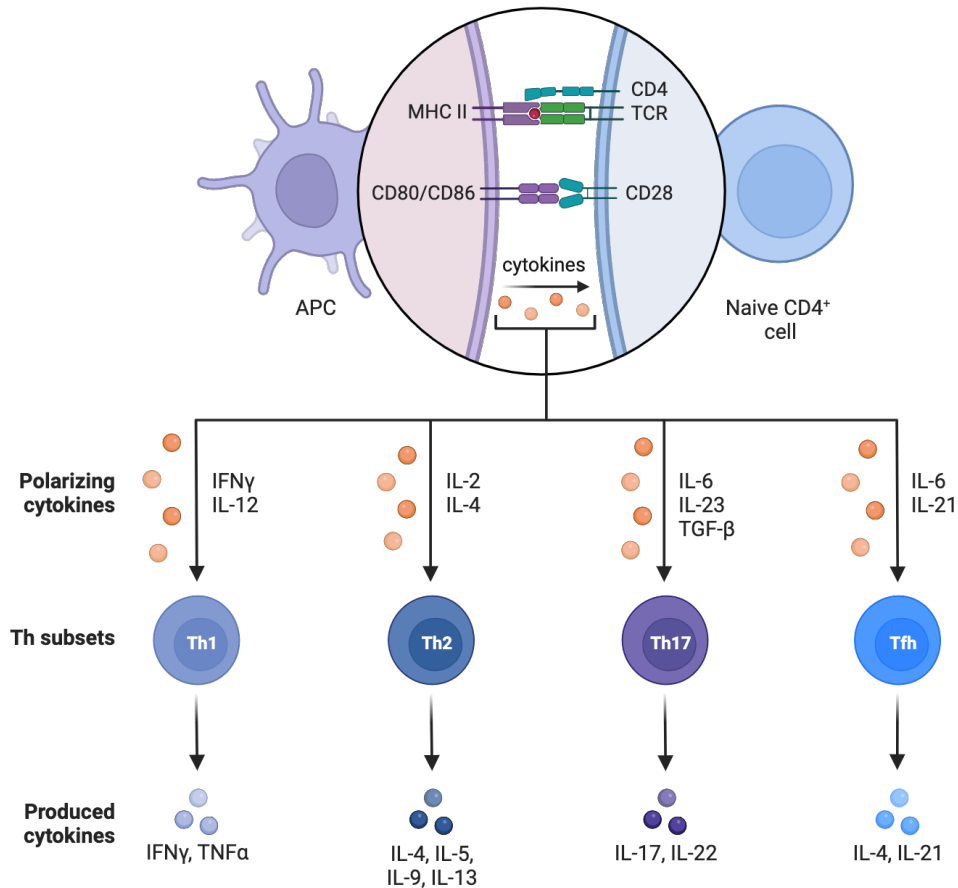
The engagement of the TCR with its specific antigen marks the initial phase of T cell activation, highly dependent on the context of the presentation.

CD8<sup>+</sup> T cells, upon recognizing their specific antigen and receiving appropriate co-stimulation, differentiate into CTLs. Key cytokines like IL-2, IL-12, and type I interferons drive this process, while CTLs are characterized by the transcription factors T-bet and Eomesodermin (Eomes) (van der Leun et al., 2020). CTLs primarily identify and eliminate infected cells by releasing perforin and granzymes, inducing apoptosis in the target cells.

On the other hand, CD4<sup>+</sup> T cells, also known as Th, have a more diverse fate upon activation (**Fig. 7**). For instance, the presence of IL-12 promotes the differentiation into Th1 cells, marked by the transcription factor T-bet. CXCR3<sup>+</sup> Th1 cells are adept at combatting intracellular pathogens by producing IFN $\gamma$ , which notably activates macrophages and NK cells that can eliminate infected cells. In contrast, Th2 differentiation is driven by IL-4 and is characterized by the transcription factor GATA3. CCR4<sup>+</sup> Th2 cells are specialized in countering extracellular parasites, mainly through the production of IL-4, IL-5, and IL-13 which stimulate IgE-producing B cells, eosinophils and mast cells, respectively. Another subset, Th17 cells, emerges in the presence of IL-6 and TGF- $\beta$ , with ROR $\gamma$ t as their defining transcription factor. CCR6<sup>+</sup> Th17 cells play a crucial role in defending against fungal and bacterial infections by secreting IL-17 (Patente et al., 2019).

Beyond classical Th1, Th2 and Th17 subsets, CD4<sup>+</sup> Th can differentiate into other less-known subsets : Th9 cells are driven by transcription factor PU.1 and are involved in allergic reactions via IL-9 production, while Th22 cells produce IL-22 and have been described in skin homeostasis and inflammation (Cui, 2019).

Upon antigen encounter, CD4<sup>+</sup> T cells can also differentiate into T follicular helper (Tfh) cells, a distinct subset that plays a crucial role in B cell maturation, antibody class switching, and GC formation (Crotty, 2014). The polarization of naïve CD4<sup>+</sup> T cells into Tfh is a multi-step process, characterized by early induction of the transcription factor Bcl-6. The most defining cytokines of Tfh are IL-21, IL-4 and IL-6, while CXCR5 is their golden surface marker, allowing their localization within lymphoid tissues.



**Figure 7. The process of Th polarization and differentiation.**

The figure highlights the interactions between a naive CD4<sup>+</sup> T cell and an APC, followed by the polarization towards various Th subsets. At the onset, an APC presents an antigen to a naive CD4<sup>+</sup> T cell through the MHC II complex, facilitated by co-stimulatory molecules like CD80/CD86 interacting with CD28 on the T cell. Concurrently, cytokines secreted in the microenvironment influence the naive CD4<sup>+</sup> T cell's polarization into specific Th subsets - Th1, Th2, Th17, and Tfh. Each subset, influenced by a unique combination of cytokines, commits to producing specific cytokines that define their functional roles in immune responses. The illustrated process underscores the intricacy and specificity of T cell differentiation in guiding adaptive immunity

Among CD4<sup>+</sup> T cells, there are also Foxp3<sup>+</sup> Tregs, which fate is already dictated once they leave the thymus; they can interact with APCs to enhance their suppressive functions. Tfr, which displays overlapping characteristics with both Tfh and Treg regarding transcription factors (Bcl-6 and Foxp3) and surface markers (CXCR5), will be further described later.

Thus, the diverse array of T cell subsets, each with its unique functions and characteristics, ensures a well-coordinated and precise immune response tailored to the specific threat at hand.

## 2. T cell memory

T cells start their journey outside the thymus as naïve cells. Phenotypically, naïve T cells are characterized by the expression of surface markers such as CD45RA — one of the 2 isoforms of the CD45 molecule, together with CD45RO — and lymph node-homing CCR7 within lymphoid tissues, since they have not yet encountered their cognate antigen. Functionally, they are poised to respond to new foreign antigens, but have not undergone clonal expansion or acquired effector functions. Different models of memory T cell generation are debated. A first model establishes that, upon antigenic stimulation, naïve T cells undergo a massive proliferative phase, differentiate into effector cells, and participate in the immediate immune response. Post resolution of the infectious threat, most of these effector T cells undergo apoptosis in a contraction phase except a subset transitioning into memory T cells. Another model postulates a direct orientation towards a memory phenotype following antigen encounter. These models are not exclusive, which highlights the plasticity of T cells under given conditions (Farber et al., 2014).

Memory T cells are phenotypically defined by the expression of CD45RO and can be either CCR7<sup>+</sup> (central memory, with the ability to traffic between lymphoid organs) or CCR7<sup>-</sup> (effector memory, established in peripheral tissues). Their hallmark feature is the ability to respond more rapidly and robustly upon re-encountering their specific antigen compared to their naïve counterparts. This heightened response is not only due to their strategic location (in tissues or in lymphoid organs), but also because of epigenetic modifications making key genes more accessible, preformed mRNA rapidly translated and metabolic reprogramming (Künzli and Masopust, 2023). Functionally, memory T cells provide long-term immunity, ensuring that the host is better prepared and more efficient in fighting a previously encountered pathogen (Sallusto et al., 2004).

### B. Tfh and Tfr: key regulators of the germinal center reaction



1. Germinal centers : spatio-temporal control of the humoral response

- a. *Secondary lymphoid organs, GC and spatial organization*

The immune system relies on a complex network of organs and tissues to monitor and respond to potential threats. Central to this network are the secondary lymphoid organs, which serve as hubs for the initiation of adaptive immune responses. They provide an environment where immune cells can interact, become activated, and coordinate their activities against pathogens. The lymphatic network plays a pivotal role in linking these organs. It is a vast drainage system that collects interstitial fluid, now termed lymph, from tissues (Petrova and Koh, 2018). As lymph flows through this system, it is filtered through secondary lymphoid organs, where any potential pathogens or antigens are detected, and finally returns to the bloodstream through a series of vessels.

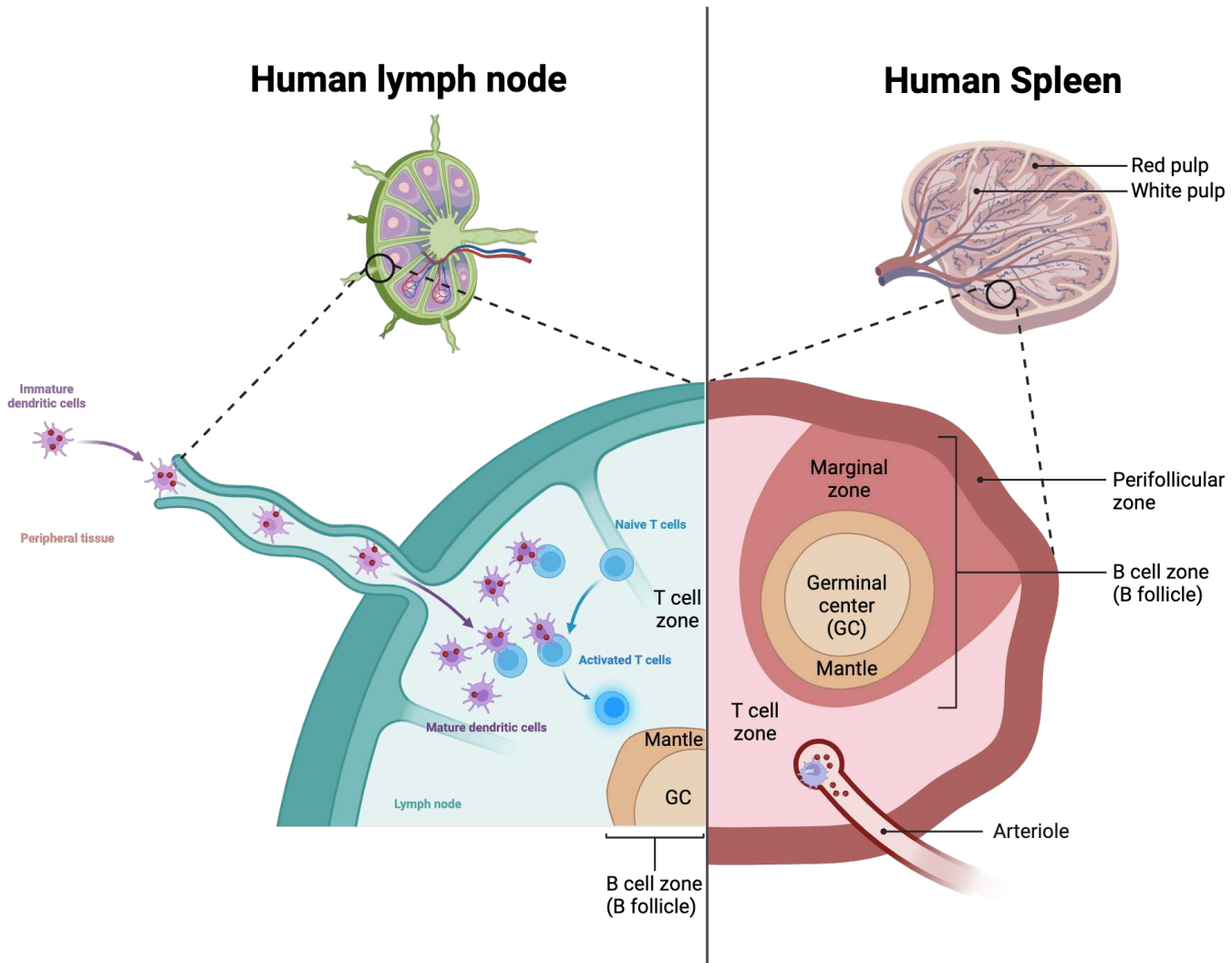
Lymphoid organs are heterogeneous and carry distinct functions according to their localization and structure. Scattered throughout the body, lymph nodes are bean-shaped structures that filter lymph. They are strategically positioned in clusters at junctions of lymphatic vessels, ensuring optimal surveillance. Within a lymph node, the outer cortex primarily contains B cells, while the inner paracortex is rich in T cells (Willard-Mack, 2006). The germinal centers (GC) within the cortex are sites where B cells proliferate and mature after encountering antigens. Unlike lymph nodes, which filter lymph, the spleen filters blood. It's divided into two main regions: the white pulp and the red pulp. The white pulp is where immune responses to blood-borne antigens are initiated, with areas rich in T cells (periarteriolar lymphoid sheaths) and B cells (follicles). The red pulp, on the other hand, is involved in filtering damaged red blood cells and recycling iron (Lewis et al., 2019). Lack of the spleen results in higher risk of *Streptococcus pneumoniae* infection, underlining the importance of draining blood-borne pathogens (Davidson and Wall, 2001). Mucosa-associated lymphoid tissue (MALT) are collections of lymphoid tissue found in mucosal surfaces, such as the gut (GALT, including Peyer's patches), respiratory tract (BALM), and more. MALT structures are frontline defense systems, detecting antigens from the external environment and initiating local immune responses (Cesta, 2006). Lastly, tonsils and

adenoids are located in the throat and nasopharynx respectively and are particularly involved in sampling airborne and foodborne pathogens.

The primary function of secondary lymphoid organs is to facilitate encounters between APCs and naïve lymphocytes (T and B cells). APCs, having detected an antigen in peripheral tissues, migrate to the nearest secondary lymphoid organ, where they present the antigen to lymphocytes. This environment boosts the likelihood of a naïve lymphocyte recognizing its specific antigen, leading to its activation and clonal expansion.

The specialized architecture of these organs supports this function (**Fig. 8**). High endothelial venules (HEV) in lymph nodes, for instance, allow naïve lymphocytes to efficiently enter from the bloodstream (Randall et al., 2008). These cells express L-selectin (CD62L) which enables their attachment to endothelial cells harboring the corresponding ligand, prior to extravasation (Galkina et al., 2007). Homing of CCR7-expressing naïve cells is completed via the detection CCL19 and CCL21 cytokines that diffuse across HEVs. Since the spleen lacks HEVs, lymphocyte entry is made passively through arterioles in the red pulp, followed by migration to the white pulp via CCR7 guidance (Chauveau et al., 2020). Within the organs, a network of fibroblastic reticular cells creates a scaffold guiding cell movements, ensuring that T and B cells pass through zones rich in their respective APCs. Indeed, lymphoid organs are organized according to a bimodal structure that is dictated by key stromal cells : follicular reticular cells (FRC) and follicular dendritic cells (FDC). FRC predominantly populate so-called "T cell zones" (or paracortex) of lymph nodes. They establish a reticular network (conduits) that serves both as a structural framework and a roadmap for T cell and APC movement as well as for free antigen sampling (Katakai et al., 2004). Crucially, FRCs release chemokines CCL19 and CCL21, which have an affinity for the CCR7 receptor, commonly expressed on naïve and central memory T cells. This interaction effectively keeps these T cells into the T cell zones (Li et al., 2021). In contrast, FDCs have a significant role in the "B cell zones" called follicles. They produce the chemokine CXCL13, creating a gradient that attracts B cells expressing the CXCR5 receptor (Rezk et al., 2013). This specific attraction ensures that B cells are concentrated within follicles, optimizing their encounter with antigens and

interaction with helper T cells, thereby facilitating an effective humoral response (Heesters et al., 2014).



**Figure 8. Structural organization and cellular migrations within human secondary lymphoid organs.**

This figure particularly focuses on lymph nodes and the spleen, showing their unique pathways for antigen entry and cellular migration. Initially, the figure highlights immature DCs capturing antigens in peripheral tissues and subsequently migrating through lymphatic vessels into lymph nodes. Within the lymph nodes, a detailed compartmentalization is exhibited, showing the specialized areas like the T cell zone where mature DCs interact with and activate naive T cells. In a contrasting depiction, the spleen's architecture is demonstrated, emphasizing its role in filtering blood-borne antigens. Here, arterioles serve as entry points, allowing the passage of circulating immune cells and antigens into specific spleen compartments such as the marginal zone. The germinal center is a common structure found in the B follicle in lymph nodes and spleens.

Within the B cell follicle can be found the GC, a very specialized structure enabling B cell maturation for high antibody production (Young and Brink, 2021). It is divided in 2 compartments: the dark zone (DZ) and the light zone (LZ). The DZ is where the rapidly

dividing CXCR4<sup>+</sup> B cells, known as centroblasts, are found. These cells undergo somatic hypermutation (SHM) in the variable regions of their immunoglobulin genes, introducing mutations to improve antibody affinity for the antigen. The LZ hosts centrocytes, which are non-dividing CXCR4<sup>-</sup> B cells, where they compete for binding to antigens presented on the surface of follicular FDCs (Weber, 2018). The LZ is where most Tfh are found after migrating from the T cell zone. B cells with the highest affinity antibodies are then selected for survival, while others undergo apoptosis. Deficiency in CXCR4 results in GC B cells unable to enter the DZ and undergo affinity maturation (Allen et al., 2004).

In addition to lymphoid cells and stromal cells, myeloid cells also play an essential role in lymphoid organs. Among them, macrophages are responsible for the phagocytosis of drained pathogens, together with the clearing of apoptotic cells like B cells from the GC, thus avoiding the persistence of self-antigens (den Haan and Martinez-Pomares, 2013).

In conclusion, secondary lymphoid organs are indispensable components of the immune system. Their strategic locations, coupled with their specialized structures, ensure that T and B cells recognize their specific antigen and mount a cooperative response.

#### *b. GC formation*

The formation of the GC is a central event in the humoral immune response to allow the production of high-affinity antibodies; it requires the cooperation between different cell types, in conjunction with molecular factors tightly regulating the process. The emergence of new technologies such as intravital imaging have rendered possible the exploration of GC dynamics *in situ*.

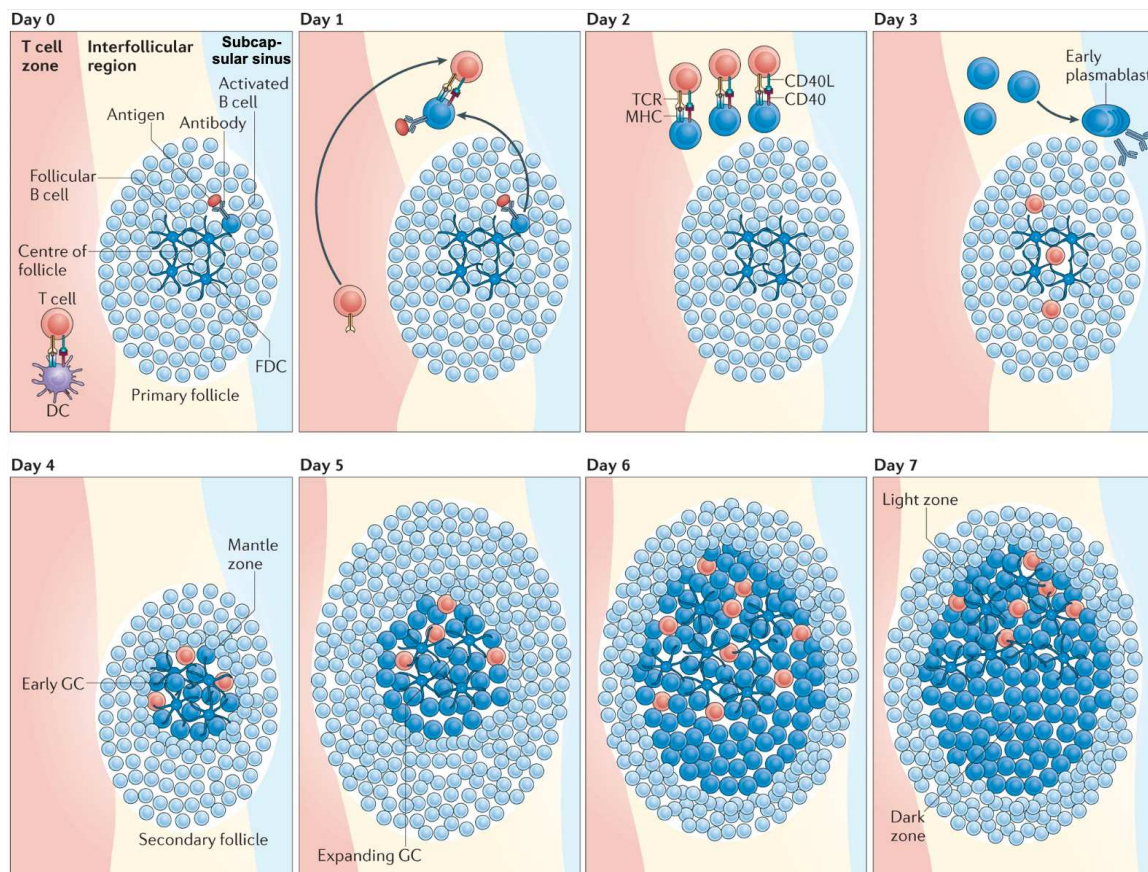
T-independent B cell responses can take place when a highly repetitive antigen binds multiple B cell receptors (BCR), thus rapidly differentiating into short-lived plasmablasts that produce low-affinity antibodies (Mongini et al., 1992). These antibodies serve as an immediate, albeit less specific, line of defense. However for the sake of this thesis, only T-dependent B cell responses will be taken into consideration.

At day 0, in the B cell follicles of secondary lymphoid organs, resting B cells await their cognate antigen, while T cells do the same in the T cell zone (**Fig. 9**). Upon encountering this antigen — often with the aid of FDCs that trap and present antigens in their native form — B cells become activated (De Silva and Klein, 2015). After one day, they migrate towards the T-B border region, where antigen-primed differentiated Tfh have migrated as well, allowing for preliminary interactions. There, the fate of these activated B cells relies largely on their affinity for the antigen: B cells that possess higher affinity BCR might take the extrafollicular route while lower affinity B cells will enter the GC reaction (Paus et al., 2006). Probably due to increased B cell antigen presentation to Tfh when BCR affinity is high, this mechanism of regulation enables the rapid production of high-affinity antibodies that do not require maturation, while B cells with low-affinity BCRs are instructed to undergo GC reaction to avoid the secretion of low-quality antibodies. Having received the go-ahead from Tfh after deeper interaction (T-B interactions will be developed in the next chapter), low-affinity B cells migrate towards the center of the follicle by day 3-4, initiating the early stages of the GC with GC-Tfh. These early steps of GC formation require the expression in B cells of transcription factors Bcl-6 (Kitano et al., 2011) and IRF4 (Ochiai et al., 2013), without which GCs will not form. These cells, now referred to as centroblasts, undergo rapid proliferation. This proliferation has the effect of displacing resting IgD<sup>+</sup> IgM<sup>+</sup> B cells to the periphery of the GC, thus forming the mantle.

As the GC matures, it assumes a distinct architecture characterized by two zones at day 6 : the DZ and the LZ. In the DZ, centroblasts are highly proliferative and undergo SHM in their immunoglobulin genes, a process that introduces random mutations to increase the diversity of the BCR (**Fig. 9**). This process is not without risk, and some B cells acquire deleterious mutations leading to reduced affinity or loss of function. Such B cells are destined for apoptosis. B cells that survive the DZ then move to the LZ. Here, as centrocytes, they interact with FDCs which have captured antigen-antibody complexes via their fragment crystallizable region (Fc) and complement receptors. Centrocytes present their newly mutated BCRs to the retained antigen, while concurrently engaging with Tfh. This double-check mechanism ensures that only B cells with enhanced affinity receive the necessary survival signals. Indeed, given the lower number of GC-Tfh, GC-B cells compete with each other by presenting more

antigens to GC-Tfh. During these interactions, signals for class-switch recombination (CSR) are also delivered, leading B cells to switch from producing IgM antibodies to other isotypes like IgG, IgA, or IgE. From the LZ, B cells face a crossroad: they can either return to the DZ for another round of mutation and selection, or they can exit the GC. Those that exit can differentiate into memory B cells or long-lived plasma cells, now equipped with high-affinity BCRs fine-tuned through the rigorous processes within the germinal center (De Silva and Klein, 2015).

In summary, the germinal center is a meticulously orchestrated environment where B cells undergo a refining process permitted by Tfh.



**Figure 9. Dynamics of germinal centers following antigen encounter.**

This figure sequentially illustrates the maturation process of B cells and T cells upon antigen recognition, over a seven-day period. Initially, both B cells in the primary follicle and T cells in the T cell zone are activated by the same antigen. They then migrate towards the interfollicular region, where they engage in interactions that lead to their full activation, with T cells acquiring Tfh characteristics and some B cells already differentiating into plasmablasts. Subsequently, Tfh migrate into the follicle, where, alongside most activated B cells, they contribute to the formation and expansion of the GC. This involves B cells proliferating and pushing aside resident follicular B cells, resulting in the establishment of an early GC characterized by a distinct structural organization into DZ and LZ. Adapted from De Silva et al. 2015.

c. *Resolution of GCs*

Understanding the resolution, or shutdown, of the GC reaction is essential for appreciating how long-lasting, protective immune responses develop and how they are regulated to avoid excessive humoral response and inflammation.

One primary mechanism believed to influence the GC shutdown is the effect of soluble antibodies on antigen availability (Arulraj et al., 2022). These antibodies can either enhance (via the effect of complement) or dampen the downstream antibody response. When produced in large quantities, they can potentially bind to and "mask" antigens in lymphoid organs, making them inaccessible to GC B cells. This can lead to reduced antigen access over time, contributing to GC termination. This feedback mechanism can shift B cell responses away from epitopes excessively targeted.

Tfr play a pivotal role in modulating GC reaction termination, since they share suppressive characteristics with Treg; these will be developed later on. Also, FDCs are thought to play a role in the resolution of the GC reaction: after one week, they display more internalization of antigen complexes and have reduced dendrite length, limiting antigen exhibition to centrocytes (Arulraj et al., 2022).

Lastly, antigen availability is a critical aspect that shapes the ongoing GC reaction (Cirelli and Crotty, 2017); when antigenic persistence is observed such as during a lymphocytic choriomeningitis virus (LCMV) infection model, an accumulation of Tfh results in sustained GC reaction and further antigen clearance (Fahey et al., 2011). On the contrary, CXCR5 deficient mice, which lacked both Tfh and B follicles, were unable to dampen antigenic persistence. Accumulation of antigens can be then linked to unspecific antibody production by B cells, and thus autoimmunity (Choi et al., 2011). When the pathogen is eliminated under the action of adaptive and innate immune cells, antigen presentation is diminished, thus inducing less Tfh and GC B cells. B cells, upon activation, require a constant stimulation to maintain their phenotype, otherwise they are eliminated in just a few days (Arulraj et al., 2021).

Altogether, these mechanisms ensure that the GC reaction and the subsequent antibody production are tightly regulated in time and in magnitude to avoid inflammation and avoid autoimmunity.

## 2. Tfh and their importance in the GC reaction

### a. *Tfh differentiation and migration to B follicles*

Tfh stands as a specialized subset expressing CXCR5 within effector CD4<sup>+</sup> T cells, primarily dedicated to assisting B cells during the humoral processes of the adaptive immune response. The evolution of naïve CD4<sup>+</sup> T cells into the Tfh lineage is governed by a complex network of molecular and cellular signals.

Tfh were firmly categorized as a distinct CD4<sup>+</sup> T cell polarization in 2009 with 3 different studies that demonstrated Bcl-6 is the master transcription factor defining the Tfh phenotype (Johnston et al., 2009; Nurieva et al., 2009; Yu et al., 2009). However, earlier studies already characterized these cells at different levels, without defining them as Tfh. First, a CD4<sup>+</sup> T cell subset expressing CD57 and low amounts of IL-2 was described in 1986 as residing in the GC (Velardi et al., 1986). In 1999, the expression of CXCR5 was observed in antigen-activated CD4<sup>+</sup> T cells (Ansel et al., 1999). Yet it was one year later that this expression was associated with a follicular localization and B cell helper functions by 2 different teams (Schaerli et al., 2000) (Breitfeld et al., 2000). Further research confirmed the properties of these cells (Kim et al., 2001).

Not all APCs have the same potential to induce Tfh polarization; it has been documented that cDC2 — a conventional DC subset — has the ability to prime this phenotype via IL-12 secretion (Krishnaswamy et al., 2017) while CD14<sup>+</sup> macrophages localize in the B follicle and enhance Tfh phenotype through activin A and TGFβ (Durand et al., 2019).

Bcl-6 is in fact a transcription repressor; it acts by inhibiting Blimp-1, its reciprocal inhibitor, thus enabling Tfh polarization. As well, Bcl-6 can bind to the promoters of Tbet and RORγt, inhibiting Th1 and Th17 polarization, respectively. IL-2 signaling acts as a strong repressor of Tfh differentiation via CD25-STAT5 pathway (Ballesteros-Tato et al., 2012). Yet, low amounts of IL-2 are produced by Tfh, which reveals an



equilibrium between pro-Tfh and anti-Tfh signals; IL-2 brings balance and Tfh polarization arises when an excess of Tfh-inducing molecules are expressed.

TCR strength during antigen presentation has been linked to Tfh differentiation (Fazilleau et al., 2009); but this association seems dependent on the multiple factors in the microenvironment since extremely strong or weak signals can diverge the T cell to adopt other helper lineages (Tubo et al., 2013).

In addition, cytokines are fundamental in orchestrating Tfh differentiation. Specifically, IL-6 and IL-21 have emerged as central players. Both cytokines, upon binding to their respective receptors, can trigger a signaling cascade culminating in the expression of Bcl-6. IL-6 induces the repression of IL-2 signaling, thus reinforcing Bcl-6 expression. IL-6 and IL-21 have been well described in murine Tfh polarization (Eto et al., 2011), although the role of IL-6 has been questioned in humans due to contradictory results (Korn and Hiltensperger, 2021).

Using *in vitro* models, our team has previously proven the ability of TCR-stimulated naive and memory CD4<sup>+</sup> T cells to differentiate into GC-like Tfh when cultured with activin A, IL-12 and IL-7 (Jeger-Madiot et al., 2022). These differentiated cells were functional given their propensity to help B cell responses, highlighting the great plasticity of Th and the importance of the microenvironment.

Among cell surface molecules, CD28 and ICOS were essential to initiate Tfh polarization via PI3K pathway, leading to CXCR5 expression and CCR7 downregulation, and thus migration of cells (Linterman et al., 2014)(Wan et al., 2021). The initially CXCR5-expressing Tfh are called **pre-Tfh**, characterized by a low expression of PD-1. By following the gradient of their ligand, CXCL13, Tfh are drawn towards the B cell follicles. This chemotactic movement ensures that these cells are strategically positioned to interact with B cells. This interaction takes place at the T-B border; studies have shown that primary contacts are initiated in the interfollicular zone, where pre-Tfh are now called **Tfh** with increased expression of PD-1 and ICOS (Kerfoot et al., 2011). ICOS on Tfh serves as a motility molecule through the interaction with bystander B cells expressing ICOS-L, responsible for guiding Tfh to the GC (Xu et al., 2013). But ICOS also acts as a costimulatory molecule (Pedros et al., 2016)

diminishing Klf2 to keep Tfh phenotype and retain Tfh in the B follicle (Weber et al., 2015).

PD-1 dampens PI3K pathway and so Tfh migration: this represents a regulatory mechanism in which ICOS and PD-1 form a balance between migration and arrest (Shi et al., 2018). Therefore, Tfh trafficking is only permitted when ICOS outbalance PD-1 expression. When Tfh reach the GC, they are called **GC-Tfh** and express high levels of PD-1. This allows GC-Tfh to be sequestered in the GC, and by inducing CXCR3 downregulation, PD-1 maintains GC-Tfh in the LZ where they can interact with B cell ongoing affinity maturation and class switching.

While GC-Tfh and GC-B cell interactions will be detailed in the next section, when GC-Tfh have accomplished their effector functions, they can leave the GC and re-circulate between different GCs, or they can acquire a memory phenotype, or else they can also exit the lymphoid organ (Arulraj et al., 2021).

*b. Tfh effector functions in the germinal center*

In the DZ, GC-B cells undergo rapid proliferation and somatic hypermutation, refining their antibody genes to improve antigen affinity. In GC LZ, B cells evaluate the updated affinity of their immunoglobulins by interacting with antigens displayed on FDCs. When a B cell detects an antigen, it captures and presents it to the limited number of GC-Tfh present in the LZ. This interaction with Tfh activates the expression of Myc in B cells, influencing their growth and the following rounds of replication in the DZ (Calado et al., 2012). PD-1 plays an important role in assuring strong GC-Tfh/GC-B interactions. A competition has been depicted between B cells and is thought to restrain the interaction to cells with the most affinity (Shi et al., 2018). The latter may proceed towards plasma cell differentiation, while weaker interaction preferentially orientates GC-B cells towards a memory phenotype (Shinnakasu et al., 2016) (**Fig. 10**).

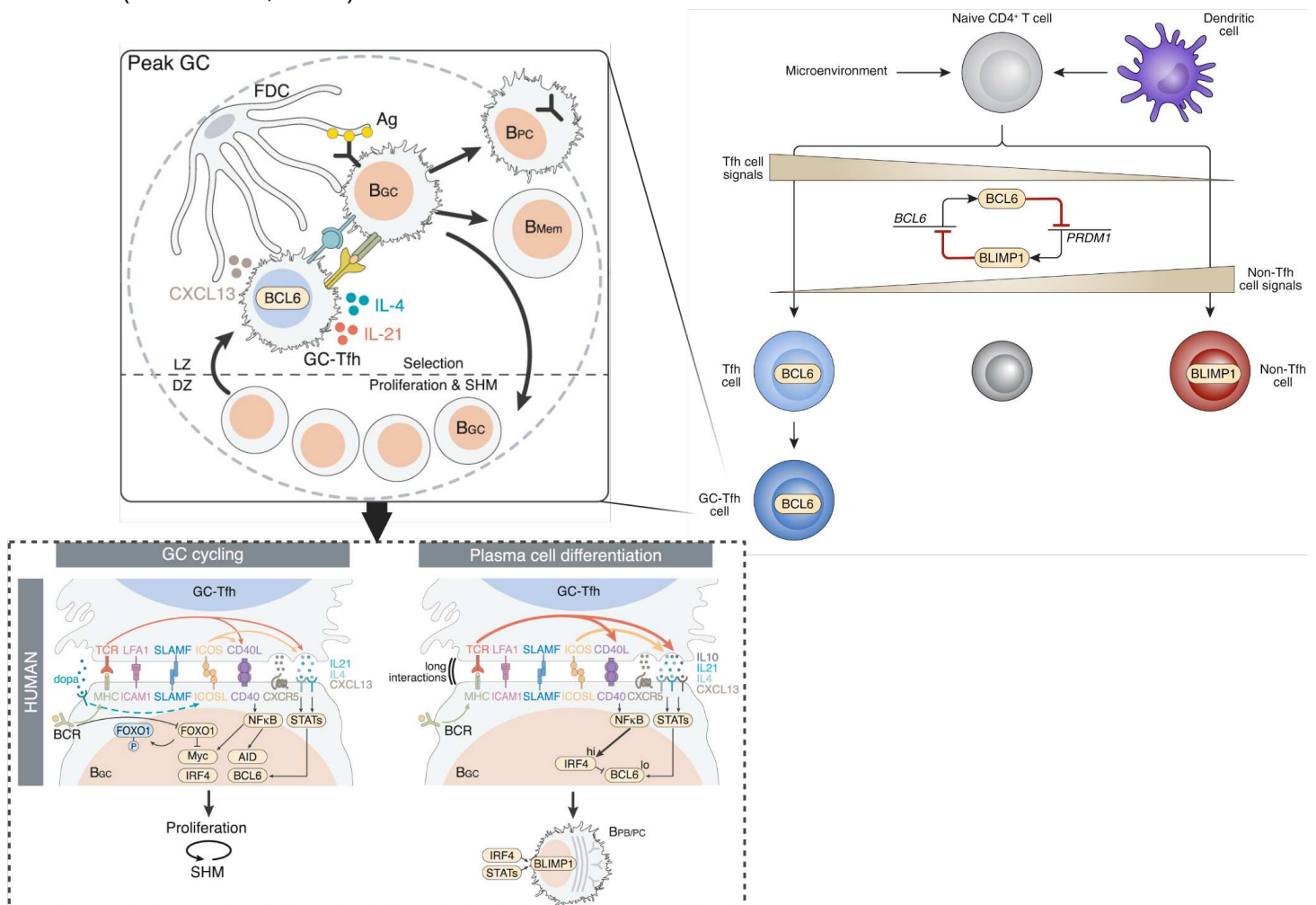
One of the most crucial molecules guiding CSR in the LZ is CD40, expressed on the surface of B cells (Mintz and Cyster, 2020). Tfh express its ligand, CD40L. Upon GC-Tfh and B cell encounter in the LZ, CD40L on the Tfh cell binds to CD40 on the B cell, delivering NF- $\kappa$ B-mediated signals for B cells to undergo CSR. Specifically for CSR, this signaling upregulates the expression of activation-induced cytidine deaminase (AID) (Dedeoglu et al., 2004). AID is an enzyme that introduces DNA breaks in the

immunoglobulin genes, initiating the process of CSR. Moreover, the interaction between CD40 and CD40L also induces the production of cytokines by GC-Tfh, providing further signals to B cells guiding them towards specific isotype switching.

Indeed, GC-Tfh produce and release various cytokines, which are not produced at the same moment and influence the production of antibodies by B cells (**Fig. 10**). An elegant paper published in 2016 detailed murine GC-Tfh heterogeneity in terms of cytokine secretion, surface marker expression and functions (Weinstein et al., 2016). Sequentially, GC-Tfh start secreting IL-21 within the LZ, close to the DZ where proliferating B cells engage contacts to check immunoglobulin affinity. Then, GC-Tfh shift towards IL-4 secretion only, passing by a double IL-21<sup>+</sup> IL-4<sup>+</sup> stage. This maturation is accompanied by CD40L and highly promotes CSR and antibody production. In detail, IL-21 signature plays a pivotal role in promoting B cell differentiation and is instrumental in CSR, particularly driving the switch to IgG subclasses as evidenced in IL-21-lacking mice during malaria infection by *Plasmodium chabaudi* (Pérez-Mazliah et al., 2015). On the other hand, IL-4 promotes switching to human IgG4 and IgE (Kubo, 2021). Other cytokines are involved in GC-Tfh function, such as IFN $\gamma$  which stimulates switching to IgG1 in mice but not humans (Dahlgren et al., 2022).

Also, GC-Tfh express the SLAM-associated protein (SAP), which is crucial for their interactions with B cells. Deficiencies in SAP can lead to impaired T-B cell interactions due to dysfunctional immunological synapse, resulting in a truncated GCs and CSR process (Cannons et al., 2010).

Antigen specificity is another critical point of Tfh cell function. It has been shown that Tfh effectively display strong TCR affinity in an antigen-specific manner (A. M. Robinson et al., 2022) (Hill et al., n.d.). Tfh are thought to primarily recognize non-self-antigens, which comforts their role in enhancing antibody production during infection (Ritvo et al., 2018).



**Figure 10. Tfh differentiation and functions in the GC.**

Tfh differentiation: DC signals and the cytokines present in the microenvironment dictate the fate of naive CD4<sup>+</sup> T cells. A network of regulatory cues, including the mutually exclusive expression of like Bcl6 and Blimp1 orientate the polarization towards Tfh phenotype. During the peak of GC activity, FDCs, B cells, and GC-Tfh intimately interact. Antigen presented by FDCs engages B cells (Bgc), driving their selection, proliferation, and SHM. GC-Tfh cells aid this process by delivering essential cytokines such as IL-4 and IL-21, signifying their pivotal helper role. The outcome of Tfh help depends on a nuanced molecular orchestration. Induction of B cell cycling is promoted by intermediated BCR affinity and intercellular interactions, while plasma cell differentiation is elicited by stronger interactions due to high affinity. Adapted from Crotty, 2019.

*c. Tfh effector functions outside the germinal center*

Noteworthy, Tfh not only have functions within the context of the GC reaction, but they also can leave lymphoid organs and carry diverse functions. Among those, a memory

phenotype can be acquired after GC resolution and memory Tfh can migrate in both lymphoid and non-lymphoid tissues (Hale and Ahmed, 2015). Memory Tfh from the bloodstream are characterized by low expression of PD-1 and increased expression of CD62L and CCR7, allowing their trafficking. The presence of circulating memory Tfh (cTfh) cells in the blood allows for a systemic distribution of memory responses, enabling rapid engagement with B cells upon re-exposure to the antigen, not only at the original site of antigen encounter but also at distant sites. An original work of Elena Brenna *et al.* focusing on human Tfh cell repertoire pointed out that cTfh are clonally related to tonsillar Tfh, whereas no sharing was found with effector Th cells (Brenna *et al.*, 2020). cTfh were heterogeneous, either displaying CXCR3 expression and clonally related to CXCR3<sup>+</sup> Tfh from the mantle/extrafollicular zone, or lacking CXCR3 expression and clonally related to CXCR3<sup>-</sup> GC-Tfh. This study brings to light the different routes for Tfh to acquire memory. Investigations have underscored that the induction of a memory Tfh phenotype can also emerge from memory Th cells from the blood through OX-40 signaling (Pattarini *et al.*, 2017). These findings confirm the high plasticity of the Tfh phenotype.

Interestingly, Hideki Ueno's team uncovered the existence of 3 human cTfh subsets that shared properties with classical Th1, 2 and 17 subsets (Morita *et al.*, 2011). These circulating subsets were later termed Tfh1, Tfh2 and Tfh17, and were characterized upon differential CXCR3 and CCR6 expression, as well as the production of the same cytokines as their Th counterparts. Nonetheless, the corresponding transcription factors were expressed but at lower levels. Tfh2 and Tfh17 were able to help naïve and memory B cells produce different antibodies, unlike their Th counterpart, showing that they retained Tfh features. The mechanisms leading to the acquisition of Th properties by cTfh is uncertain. Though, taking into account the results from E. Brenna *et al.*, CXCR3<sup>+</sup> cTfh are supposed to derive from not fully mature Tfh, which can be associated to the Tfh1 B cell-unhelpful subset. On the other side CXCR3<sup>-</sup> cTfh, thought to be derived from GC-Tfh, are supposedly cTfh2 or cTfh17 which corroborates their capacity to support antibody production. Of note, this model doesn't rule out the other model that proposes a Th effector phenotype prior to becoming cTfh, both not being exclusive. Essentially, the presence of diverse cTfh subsets facilitates a localized antibody response tailored to the affected tissue, aligning the cytokine profile with the specific pathogen in question. For example, Tfh1, which primarily help macrophages

fight against intracellular pathogens, might not necessitate antibody production as a primary mechanism.

### 3. Tfr heterogeneity and role in GC regulation

For years, Tfr were primarily characterized as a specialized subset of thymic-derived Treg, dedicated to exerting control over both Tfh and B cells within the GC, thereby directly influencing antibody production. This painted a binary picture, positioning Tfh and Tfr on opposite ends of a regulatory balance. However, recent scientific advances have uncovered a more intricate landscape, revealing the heterogeneity of Tfr. This newfound understanding speaks to the diverse developmental trajectories Tfr undergo and the varying effector and regulatory roles they play, suggesting the dynamic interplay within germinal centers is more multifaceted than traditionally believed.

#### *a. Tfr development and localization within lymphoid tissues*

The nuanced understanding of Tfr, which now occupies a central position in the humoral response, has evolved gradually. Early inklings about the existence of a regulatory subset in lymphoid tissues emerged from a 2004 study, which observed that regulatory cells could migrate to the B follicles following stimulation, and carried suppressive properties over GC-Tfh and B cells (Lim et al., 2004). This hinted at the profound regulatory capacities of a subset of T cells within lymphoid tissues. Nevertheless, the significant breakthrough in our understanding of Tfr occurred in 2011. This was a defining year for Tfr cell research as three separate articles collectively described these cells as a Foxp3<sup>+</sup> and Bcl-6<sup>+</sup> population suppressing GC reactions (Chung et al., 2011) (Linterman et al., 2011) (Wollenberg et al., 2011).

This discovery unambiguously identified Tfr as distinct entities with a unique lineage. Delving into their developmental arc, it's evident that Tfr emerge through a multi-stage differentiation process. This maturation is guided by specific signals, notably from costimulatory molecules like CD28, ICOS, SAP, occurring during APC contact (Linterman et al., 2011). Central to this differentiation is the Tfh-specific transcription factor Bcl-6, pivotal for the Tfr cell's identity.

A particularly intriguing aspect of Tfr cell biology is their distinct expression patterns, different between mice and humans, setting them apart from traditional Tregs. One of the notable markers differentiating them in mice is the downregulation of the IL-2 receptor alpha chain, CD25. This downregulation isn't a monolithic process. Initial Tfr cell populations still express CD25, but as they differentiate further, the expression dampens, leading to more mature CD25<sup>-</sup> Tfr (Wing et al., 2017). Microscopic examinations of vaccinated mice lymphoid organs have provided insights into this differentiation trajectory. In these studies, a majority of Tfr residing in the follicle and near the T-B border retained CD25 expression. In contrast, the germinal center-resident Tfr predominantly lacked CD25. This difference is further reinforced by their differential chemokine receptor profile. The origin of these Tfr, when tracked using adoptive cell transfer techniques in mouse models, is intriguing. Both CD25<sup>+</sup> Tfr and CD25<sup>-</sup> Tfr populations seem to emerge from naïve CD25<sup>+</sup> Foxp3<sup>+</sup> Tregs, while minimal evidence suggests their development from CD25<sup>-</sup> Foxp3<sup>-</sup> T-cells.

When transitioning our understanding from mice to humans, the picture gets even more complex. A major portion of mouse Tfr downregulate CD25, but this phenomenon appears much less prevalent in human Tfr. Studies focusing on human mesenteric lymph nodes have revealed a concentration of Tfr, which predominantly express CD25, primarily around the T-B border, but not extensively within the GC (Sayin et al., 2018). Notably, while a significant portion of Tfr in human lymph nodes expresses CD25, only a minority, approximately 3-5%, is found in the germinal center, suggesting a marked difference in Tfh/Tfr ratios within the GC between species (Wing et al., 2017). This might underscore a critical functional role of Tfr outside the GC in humans. Additionally, human lymph nodes treated with rituximab (a CD20<sup>+</sup> B cell depleting therapy) demonstrated that Tfr can exist even without GCs, further hinting at their distinct functional attributes (Wallin et al., 2014).

Tfr differentiation from Tfh precursors has been highlighted in some studies; for instance in lupic patients, Tfh could become Tfr under the effect of IL-2 (Hao et al., 2021), which is in accordance with human studies manifesting CD25 expression in Tfr. Another article showed that the terminal stage of GC-Tfh differentiation passes through an ectopic expression of Foxp3, which was accompanied by a decrease in

GC size and thus resolution of the humoral response (Jacobsen et al., 2021). This suggests the emergence of a Tfr population at later stages of Tfh differentiation to promote the end of GC reaction. Also, TCR overlap between both Tfh and Tfr has recently been characterized, even in mice (Ke et al., 2023). A groundbreaking study, did confirmed these results and proved that some Tfr share their TCR with Treg (natural "nTfr") while other share their TCR with Tfh (induced "iTfr") (Le Coz et al., 2023). iTfr and nTfr were distinguished by CD38 expression, higher in iTfr, which shared many surface markers with Tfh, derived from an intermediate CD25<sup>hi</sup> Tfh stage, and could contribute to B cell help for antibody production due to their localization in the GC. On the contrary, nTfr were "elite suppressors", found rather at the T-B border.

Tfr differentiation requires ICOS engagement. ICOS loss causes decreased NFAT2 activation, and thus decreased CXCR5 expression (Panneton et al., 2023). NFAT2 being an inducer of CXCR5, ICOS stimulation correlates with both NFAT2 and CXCR5 expression. This costimulation blocks Klf2 expression to promote Bcl-6 and follicular phenotype orientation, with the promotion of NFAT2 nuclear localization. CXCR5 expression, like in Tfh, enables their migration towards the B follicle. However, unlike Tfh, Tfr display rather intermediate levels of PD-1. Tfr do not require PD-1 expression to be located within the GC, and PD-1 has even been associated with negative regulation of Tfr phenotype in murine models (Sage et al., 2013). PD-1 expression in Tfr appears to counterbalance their activation, since they are supposed to be primed more rapidly and in a bystander fashion (Ritvo et al., 2018). These observations underline the differences that exist between Tfh and Tfr differentiation, despite sharing multiple markers.

#### *b. Dual functions of Tfr in the humoral response*

Tfr are more than mere regulators in light of their heterogeneous developmental paths; they play a multifaceted role in shaping humoral responses. Loss of Tfr is generally associated with disrupted B cell response, antibody production and therefore, risk of autoimmunity (Fu et al., 2018). However, some models have shown that Tfr deficiency, despite increasing Tfh amounts and GC-B cell activation, led to decreased antigen-specific antibody response (Linterman et al., 2011). These observations highlight that Tfr do not have a caricatural role in the suppression of the GC reaction, but instead



have a more finely equilibrated function navigating between anti-inflammatory signals, antigen specificity and timecourse of the GC reaction.

One of the primary mechanisms through which Tfr exert their influence is the secretion of specific cytokines, like TGF- $\beta$ . This cytokine, despite being an important suppressor of Tfh responses (McCarron and Marie, 2014), is instrumental in directing B cells towards an IgA isotype switch, thereby guiding the overall character of the humoral response against pathogens (This and Paidassi, 2022). Similarly, IL-10 production by Tfr has been shown to promote the GC reaction by favoring GC-B cell circulation in the DZ (Laidlaw et al., 2017). Another study suggested that this Tfr-derived IL-10 can have an impact on antigen-specific antibody response associated with IgE CSR (Xie et al., 2020). These findings were surprising given that in the periphery, IL-10 has immunosuppressive functions notably on Th functions (Rubtsov et al., 2008), and this showed that Tfr effector functions differ from Treg and are dependent on the environment (**Fig. 11**).

Yet, the regulatory functions of Tfr in the GC are not limited to simply modulating isotype switching. These cells have a vital role in maintaining self-tolerance, a mechanism of utmost importance to prevent autoimmunity (**Fig. 11**). Specifically, Tfr act to suppress B cells that recognize self-antigens, like nuclear factor proteins, via direct cell interactions (Ke et al., 2023). Furthermore, Tfr express an array of molecules like FasL, FGL2, Granzyme B, and neuritin, which act in concert to inhibit B cells, and in some instances, even instigate their apoptosis. This strategic inhibition ensures that aberrant B cell activity is kept in check, preserving the delicate equilibrium of the germinal center.

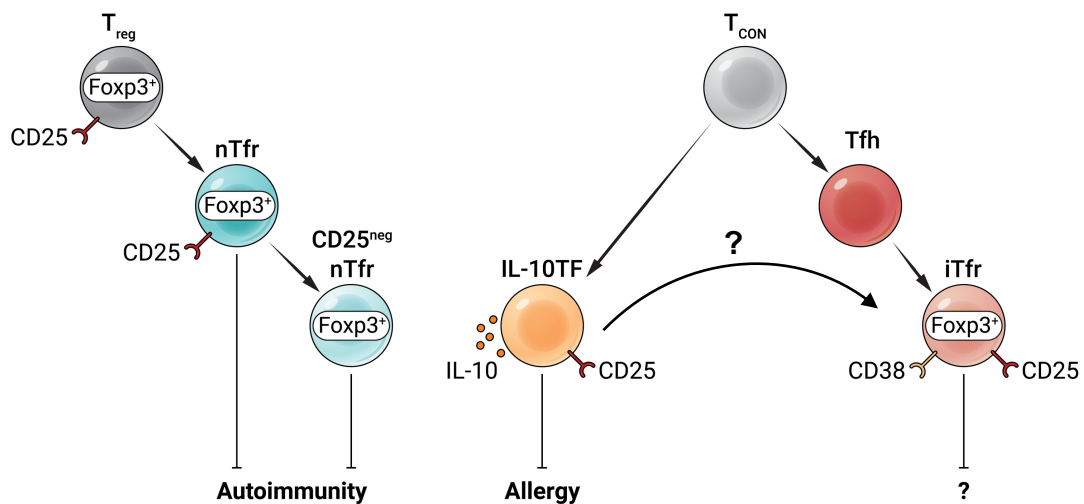
Central to Tfr's suppressive repertoire is CTLA-4, a molecule of paramount importance. It operates by depleting the CD28 ligands, CD80 and CD86, from antigen-presenting cells, which, in turn, dampens the co-stimulatory signals crucial for Tfh cell activation (Sage et al., 2014b). This function becomes especially significant during the nascent stages of Tfh cell formation at the T-B border, by only allowing highly activated T-B conjugates to interact when costimulation signals exceed coinhibitory ones. Delving deeper into the regulatory palette of Tfr, these cells also express CD39 and CD73 ectonucleotidase enzymes (Deaglio et al., 2007). Together, these enzymes form

a duo that eliminates ATP, a known DAMP, from the surrounding milieu and produce anti-inflammatory adenosine. CD25 expression, known to be a regulatory mechanism in Treg by depriving Th from IL-2 essential for their proliferation (Pandiyan et al., 2007), has a different role in Tfr. Indeed, given its suppressive impact on Bcl-6-mediated follicular polarization, its expression by Tfr rather contributes to stabilizing Tfh phenotype and modulating Tfr own commitment. Not to be overlooked, Tfr employ LAG3, a powerful inhibitory mechanism, to restrain Tfh, ensuring they do not get activated too rapidly (Roman M. Chicz et al., 1992). Tfr's regulation is thought to also extend to IL-1-mediated priming of Tfh, since the IL-1 decoy receptor, IL-1R2, is expressed by Tfr (Ritvo et al., 2017a).

Antigen recognition patterns in Tfr present another layer of regulation. Reports suggest a bias in Tfr antigen recognition towards self-antigens in mice, with a possible bystander activation that could allow suppress of self-reactive response during T cell activation (Maceiras et al., 2017). This observation aligns well with the notion that Tfr share a common TCR repertoire with Treg cells, further emphasizing their role in self-tolerance. On the other side, Tfr derived from Tfh could have a more balanced role in contact with B cells, by promoting antigen-specific responses while favoring the termination of the GC reaction (Graca et al., 2023).

Remarkably, Tfh can also express some of the abovementioned molecules classically attributed to regulatory T cells, like CTLA-4 or CD25 (Crotty, 2019). This highlights that upon strong TCR engagement, there is a negative feedback induction to prevent excessive inflammation: for instance CD25 will bind IL-2 to reduce Bcl-6-mediated Tfh phenotype and avoid over-activation.

In addition to GC-related functions, Tfr can have a role outside lymphoid organs. Much like Tfh, cTfr have been identified. These cTfr seem to harbor a memory phenotype (Sage et al., 2014a). While our understanding of cTfr remains limited when compared to their cTfh counterparts, what has become evident is their association with the formation of ELS (Pedroso et al., 2022). Furthermore, the cTfr have also been linked to the pathogenesis of various immune disorders, a topic further developed in subsequent sections. Their action is still unclear, since they have been associated with both suppression of B cell antibody production (Wallin et al., 2014) and a lack of suppressive capacities (Fonseca et al., 2017). This highlights that the localization and the microenvironment of cTfr are in may be essential in defining their functional properties.



**Figure 11. Tfr heterogeneity is associate with different differentiation lineages and functions.**

Distinct populations of Tfr, each with specialized functions, operate within the B follicle and the GC. Natural Tfr (nTfr) cells, predominantly found in the mantle, originate from Treg, maturing with a potential loss of CD25 expression and primarily function to prevent autoimmunity mediated by Tfh activation. In contrast, induced Tfr (iTfr), deriving from Tfh, predominantly localize within the GC. They exhibit markers such as CD38 and CD25, retaining some capacity to facilitate B cell assistance while also producing IL-10. Their precise role, however, remains to be fully elucidated. Lastly, there exists a unique subset of Tfh known as IL-10TF cells. These cells, thought to be precursor of Tfr while not expressing Foxp3 (yet), are significant producers of IL-10 and play a role in immunoglobulin E-mediated mechanisms. Adapted from Graca et al., 2023.

In sum, Tfr are multifunctional entities that wear multiple hats in the humoral response. They are not solely suppressive and strongly differ from Treg; rather, they acquire helper functions that finely tunes the GC response. From guiding B cell isotype switching to maintaining self-tolerance and even having a role in improving the quality of the response, their impact on the humoral landscape is both profound and nuanced.

## C. Tfh and Tfr involvement in antibody-mediated pathogenesis

### 1. Implications of Tfh and Tfr dysfunction autoimmunity

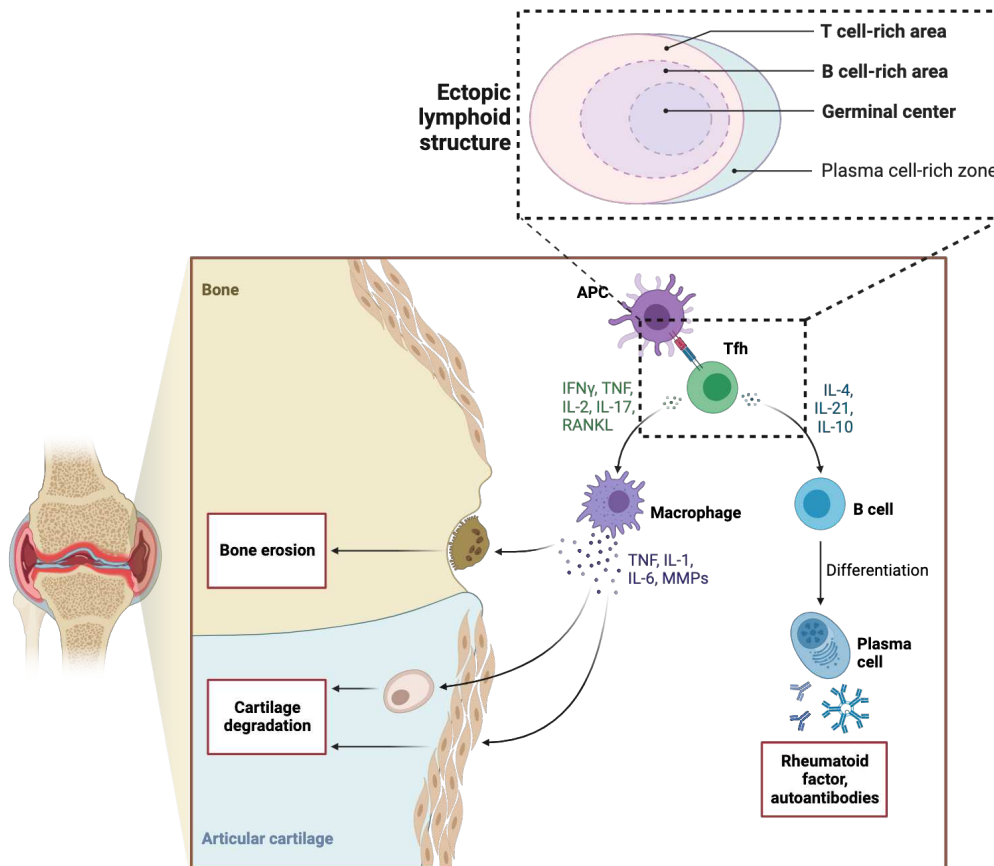
Considering Tfh and Tfr pivotal roles in controlling GC reactions and subsequent antibody production, they are of great interest in the context of autoimmunity mediated by the humoral response. Due to the easiest access to blood samples in patients, the involvement of Tfh and Tfr has mainly been characterized by the study of their circulating counterparts.

Rheumatoid arthritis has been associated with increased cTfh, which could promote anti-cyclic citrullinated peptide (CCP) autoantibodies via an augmented IL-21 secretion (Liu et al., 2012). The quantitative balance between cTfh and cTfr can seemingly be impaired during disease onset, as reported in patients with higher cTfh and lower cTfr, which correlated to CXCL13 levels (Cao et al., 2020). The presence of follicular-related cytokines within the bloodstream has a direct link with ongoing GC activity, and CXCL13 has been proposed as a biomarker to evaluate this activity (Havenar-Daughton et al., 2016). While Tfh/Tfr imbalance has also been documented in SLE (Jacquemin et al., 2015) (Xu et al., 2017), the restoration of a normal balance — including in lymphoid tissues — through OX-40 stimulation in a murine lupic model was associated with an improved outcome, with reduced autoantibody titers (Kumar et al., 2022). This mechanism is due to OX40-dependent expansion of Treg and Tfr, and thus downregulation of STAT3 in Tfh, which is an important transcription factor for their differentiation. Investigations have also pointed out the potential role of defective cTfr defective in the physiopathology of multiple sclerosis (Dhaeze et al., 2015). In granulomatosis with polyangiitis (GPA), a vasculitis that in some cases can display autoantibodies, circulating T cells with a CXCR5<sup>+</sup> Bcl-6<sup>+</sup> follicular phenotype have been found augmented (Abdulahad et al., 2013).

Some studies have focused on tissue infiltration in the context of Tfh and Tfr, rather than sticking to peripheral blood. Multiple sclerosis has been documented to display Tfh infiltration in the cerebrospinal fluid of patients, which was associated to a pathological role in a murine model of the disease (Schafflick et al., 2020). Lymphocyte

infiltration in non-lymphoid tissues can lead to the formation of ELS. They correspond to organized immune cell aggregates that form during chronic inflammation and resemble GCs found in secondary lymphoid organs. These ELS can support local antibody production by B cells, which is often detrimental in the context of autoimmunity. One well-documented pathology displaying ELS with Tfh and Tfr aggregates is Sjögren's syndrome. It has surprisingly been shown that the cTfr/cTfh ratio was positively correlated to ELS formation with B cell infiltrates (Fonseca et al., 2018), while absolute numbers of PD-1<sup>+</sup> ICOS<sup>+</sup> cTfh correlated with disease activity. This study highlights the complexity and diversity of disease pathophysiology solely regarding Tfh and Tfr relative proportions. Finally, RA patients can develop ELS in the synovium of inflamed joint, in which follicular-like populations have been characterized, with CXCL13, PD-1, ICOS and IL-21 (Rao et al., 2017) (**Fig. 12**). The production of IL-10 was also detected and associated to B cell expansion, thus suggesting an involvement of Tfr in ELS and pathophysiology.

Altogether, a dysregulation of Tfh and Tfr has clearly been established in the onset of autoimmune diseases. Their characterization has often been performed under the reductive prism of the Tfh/Tfr ratio among circulating cells. Although some findings establish strong correlations between this ratio and disease activity in few conditions, its relevance is questioned given the different impact that Tfh and Tfr proportions can have in local ELS.



**Figure 12. The role of ELS : example RA pathogenesis.**

ELS, shown in the inset, are organized lymphoid aggregates that form in the synovial tissues affected by RA. Within the ELS, various immune cells such as Tfh cells and B cells engage in interactions, reminiscent of those occurring in the GCs of secondary lymphoid organs. This facilitates the local production of autoantibodies, including rheumatoid factors. In the main illustration, the sequence of pathological events facilitated by the ELS in the RA-affected joint is depicted. The immune cells from ELS contribute to the production of inflammatory cytokines like IFN $\gamma$ , TNF, IL-2, IL-17, and RANKL. These cytokines perpetuate a local inflammatory environment, leading to the activation of macrophages, synovial fibroblasts and osteoclasts, resulting in cartilage degradation and bone erosion. The immune cells, particularly B cells, also contribute to the local production of autoantibodies, which further amplifies the autoimmune response within the joint. Thus, the ELS serve as crucial hubs of immune cell interaction and activation, fueling the persisting inflammation and joint damage observed in RA.

## 2. Altered Tfh and Tfr in other physiopathological contexts

Understanding Tfh and Tfr dysregulation in autoimmunity cannot be separated from their involvement in other pathological conditions. For instance, cancer treatments such as immune checkpoint blockade can unleash autoimmunity as an adverse event (Khan and Gerber, 2020). Indeed, ELS can be found in cancer (Sato et al., 2023), with a contribution of Tfh and B cells in generating anti-tumor antibody-mediated immunity

(Meylan et al., 2022). In this particular context, the role of tolerance is challenged by the necessity of mounting an antibody response against tumor antigens — which can originally be self-antigens being mutated — without attacking normal cells, notably in the context of checkpoint blockade. Tfh have therefore been associated with improved anti-cancer activity like in breast cancer, CXCL13 being a strong biomarker of effective response (Gu-Trantien et al., 2017).

A striking example of Tfh involvement comes from the domain of vaccination. An effective vaccination strategy harnesses the potential of Tfh to boost antibody production, such as in influenza vaccination, while age-related immunosenescence dampens Tfh function and humoral responses (Hill et al., n.d.). This shows the potential of tailoring Tfh responses to optimize vaccine efficacy. However, the story of Tfh in vaccination acquires added complexity when considering human immunodeficiency virus (HIV). A novel vaccination strategy against HIV confirmed the power of Tfh in eliciting protective responses; mice subjected to intranasal and intravaginal injections of virus-like particles (VLP) witnessed an expansion of Tfh, subsequently fostering an antibody-mediated neutralizing response against the virus (Vazquez et al., 2023). Yet, the interplay between Tfh, Tfr, and HIV is intricate. Given their expression of CD4, both Tfh and Tfr emerge as direct targets of HIV infection. The virus not only induces an elevation in their numbers within GCs but also imparts an aberrant phenotype to these reservoir cells (Colineau et al., 2015). This duality underscores the nuanced role of Tfh and Tfr in HIV's pathogenesis and vaccine strategies, challenging our approaches to therapeutic interventions.

Further, Tfh have exhibited both positive and negative input in the domain of transplantation. For instance, in a heart transplantation study, donor-derived Tfh have been reported to facilitate the generation of alloantibodies against recipient MHC I, hinting at their potential pathogenicity (Charmetant et al., 2022). In contrast, during kidney transplantation, while Tfh were found aiding antibody-mediated rejection of donor grafts, their counterpart, Tfr, counterbalanced this detrimental effect (Mohammed et al., 2021).

The challenges mentioned in the first part of the introduction become even more relevant when considering Tfh and Tfr heterogeneity. While their study has focused

on the dichotomic help/suppression functions that they supposedly carry, recent findings highlight the complexity of these cells (Le Coz et al., 2023). Their diverse phenotype, functions, localization and evolution require more sophisticated approaches to unravel their properties. These include transcriptomics and epigenomics to understand their expression patterns, as well as proteomics to decipher their secretome and metabolic features (Bittner and Zipp, 2023). High-dimensional imaging techniques, like imaging mass cytometry, are able to capture Tfh and Tfr phenotype and position *in situ* (Ioannidou et al., 2021). However, as opposed to animal models, human studies can hardly follow Tfh and Tfr subsets across time and space within an individual (Moran and Phan, 2017). This is why new opportunities are rising, such as trajectory inference and machine learning (Tripathi et al., 2023). By only considering some parameters (like time-course samples, gene or protein expression), mathematical models are able to predict differentiation trajectories, patient disease classification, patient response to treatment (Kong et al., 2022). Noteworthy, TCR sequencing is also emerging in the field of T cells in physiopathology : it serves as a tracing marker between populations, and antigen specificity could be predicted using novel machine learning algorithms, which is of utmost interest in infection, cancer, alloimmunity and autoimmunity (Katayama et al., 2022). Overall, main challenges are to find the best and the least parameters to explain and predict most information, thereby limiting costs and time.

In conclusion, the multifaceted roles of Tfh and Tfr extend across an intricate range of antibody-mediated physiopathological conditions. From autoimmunity intertwined with cancer to the overlap between autoimmunity and vaccination, the landscape is vast and interconnected. This reinforces the imperative for a detailed, disease-specific understanding of Tfh/Tfr regulation, equipping us with the insights necessary to harness their potential for therapeutic advancements.



## PART III. IL-1B INVOLVEMENT IN INFLAMMATION, AUTOIMMUNITY, AND T CELL RESPONSES

IL-1 $\beta$  operates at the epicenter of inflammatory cascades, with the multifaceted roles and regulatory mechanisms dictating its influence on both localized and systemic immune responses, tissue integrity, and homeostasis. Despite its role has long been strictly circumscribed to inflammatory processes, the narrow association between autoimmunity and inflammation, as highlighted in the first 2 parts, interrogates its contribution to autoreactive adaptive responses.

### A. Role of IL-1 $\beta$ in inflammatory processes and cytokine signaling

#### 1. Overview of IL-1 $\beta$ as a pro-inflammatory cytokine

The IL-1 family is a group of 11 cytokines — among which IL-1 $\alpha$  and  $\beta$ , and IL-1 receptor antagonist (IL-1Ra) — playing central roles in the regulation of immune and inflammatory responses, characterized by a functional diversity, including promoting inflammation (i.e., IL-1 $\alpha$ , IL-1 $\beta$ ) and limiting or resolving inflammatory reactions (i.e., IL-1Ra), each member having distinct mechanisms of production, release, and activity (Dinarello, 2018).

The synthesis and activation of IL-1 $\beta$  are multi-stage processes influenced by a range of cellular and environmental factors. The synthesis of pro-IL-1 $\beta$  is often initiated in response to immune triggers such as PAMPs and DAMPs. These signals activate pathways like nuclear factor-kappa B (NF- $\kappa$ B), leading to the increased transcription and translation of pro-IL-1 $\beta$ .

The processing of pro-IL-1 $\beta$  into its active form is a separate event, typically occurring after the cell is exposed to a second set of signals. The activation of inflammasomes, such as NLRP3, is central to this process. Inflammasomes are multiprotein intracellular complexes that detect pathogenic microorganisms and stressors, initiating the activation of inflammatory responses. They respond to a diverse array of stimuli including ATP, pore-forming toxins, and crystalline substances, leading to the

activation of caspase-1. Caspase-1 then cleaves pro-IL-1 $\beta$ , converting it into its active form ready for secretion (Chan and Schroder, 2020).

In contrast, IL-1 $\alpha$  has a distinct synthesis and activation pathway. IL-1 $\alpha$  is often present in its active form within cells and does not require cleavage by caspase-1 for activation. It can be released upon cellular necrosis and exert its effects in an autocrine or paracrine manner.

Primary sources like macrophages, dendritic cells, and epithelial cells are induced to release IL-1 $\beta$  in response to specific triggers, establishing a localized or systemic inflammatory environment. The cytokine targets IL-1Rs-expressing cells, thus initiating a series of cellular events that augment immune responses.

In acute inflammatory scenarios, IL-1 $\beta$  acts rapidly and potently. It amplifies vascular permeability, leading to an influx of immune cells at the infection or injury site. The cytokine enhances the expression of adhesion molecules on endothelial cells, facilitating the transmigration of neutrophils and macrophages into the inflamed tissue. These infiltrated cells are then activated and release additional inflammatory mediators, creating a feedback loop that intensifies the inflammatory response (Mantovani et al., 2019).

Beyond localized inflammation, IL-1 $\beta$  orchestrates systemic inflammatory responses. It impacts the central nervous system, particularly the hypothalamus, instigating fever as a host defense mechanism. The cytokine induces the liver to produce acute-phase proteins like C-reactive protein (CRP) and serum amyloid A (SAA), markers often elevated during inflammatory and infectious processes (Weinstein and Taylor, 1987).

Additionally, IL-1 $\beta$  exerts effects on hematopoiesis, influencing the production and release of immune cells from the bone marrow, such as neutrophils, monocytes, and dendritic cell precursors. The cytokine can also induce systemic metabolic changes, impacting glucose metabolism and insulin sensitivity, which is notably observed in inflammatory conditions like type 2 diabetes (T2D) (Gabay et al., 2010).

## 2. IL-1 $\beta$ signaling pathway and downstream targets

IL-1 $\beta$  acts on a broad spectrum of cells that display the corresponding receptors on their surface. IL-1 receptors (IL-1Rs) are meticulously expressed to ensure finely balanced immune responses.

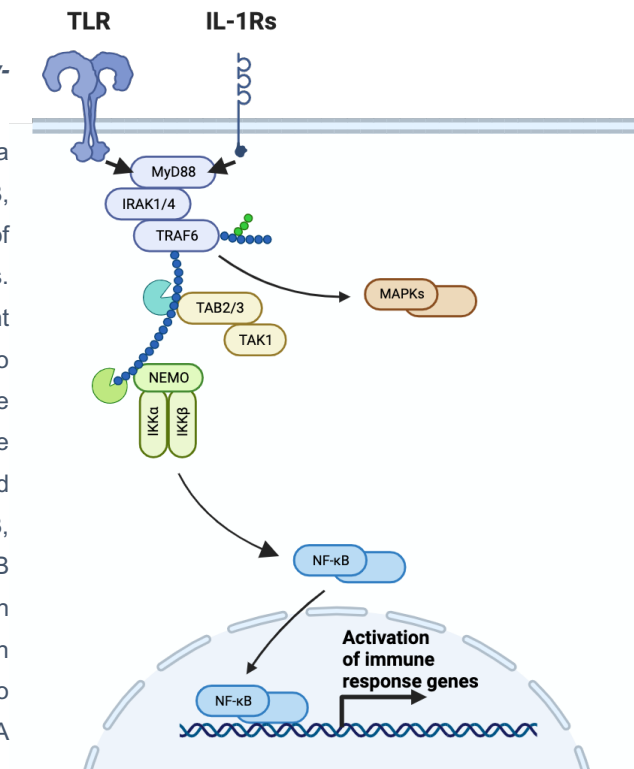
IL-1R1 is the agonist receptor of IL-1 $\beta$  and  $\alpha$ ; upon binding to IL-1 $\beta$ , it undergoes a conformational change, setting the stage for the subsequent recruitment of the IL-1 receptor accessory protein (IL-1RAcP). This complex interaction between IL-1 $\beta$ , IL-1R1, and IL-1RAcP forms a high-affinity signaling assembly, summoning downstream adapter molecules. MyD88, a pivotal adapter protein, is the first to answer this call (**Fig. 13**). Its recruitment to the IL-1R1-IL-1RAcP complex acts as a nexus, channeling signals deeper into the cellular machinery. IRAKs (Interleukin-1 Receptor-Associated Kinases) are quickly phosphorylated and activated, forming a bridge to the next molecular relay in the cascade, TRAF6. As the baton is passed, an ubiquitination event follows, activating TAK1, a kinase with the capacity to unlock the gates to two pathways: NF- $\kappa$ B and MAPK (Jiang et al., 2002).

The NF- $\kappa$ B pathway, recognized as a principal regulator of inflammation, orchestrates the transcription of numerous pro-inflammatory genes, thus shaping the cellular response to extrinsic challenges. In tandem, the MAPK pathway governs cellular processes such as proliferation and differentiation, meticulously regulating the synthesis of assorted cytokines and chemokines (Shi and Sun, 2018) (**Fig. 13**).

Key targets of IL-1 $\beta$  include immune cells such as macrophages, dendritic cells, B cells, and T cells, as well as non-immune cells like endothelial cells (Dinarello, 2018). IL-1 $\beta$  signaling orchestrates a complex downstream transcriptional program that profoundly influences immune and inflammatory responses. Its activation leads to the upregulation of various pro-inflammatory mediators, such as cytokines (i.e., IL-1 $\beta$  itself, IL-6, TNF- $\alpha$ ) and chemokines (i.e., CCL2, CXCL8). The expression of adhesion molecules like ICAM-1 and VCAM-1 is elevated, facilitating leukocyte movement. Furthermore, it induces the synthesis of enzymes like COX-2 and iNOS, leading to prostaglandin and nitric oxide production, respectively. To ensure tissue integrity, anti-apoptotic proteins like members of the Bcl-2 family are also transcribed. Meanwhile, feedback mechanisms, such as the upregulation of suppressors of cytokine signaling, are initiated to modulate the inflammatory cascade.

**Figure 13. IL-1 binding leads to NF- $\kappa$ B pathway-mediated inflammation.**

When IL-1 $\beta$  binds to its receptors (IL-1Rs), it initiates a signaling cascade that leads to the activation of NF- $\kappa$ B, a transcription factor essential for the expression of genes involved in immune and inflammatory responses. Upon binding its receptor IL-1Rs, there is a recruitment of adaptor proteins, like MyD88, which is common to TLR pathway. MyD88 recruits IRAK1/4, leading to the engagement of TRAF6. TRAF6 activation facilitates the activation of transforming growth factor-beta-activated kinase 1 (TAK1), which in association with TAB2/3, activates downstream kinases including IKKs (I $\kappa$ B kinase) and MAPKs (mitogen-activated protein kinases). The activation of IKKs leads to the degradation of I $\kappa$ B (inhibitor of  $\kappa$ B), allowing NF- $\kappa$ B to translocate into the nucleus. In the nucleus, NF- $\kappa$ B binds to specific DNA sequences and activates the transcription of immune response genes, thus modulating the expression of cytokines, chemokines, and other proteins essential in the inflammatory response.



### 3. Regulation of IL-1 $\beta$ signaling

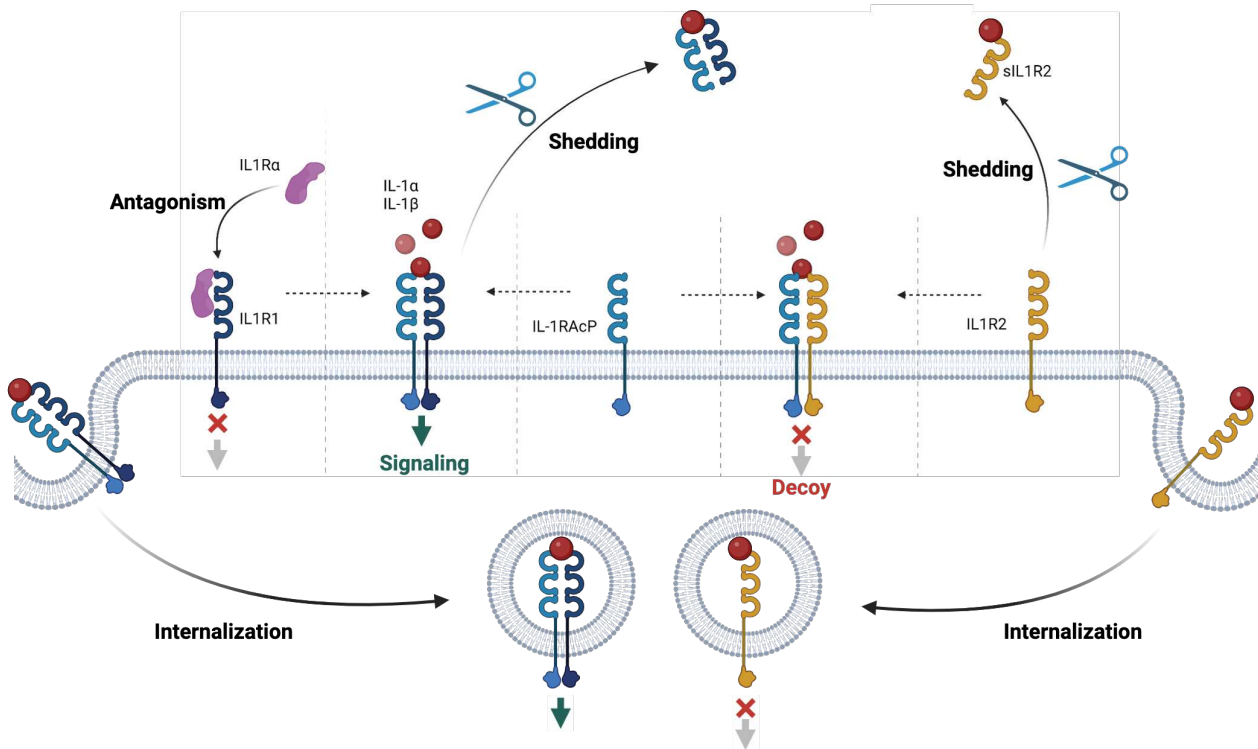
To avoid excessive IL-1 $\beta$ -mediated signaling, cells can express modulatory molecules on their surface (**Fig. 14**). The IL-1R2, lacking an intracellular domain, avidly binds to IL-1 $\beta$ , yet refrains from signaling, serving as a decoy receptor that sequesters superfluous IL-1 $\beta$ . This mechanism is pivotal in preventing uncaptured inflammatory signaling. In a complementary fashion, IL-1Ra competes with IL-1 $\beta$  for binding to IL-1R1. By associating with IL-1R1, IL-1Ra inhibits signal propagation, thereby acting as an intrinsic equilibrium to the pro-inflammatory potential of IL-1 $\beta$ .

Among regulatory tactics developed by IL-1Rs-expressing cells is the preferential binding of IL-1 $\beta$  to the decoy receptor IL-1R2 (Arend et al., 1994). This higher affinity interaction acts as a buffer, preventing excessive activation by delaying the direct agonism of IL-1 $\beta$  with IL-1R1.

Adding layers to this regulation, both IL-1R1 and IL-1R2 receptors can be cleaved from the cell surface and secreted into the extracellular environment (Slack et al., 1993; Symons et al., 1995; Orlando et al., 1997; Elzinga et al., 2009). The shedding of soluble IL-1R1 (sIL-1R1) and IL-1R2 (sIL-1R2) occurs when too much activation and too many inflammatory signals are perceived (**Fig. 14**). Once in the soluble state, these receptors act as molecular sponges, capturing IL-1 $\beta$  in the milieu and preventing its binding to cell-associated receptors. Soluble receptors can even be detected in the blood and possibly carry regulatory properties (Svenson et al., 1993).

Upon IL-1 $\beta$  binding, both IL-1R1 and IL-1R2 can undergo internalization (**Fig. 14**). For IL-1R2, this process culminates in simple sequestration, effectively removing the ligand-receptor complex from the cell surface (Bourke et al., 2003). In contrast, the internalization of IL-1 $\beta$ -bound IL-1R1 carries a dual purpose and has been largely described in human cell lines. On one hand, it acts as a regulatory step, reducing the availability of IL-1R1 on the cell surface and thus limiting further IL-1 $\beta$  binding. Simultaneously, this internalization facilitates signaling from within endosomal compartments, ensuring that the cellular response to IL-1 $\beta$  is both rapid and finely

tuned. Of note, IL-1R1 internalization is a process notably mediated by the action of the Tollip ubiquitin-ligase, leading to receptor recycling (Brissoni et al., 2006).



**Figure 14. Interactions and regulation of IL-1 $\beta$  signaling.**

IL-1 $\beta$  initiates its biological functions by binding to IL-1R1. For effective signal transduction to occur, the recruitment of the IL-1 receptor accessory protein (IL-1RACp) is necessary, forming a complex that activates intracellular signaling pathways. Inhibition of this signaling is facilitated through multiple mechanisms. IL-1Ra acts as a competitive antagonist, binding to IL-1R1 and preventing the interaction of IL-1 $\beta$  with the receptor. Membrane-bound IL-1R2 acts as a decoy, capturing IL-1 $\beta$  and preventing it from interacting with IL-1R1. However, binding to IL-1R2 does not initiate intracellular signaling, serving to sequester the ligands away from the active signaling receptor. Soluble sIL-1R1 and sIL-1R2 contribute to anti-inflammatory activity by binding and sequestering IL-1 $\beta$  in the extracellular environment, preventing it from interacting with membrane-bound receptors. Both receptors can be internalized, limiting cell sensibility to IL-1 $\beta$ , but still enabling signal transduction for IL-1R1. Adapted from Liu et al., 2022.

## B. IL-1 $\beta$ in autoimmune diseases: modulating humoral immunity

### 1. Effects of IL-1 $\beta$ on antigen presentation and antibody production

Focusing on its influence on innate cells controlling antigen presentation, IL-1 $\beta$  acts as a modulator of APC activation. Both dendritic cells and Langerhans cells, crucial players in the antigen presentation paradigm, are affected by IL-1 $\beta$ , leading to changes

in T cell polarization and, by extension, antibody production (Van Den Eeckhout et al., 2021). Further expanding on its multifaceted role, a unique subset of monocytes residing in secondary lymphoid organs produces IL-1 $\beta$ . This production is accompanied by autocrine effects, which have been demonstrated to be influential in shaping antibody-producing germinal center reactions (Barbet et al., 2018).

IL-1 $\beta$  not only exerts a profound influence on innate immune responses but also has distinct effects on autoantibody production and the activation of various immune cells that sustain humoral immunity. Regarding its impact on antibody production, studies in murine models revealed that IL-1 $\beta$  is necessary for T-dependent B cell antibody production (Nakae et al., 2001a). In contrast, T-independent antibody production remained uninfluenced by IL-1 $\beta$ , suggesting a selective impact on T cells or on a B cell subset. Of note, IL-1 $\alpha$  did not seem to share these effects. The enhancement of T-dependent antibody production by IL-1 $\beta$  can be attributed to its role in upregulating CD40L and OX40 expression on T cells, which subsequently support T cell assistance during germinal center interactions (Nakae et al., 2001b). Direct effects of IL-1 on early activated B cells have also been described via the expression of IL-1Rs (Tanaka et al., 1989). B cells even have the capacity to produce their own IL-1 $\beta$ , which is thought to have autocrine functions and to signal to neighboring cells (Bertoglio, 1988). Indeed IL-1 $\beta$  is proven to favor the proliferation and activation of human B cells (Hoffmann et al., 1984).

## 2. Association of IL-1 $\beta$ with autoimmune diseases

In the context of autoimmune diseases, IL-1 $\beta$ 's role is particularly relevant given its association with chronic inflammation. In particular, it drives the persistent activation of immune cells and stromal cells within affected tissues.

In rheumatoid arthritis, IL-1 $\beta$  plays a prominent role. Its continuous signaling is a major contributor to synovial hyperplasia, a thickening of the synovial membrane. This persistent action further accelerates joint erosion, marking the relentless progression of inflammatory arthritis. On a therapeutic front, the use of Anakinra, a synthetic IL-1 receptor antagonist that mimics IL-1Ra, has been approved for RA treatment

(Fleischmann et al., 2003), as well as in juvenile idiopathic arthritis (Allantaz et al., 2007). Additionally, it's noteworthy that disease severity in RA and SLE patients has been observed to decrease in those with elevated levels of IL-1R2 in their serum, indicating an ongoing systemic regulation of IL-1 $\beta$  signaling (Jouvenne et al., 1998)(Italiani et al., 2018). Soluble IL-1R1 has also shown potential as a therapeutic option, offering a reduction in inflammation associated with RA (Barksby et al., 2007). Similarly, sIL-1R1 has been recognized as a valuable treatment in Cryopyrin-Associated Periodic Syndromes (CAPS) (Gillespie et al., 2010). Such interventions emphasize the potential of directly targeting the IL-1 $\beta$  pathway in managing the adverse effects of autoimmune disorders.

Regarding skin autoimmune disorders, IL-1 $\beta$ 's contribution to psoriasis is significant. It is implicated in both the excessive proliferation and the aberrant differentiation of keratinocytes (Sun et al., 2017), as well as the activation of Th17-like cells (Cai et al., 2019). This dual action culminates in the emergence of characteristic psoriatic plaques.

Multiple sclerosis offers another dimension to IL-1 $\beta$ 's influence in autoimmune conditions. Elevated levels of IL-1 $\beta$  have been detected in both the cerebrospinal fluid (CSF) and serum of MS patients (Dujmovic et al., 2009). A compelling observation in experimental models is that IL-1R1-deficient mice displayed an absence of Th17 cells, resulting in heightened resistance to experimental autoimmune encephalomyelitis (EAE), a model for MS.

Other conditions within the vasculitis category have been involved in IL-1 $\beta$ -mediated pathophysiology; Takayasu arteritis patients had increased production of IL-1 $\beta$  and sIL-1R2 (Kabeerdoss et al., 2022), and granulomatosis with polyangiitis (GPA) displayed increased proportions of Th17, which are known to require IL-1 $\beta$  for their differentiation (Wilde et al., 2012).

In addition, several clinical trials have involved IL-1 $\beta$  targeting within the autoimmune disease spectrum such as Sjögren's syndrome (Anakinra) (Norheim et al., 2012), T1D and MS (Rilonacept) and urticarial vasculitis (anti-IL-1 $\beta$  antibody) (Dinarello et al., 2012), without reporting significant effects yet.



### C. Impact of IL-1 $\beta$ on T Cell Differentiation and Function

Despite a clear association between autoimmunity and IL-1 $\beta$ , studies have mainly focused on its pro-inflammatory role on innate cells and stromal tissue compartments, leading to therapeutic strategies targeting its signaling. Hence, very limited interest has been provided to the impact on the key players of the GC reaction, a hallmark of antibody-mediated autoimmunity.

#### 1. IL-1 $\beta$ involvement in CD4<sup>+</sup> helper T cell differentiation and function

Since its discovery, IL-1 $\beta$  interaction with multiple immune cell types has been studied, including CD4<sup>+</sup> T cells, in different settings comprising murine models, human cell lines and human-derived primary cells.

A direct activation of CD4<sup>+</sup> T cells has been observed when cultured with IL-1 $\beta$  (Ben-Sasson et al., 2009). This activation induced IL-4 and IL-17 production and was associated with an increased antibody production *in vivo*, in mice, while the blocking of IL-1 $\beta$  or knocking out IL-1R1 receptor led to a deficit of response to bacterial antigens. Another study showed that the effect of IL-1 $\beta$  on T cell priming was antigenic-specific and not linked to IL-1 $\alpha$ , and was also mediated by an effect on DCs (Nambu et al., 2006). These studies indicate that IL-1 $\beta$  has the capacity to orientate Th polarization.

Among helper T cells, the Th17 subset may be the most well described in light of the IL-1 $\beta$  pathway. In addition to their role for combatting extracellular pathogens, they also play a role in the pathogenesis of various autoimmune diseases, as mentioned earlier. Interestingly, IL-1 $\beta$  has been identified as a key player in promoting human Th17 differentiation, along with a decreased IL-10 signaling which potentiates Th phenotype (Zielinski et al., 2012) (Ghoreschi et al., 2010). Additionally, the differentiation of Th17 cells results in the differential expression of IL-1 receptors IL-

1R1 and IL-1R2 (Lee et al., 2010). The nuanced responsiveness to IL-1 $\beta$  appears to be critical to drive helper T cell fate.

The transcription factor Foxp3, being expressed during early activation of effector T cells, has intriguingly been found to enhance the transcription of IL-1R2 directly within Th17 cells (Kim et al., 2021). This action tempers IL-1 $\beta$  signaling during antigen encounter by Th17, underlining the pivotal role of this cytokine in effector functions. Conversely, IL-1 $\beta$  can impede the transdifferentiation from Th17 cells to Tregs, which has been described as a key mechanism for the resolution of inflammation and to avoid autoimmunity (Gagliani et al., 2015). These observations confirm IL-1 $\beta$ 's power in dictating T cell fate and regulating autoimmunity (Lee, 2018).

Circulating Treg, upon examination *ex vivo*, were found to express IL-1R1. These cells are theorized to be early intermediates in an IL-1 $\beta$ -directed differentiation pathway (Raffin et al., 2011). Such a path, under the influence of IL-1 $\beta$ , might lead to these naïve Foxp3<sup>+</sup> Tregs transitioning to Th17 effector cells. Other studies have shown the expression of IL-1R1 and IL-1R2 in circulating Treg (Tran et al., 2009) as well as in lymphoid organs (Massoni-Badosa et al., 2022). The expression of IL-1Rs by Treg was induced by Foxp3, and associated with higher suppressive activity and activation. This underscores IL-1 $\beta$ 's role in not only promoting effector responses but also potentially modulating regulatory responses.

In murine models, the *in vivo* presence of IL-1 $\beta$  has been shown to heighten CD4<sup>+</sup> T cell activation and differentiation within lymphoid tissues upon antigen encounter (Ben-Sasson et al., 2013). More so, IL-1R1 signaling in these models was found to be primordial for optimal CD4 T cell functionality, including cytokine production and the expression of co-stimulation molecules (Jain et al., 2018). This emphasizes the role of IL-1 $\beta$  in driving T cell responses post-antigen recognition.

Unexpectedly, T cells have also been documented to produce their own IL-1 $\beta$  under the precursor form, leading to an accumulation in the cytoplasm until cleavage instructions are given (Pulugulla et al., 2018). This autocrine production of IL-1 $\beta$  highlights the intricate autoregulatory loops embedded within T cell functionality.

In conclusion, IL-1 $\beta$ 's involvement in the CD4<sup>+</sup> helper T cell landscape is multi-faceted, dictating differentiation pathways, influencing functional outputs, and promoting or inhibiting specific T cell subsets based on the context. Its impact on Th17 and Treg dynamics, in particular, highlights the cytokine's centrality in balancing effector and regulatory responses, a balance that is essential for immune homeostasis and combating diseases.

## 2. Evidence for an impact of IL-1 $\beta$ on Tfh and Tfr cell properties

The IL-1 $\beta$  pathway and its potential effects on Tfh and Tfr are recent areas of immunological research. The majority of insights have been gathered from murine studies, with limited evidence in humans. Nevertheless, the available data indicate a notable interplay between IL-1 $\beta$  and Tfh/Tfr functionalities.

In mice, Tfh are thought to predominantly express IL-1R1 with limited IL-1R2 expression (Ritvo et al., 2017b). Notably, when mice were immunized with IL-1 $\beta$ , there was a marked increase in Tfh activity. This was accompanied by an increase in antigen-specific antibody production, signifying a pivotal role of IL-1 $\beta$  in mediating Tfh cell activity. Conversely, Tfr may exhibit expression of IL-1R2 and IL1Ra, but with a more restricted expression of IL-1R1. This differential receptor expression between Tfh and Tfr underlines the divergent functionalities these cell subsets might exhibit in response to IL-1 $\beta$ . In accordance with these observations, a peanut allergy mouse model showed limited Tfh cell response following immunization of IL-1R1-deficient mice (Dolence et al., 2018).

In murine models again, bacterial infections instigated the production of IL-1 $\beta$  by a specific subset of monocytes situated in the T cell zone. This monocyte-derived IL-1 $\beta$  promoted Tfh differentiation and functionality through direct IL-1R1 signaling, specifically influencing the GC response and accordingly impacting other helper T cell subsets (Barbet et al., 2018).

Another significant clue is the role of IL-1Ra. Two studies showed that murine IL-1Ra inhibited T follicular helper (Tfh) cell expansion and subsequently impacted GC-

dependent humoral immunity (Lin et al., 2021). Another mouse model underscored that IgA antibody response, especially in response to bacterial challenges, required myeloid differentiation primary response 88 (MyD88) signaling within Tfh (Kubinak et al., 2015).

In human studies, cTfh cells from healthy individuals were notably responsive to IL-1 $\beta$  stimulation via the NF- $\kappa$ B pathway, and this responsiveness was reversed by Anakinra (Kothari et al., 2021). Intriguingly, the IL-1 $\beta$ -responsive populations are high in CCR6, which resonates with the known pro-Th17 effects of IL-1 $\beta$ .

When both IL-1 $\beta$  and TGF- $\beta$  were present in human Tfh cultures, there was an enhanced Tfh cell functionality and maturation, underscored by elevated expression of the *BCL6* transcription factor, crucial for Tfh cell identity (Schmitt et al., 2014).

Overall, while murine studies have provided a wealth of knowledge about the role of IL-1 $\beta$  in shaping Tfh and Tfr properties, human data remains limited. Most studies have focused on Tfh and antibody production, the former being more abundant in both lymphoid organs and the blood, whereas Tfr have received very little attention. Elucidating the nuanced modulation of Tfh and Tfr by IL-1 $\beta$  in human lymphoid tissues can shed light on GC reactions and their role in autoantibody production during autoimmunity.



## Thesis objectives

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Current immunological research highlights the intricate mechanisms the immune system employs to safeguard the body's integrity. Both innate and adaptive immunity function as interdependent systems crucial for a rapid, efficient, and targeted response against pathogens. While central lymphocyte ontogeny promotes self-tolerance, it alone isn't enough; peripheral mechanisms play a complementary role in ensuring the proper function of T cells.

Despite the adaptive immune system's intricate protection, occasional deviations can disrupt its balance. Such anomalies may originate from genetic factors like mutations or allelic susceptibility in antigen-presenting molecules, or from somatic events like infections and environmental influences. When tolerance fails, T cell mechanisms can prompt abnormal cytotoxic and helper responses. The latter can compromise humoral immunity. As a result, various autoimmune diseases emerge, whose prevalence has surged in the past century (Miller, 2023), attributed to heightened environmental exposure, posing significant challenges for individuals and society alike.

In this context, the germinal center reaction, which facilitates and modulates the antibody response, holds significant interest. While B cells produce antibodies, CD4<sup>+</sup> T cell subsets remain pivotal in orchestrating the antibody response's onset, maintenance, and halting. Tfh and Tfr, residing within and around germinal centers, play specialized helper and regulatory roles that determine the antibody response's magnitude and efficacy. Their differentiation imprints them with specific traits: precise positioning, movement patterns, co-stimulatory and immune checkpoint molecule expression, and cytokine secretion, all finely tuned to the targeted antigen's specificity.

Historically, Tfh and Tfr were understood through a binary lens: Tfh as helpers and Tfr for immunosuppression. This perspective derived from their precursor cells: conventional T cells for Tfh and regulatory T cells for Tfr. Yet, recent research reveals notable heterogeneity within Tfh, complicating our comprehension of their roles.

Similarly, emerging insights are reshaping our understanding of Tfr, given their varied phenotypes, functions, and origins.

The importance of unraveling germinal center regulation becomes evident when considering Tfh and Tfr's roles in autoimmunity. These cells are linked to the emergence of several autoimmune diseases, either due to imbalances in their blood proportions or through ELS that locally sustain detrimental responses.

Such structures in autoimmune settings spotlight the interplay between inflammation, pathogenic antibody production, and the balance of innate and adaptive immune responses. Interleukin-1 $\beta$ , a principal pro-inflammatory cytokine, not only elevates inflammation but also influences CD4<sup>+</sup> T cell differentiation. Increasingly, IL-1 $\beta$  appears to modulate Tfh and Tfr phenotypes during germinal center reactions through specific patterns of expression of IL-1 antagonist and decoy receptors. However, much of this understanding relies on mouse models and circulating Tfh/Tfr from human blood, as their lymphoid-resident counterparts in human samples are difficult to access.

Therefore, the aim of this project is to decipher how IL-1 $\beta$  modulates human Tfh and Tfr in the context of physiological germinal center reaction, as well as in pathological autoantibody production.

To that aim, we first collected 3 types of human secondary lymphoid organs to characterize Tfh and Tfr heterogeneity based on the expression of IL-1 receptors using flow cytometry. Given the distinct maturation stages of Tfh/Tfr within these organs, we treated their differentiation as a meta-organ process and examined trajectories in conjunction with IL-1 receptors' expression. Secondly, in light of the differential trajectories upon IL-1 receptors' expression, we investigated the functional impact of IL-1 $\beta$  on Tfh and Tfr activation *in vitro* using single-cell transcriptomics and immunophenotyping. Lastly, we investigated whether patients with autoantibodies displayed abnormal IL-1 $\beta$  levels which translated into variations in circulating Tfh and Tfr. This was achieved by exploiting a cohort of patients with various autoimmune and inflammatory diseases.

In this project, a substantial and complex set of data was generated, necessitating the application of advanced analytical methods. I acquired and applied skills in bioinformatics and systems immunology to analyze the data comprehensively. These analytical approaches were essential for handling both low and high-dimensional data, enabling a precise and unbiased characterization of the cells and mechanisms under study.

For flow cytometry data, various techniques such as dimensionality reduction, unsupervised clustering, and trajectory inference were utilized for a more thorough analysis. Transcriptomics data were examined using similar strategies, and visualization techniques like volcano plots and heatmaps were used for better representation of high-dimensional data.

Employing these technical analyses was crucial for the successful completion of the project's objectives. They allowed for a detailed and comprehensive examination of the data, ensuring that the project's goals were met with accuracy and reproducibility.





## Materials and methods

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### **Human secondary lymphoid organ collection**

Tonsils were collected from children suffering from recurring sore throats but otherwise immunocompetent at Necker Hospital. The collection and use for following assays of these tonsils considered as surgical waste was authorized by the ethics committee of Sorbonne University (CER-2021-043). Paired mesenteric lymph nodes and spleens were obtained from adult organ donors within the digestive surgery department at Pitié-Salpêtrière Hospital. Their collection was authorized by the protocol PFS-14-009 by Assistance Publique des Hôpitaux de Paris. For 6 organ donors, blood samples were also collected and underwent SARS-Cov2 serology tests targeting IgG anti-N and IgG anti-S.

### **Treatment of secondary lymphoid organ samples**

Mesenteric lymph nodes, spleens and tonsils were all treated following the same protocol. First, fresh samples were collected and shortly washed with ethanol, sliced in pieces and then transferred in C tubes (Miltenyi) containing RPMI-1640 medium (Sigma-Aldrich) complemented with 10% of Foetal Bovine Serum (FBS) (complete medium), antibiotics (100 U Penicillin ; 100µg Streptomycin) and L-Glutamine. Cell dissociation was carried out with gentleMACS dissociator (Miltenyi) and tube content was filtered through 70µm cell strainers. Mononuclear cells were retrieved using a density gradient (Ficoll). Cell viability was assessed by trypan blue coloration. The cell suspensions were frozen in cryotubes with SVF and 10% DMSO and stored at -80°C for short-term experiments and in liquide nitrogen for long-term preservation. All manipulations involving these samples were performed in a biosafety level 2 laboratory and a sterile environment.

### **Ex vivo immunophenotyping of human secondary lymphoid organs**

Mesenteric lymph nodes, spleens and tonsils cell suspensions were thawed and left to rest overnight. The cells were then stained with the following anti-human antibodies from Miltenyi: CD3-PerCP-Vio700 (1/100); ICOS-Vioblue (1/100); CXCR5-PE-Vio770 (1/500); CD4-APC-Vio770, from BioLegend: CD38-BV711 (1/400); CD25-APC (1/160), from BD Biosciences: HLA-DR-BV786 (1/20); CD19-PE-CF594 (1/200), from R&D Systems: IL1R1-PE (1/80); IL1R2-AF700 (1/80), from Beckman Coulter: PD1-PE-Cy5.5 (1/320). The staining was done in 1X PBS with 5%FBS and incubated in the dark for 30 minutes. The cells were fixated using the BD Pharmingen Transcription

Factor kit as previously described. The cells were stained intracellularly with the following anti-human antibodies from BD Biosciences: Ki67-BUV395 (1/160); FoxP3-AF488 (1/20) and from BioLegend: CTLA4-BV605 (1/40). The cells were then incubated for 50 minutes at 4°C. The cells were washed again with the washing buffer, resuspended in 1X PBS and analyzed using the Flux Cytoflex LX (Beckman Coulter).

### **Cell sorting**

Frozen cells were thawed in complete medium, washed twice and live cells were counted with a trypan blue coloration and incubated at 37°C and 5% CO<sub>2</sub> overnight. Cells were stained for viability using 405/520 Viability (Miltenyi). Then, cells were stained with the following anti-human antibodies from Miltenyi: CD8-FITC (1/100); CD14-PerCP (1/40); CXCR5-PE-Vio770 (1/500); CD19-APC (1/500); CD4-APC-Vio770 (1/500). After a 20-minute incubation at room temperature in the dark, the cells were washed with 1X PBS 5% FBS. The cell sorting was performed on BD FACSAria II (BD Bioscience). Tfol (CD19<sup>-</sup> CD4<sup>+</sup> CXCR5<sup>+</sup>CD14<sup>-</sup> CD8<sup>-</sup>) and B cells (CD19<sup>+</sup> CD14<sup>-</sup> CD8<sup>-</sup> CD4<sup>-</sup>) were then sorted.

### **Tonsillar whole-cell and Tfol-sorted cultures *in vitro***

Following cell sorting, whole-cell or sorted Tfol suspensions were centrifuged and resuspended in complete medium with activation beads anti-CD3/CD28 T Cell TransAct™ (Miltenyi) at the concentration of 1µl for 80 000 cells. Following stimulation, cells from each sample were disposed in a 48-well plate (500 000 cells for whole-cell suspensions) or in a 96-well plate (100 000 cells for Tfol-sorted suspensions). Three conditions were studied: stimulation only, stimulation + IL-1β (0,5µg/mL) and stimulation + Anakinra (1µg/mL). The plates were incubated at 37°C and 5% CO<sub>2</sub> for 24 hours to preserve the early activation of T follicular and B cells. After a 24-hour incubation, culture supernatants were transferred into Eppendorf tubes and conserved at -20°C. Cell pellets were then resuspended in 1X PBS.

### **Immunophenotyping of tonsillar whole-cell *in vitro* cultures**

After a 24-hour incubation, all cells were washed and stained for viability using 405/420 Viability (Miltenyi). *Ex vivo* cells (as control) and whole-cell suspensions were stained with the following anti-human antibodies from Miltenyi: ICOS-Vioblue (1/100); CD3-PerCP-Vio700 (1/100); CXCR5-PE-Vio770 (1/500); CD19-APC (1/500); CD4-

APC-Vio770 (1/500), from BD Biosciences: HLA-DR-BV786 (1/20), from R&D Systems: IL1R1-PE (1/80); IL1R2-AF700 (1/80), from BioLegend: CTLA4-BV605 (1/40), from Beckman Coulter: PD1-PE-Cy5.5 (1/320). The staining was done in 1X PBS with 5% FBS and incubated in the dark for 30 minutes. The cells were washed and fixated using the BD Pharmingen Transcription Factor kit following manufacturer's protocol and incubated either for 45 minutes or overnight 4°C. The cells were washed with the kit washing buffer and stained with the intracellular mix containing the following anti-human antibodies from BD Biosciences : Ki67-BUV395 (1/160) and Foxp3-AF488 (1/20). The cells were then incubated for 50 minutes at 4°C. The cells were washed again with the washing buffer, resuspended in 1X PBS and analyzed using the Flux Cytotflex LX (Beckman Coulter).

### **Immunophenotyping of tonsillar Tfol-sorted *in vitro* cultures**

After a 24-hour incubation, all cells were washed and stained for viability using 405/420 Viability (Miltenyi). Tfol were stained with the following anti-human antibodies from Miltenyi : ICOS-Vioblue (1/100) ; CD3-PerCP-Vio700 (1/100) ; CD39-PE-Vio615 (1/200) ; CXCR5-PE-Vio770 (1/500) ; CD4-APC-Vio770 (1/500), from BD Biosciences : CD126-BUV805 (1/160) ; GITR-BV650 (1/80) ; HLA-DR-BV786 (1/20) ; CD40L-PE-Cy5 (1/160), from BioLegend : CTLA4-BV605 (1/40) ; CD73-BV711 (1/160) ; CD25-APC (1/160), from R&D Systems : IL1R1-PE (1/80) ; IL1R2-AF700 (1/80) and from Beckman-Coulter : PD1-PE-Cy5.5 (1/320). The staining was done in 1X PBS with 5% FBS and incubated in the dark for 30 minutes. The cells were washed and fixated using the BD Pharmingen Transcription Factor kit following manufacturer's protocol and incubated either for 45 minutes or overnight 4°C. The cells were washed with the kit washing buffer and stained with the intracellular mix containing the following anti-human antibodies from BD Biosciences : Ki67-BUV395 (1/160) and FoxP3-AF488 (1/20). The cells were then incubated for 50 minutes at 4°C. The cells were washed again with the washing buffer, resuspended in 1X PBS and analyzed using the Flux Cytotflex LX (Beckman Coulter).

### **Supervised analysis of flow cytometry data**

Raw cytometry data were manually compensated using CytExpert software (Beckman Coulter). Manual gating was performed using FlowJo software (BD) and geometric

mean fluorescence intensity was extracted for each population of interest. Additional gates for marker positivity were created and extracted for each population of interest.

### **Unsupervised analysis of flow cytometry data and trajectory inference**

The following cell populations were exported from FowJo as unique FCS files per sample : CD4<sup>+</sup> CXCR5<sup>+</sup> Tfol, CD4<sup>+</sup> Foxp3<sup>+</sup> regulatory cells, or the combination of both. All subsequent analyses were performed using R (version 4.3). Fluorescence intensity of all cells was logicle-transformed, and markers used for gating only were discarded (Viability, CD3, CD19, CD4). UMAP dimensionality reduction using all markers was performed with *uwot* R package. Clustering was performed on marker fluorescence with kmeans algorithm, and resulting clusters were grouped to reduce the final number of clusters via both hierarchical-based and supervised metaclustering. Heatmaps were made with median expression values using *ComplexHeatmap* R package. Dimensionality reduction at the sample level was achieved with median expression values per marker using MDS and PCA, which functions *isoMDS* (MASS R package) and *prcomp* (stats R package). Further visualizations were made using *ggplot2* R package.

Trajectory inference was conducted with a random downsampling of 70,000 cells using slingshot R package. All markers were included to calculate most likely lineages (*getLineages*) by setting starting cluster as either *pre-Tfh* or *resting Treg*. Pseudostate values were attributed to each cell using *getCurves* with *approx\_points* parameter as 50. For the lineage starting with *pre-Tfh*, we selected the one passing through *Tfh* and *GC-Tfh* sequentially, and we removed overlapping pseudostate values exceeding the median pseudostate value of the following cluster. For the lineage starting with *resting Treg*, we selected the one passing through *activated Treg* without considering Tfh clusters, since no Treg-to-Tfh differentiation has been scientifically documented (Crotty, 2019). Pseudostate and average marker expression were then performed using *ggplot2* R package.

### **Single-cell RNA library construction and sequencing**

After thawing of total lymphoid tonsillar cells, the cells were either stimulated with anti-CD3 and anti-CD25 beads or left untreated during 24 hours. Live Viability negative cells were sorted using BD FACS Aria 2 (Beckton-Dickinson). After isolation of total lymphoid cells in culture by flow cytometry from tonsils, a single-cell library

construction was performed using Chromium Next GEM Single Cell 5' Library and Gel Bead kit v1.1 (PN-1000165), Chromium Next GEM Chip G (PN-1000127), Chromium Single-Cell 5' Library construction kit (16 reactions) (PN-1000020) and Single Index kit T set A (96 reactions) (PN-1000123) according to the manufacturer protocol. Single cell suspensions obtained from 20 000 cells with barcoded gel beads and partitioning oil were loaded onto Chromium Next GEM Chip G in order to generate single-cell bead-in-emulsion. Full-length cDNA along with cell barcode identifiers were then PCR-amplified to generate 5' Gene Expression (GEX) libraries. These libraries were sequenced using NovaSeq 6000 (Illumina) to achieve a minimum of 23 000 pair-end reads per cell for GEX. Reads were subsequently aligned using Cell Ranger v6.1.1 to the GRCh38 human reference genome.

### **Single-cell RNA-seq analysis**

The resulting expression matrices were analyzed thanks to the R environment. Only cells considered high quality were selected for the subsequent analysis. The criteria for these high-quality cells were a rate of mitochondrial genes under 10%, and a number of unique molecular identifier (UMI) comprised between 200 and 15 000. Regularized negative binomial regression, based on the 3000 most variable genes, was chosen to normalize the count matrix with the SCTransform feature. A dimensionality reduction was performed through principal component analysis thanks to the RunPCA again based on the 3000 genes that varied the most. An elbow plot allowed for visual identification of contributive components. The FindNeighbors function and additional contributive PCA components were used to determine nearest neighbors and a clustering of the cells was obtained with the Louvain clustering algorithm along with FindClusters feature. Resolution of the clustering algorithm was done relying on a bootstrapping strategy, the resolution generating the highest number of clusters with co-assignment probabilities above 5% was retained. Differential gene expression analysis was carried out through the FindMarkers function and a correction of the p-values was done using the p.adj base function following the false discovery rate (FDR) procedure. Volcano plot, Heatmap and Venn Diagram visualizations were done respectively with the ggplot2 (v3.4.0) R package, the Complex Heatmap (v2.14.0) R package and the ggven R package (v0.1.9)



### **Transimmunom cohort : study design and blood sample treatment**

Autoimmune and autoinflammatory patients as well as healthy volunteers were enrolled within the Transimmunom Observational Trial (NCT02466217, Lorenzon et al., 2018). In the context of our study, we only considered conditions with at least 10 individuals: ankylosing spondylitis (n = 56), antiphospholipid syndrome (n = 23), Behçet disease (n = 38), Crohn disease (n = 10), GPA (n = 14), osteoarthritis (n = 47), RA (n = 90), SLE (n = 33), type 1 (n = 59) and 2 (n = 30) diabetes, Takayasu arteritis (n = 22), SjS (n = 17) and healthy (n = 57). Blood samples were collected from all individuals to undergo multiple assays, performed by the clinical team of the lab. Briefly, the sera were used to quantify the following autoantibodies using ELISA: antinuclear antibodies (anti-nuclear DNA, anti-SSA60, anti-SSA52, anti-Ro52, anti-SSB, anti-Sm, anti-RNP, anti-PmScl), anti-Ku, anti-TIF1, anti-cardiolipin IgG and IgM, anti-B2GP1 IgG and IgM, anti-Saccharomyces cerevisiae IgG and IgA, ANCA, anti-MPO, anti-PR3, anti-CCP, Latex, Waaler Rose and anti-MBG. The sera were also used to quantify cytokines and soluble receptors, of which we only considered the following: IL-10, IL-6, IL-4, IL-21, IL-21, IL-1Ra, sIL-1R1 and sIL-1R2. For flow cytometry, 13 panels of 10 markers we used (Pitoiset et al., 2018), of which only Tfh and Tfr proportions and absolute numbers were considered. For bulk transcriptomics, peripheral blood mononuclear cells were isolated using a density gradient (Ficoll) and enriched for CD4<sup>+</sup> T cells. T<sub>REG</sub> (CD4<sup>+</sup> CD127<sup>-</sup> CD25<sup>+</sup>) and T<sub>EFF</sub> (CD4<sup>+</sup> CD127<sup>+</sup> CD25<sup>-</sup>) were separated using magnetic beads. CD4 and Foxp3 antibodies were used to undergo a purity challenge and only samples with a purity rate above 80% were validated and used for the subsequent assays. Following RNA extraction, quality control and library construction, sequencing was achieved using Illumina technology.

### **Statistical analysis**

All statistical tests for 2-way comparisons were non-parametric: either Mann-Whitney test (unpaired comparisons) or paired Wilcoxon test (paired comparisons). Statistics provided in the results section are under the form of median  $\pm$  (1.58×IQR)/ $\sqrt{n}$  ; IQR: interquartile range.

## Results

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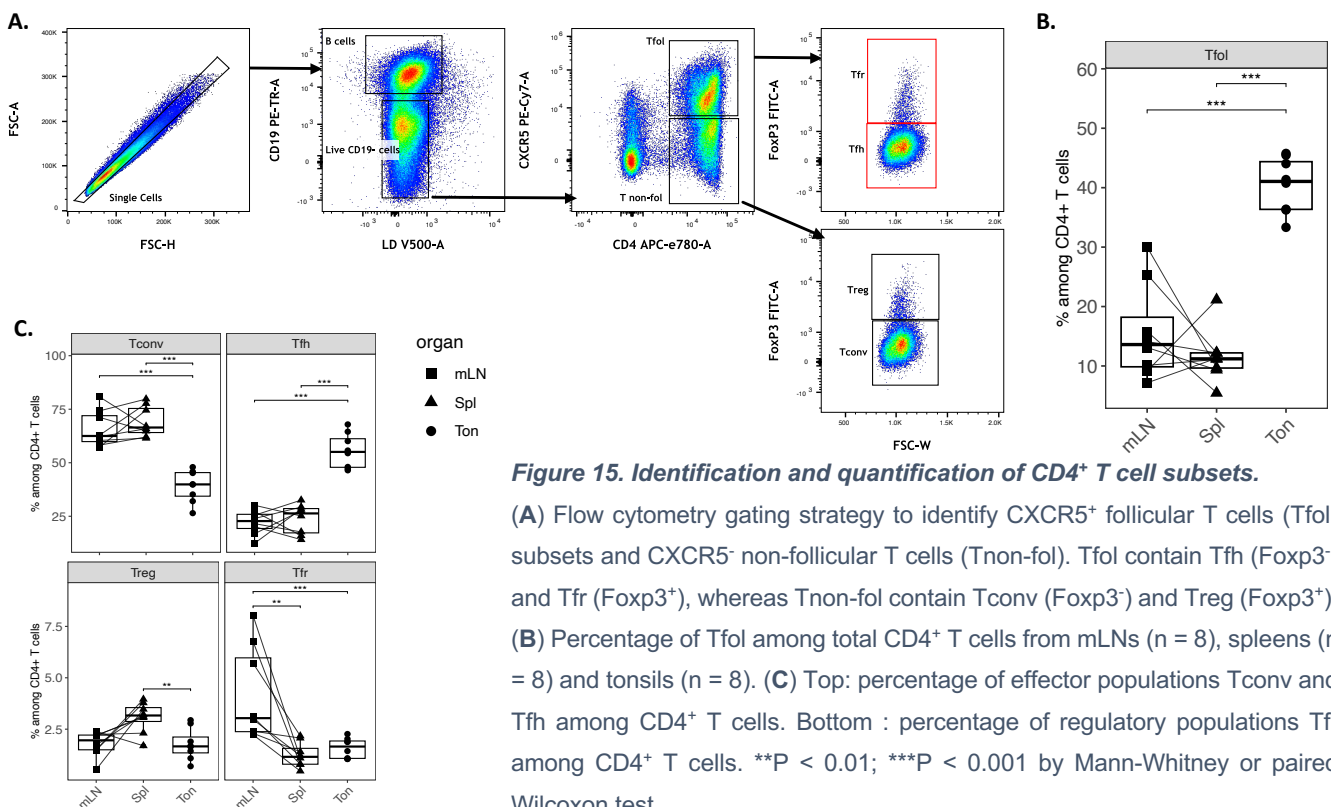


## PART I. EXPRESSION OF IL-1R $\alpha$ SHAPE TFH MATURATION AND PREDICT DUAL ORIGINS FOR TFR EMERGENCE

### A. Human secondary lymphoid organs differ in their follicular T cell composition and phenotype

To characterize the differences between follicular T cells (Tfol) from different secondary lymphoid organs (SLO), we collected 8 pediatric tonsils, as well as 8 paired spleens and mesenteric lymph nodes (mLNs) from healthy donors. They represent 3 major types of SLO: mucosa-associated, blood-draining and lymph-draining organs respectively. Using flow cytometry, we identified 4 major CD4<sup>+</sup> T cell subsets based on CXCR5 and Foxp3 expression: Tfh and Tfr (in Tfol), Tconv and Treg (in Tnon-fol) (**Fig. 15A**).

The *ex vivo* abundance of CXCR5<sup>+</sup> Tfol among CD4<sup>+</sup> T cells was significantly higher in tonsils than spleens and mLNs ( $P < 0.001$ ) (**Fig. 15B**). This results from an enriched Tfh population as opposed to Tconv abundance ( $P < 0.001$ ) (**Fig. 15C**). Spleens differed in the abundance of regulatory cells, displaying increased Treg frequency ( $P$

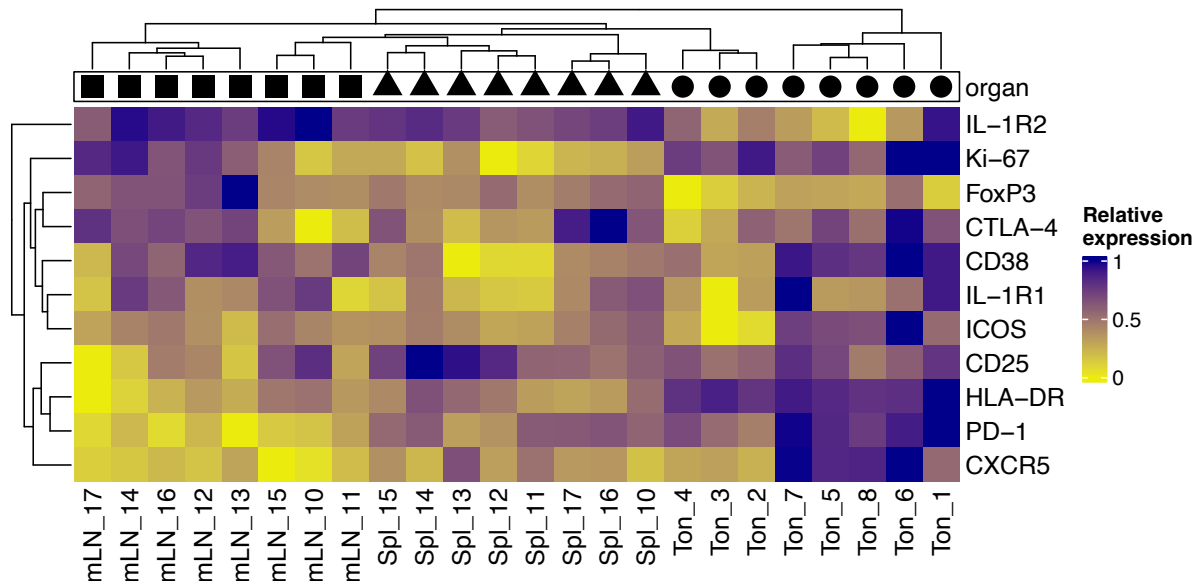


**Figure 15. Identification and quantification of CD4<sup>+</sup> T cell subsets.**

(A) Flow cytometry gating strategy to identify CXCR5<sup>+</sup> follicular T cells (Tfol) subsets and CXCR5<sup>-</sup> non-follicular T cells (Tnon-fol). Tfol contain Tfh (Foxp3<sup>-</sup>) and Tfr (Foxp3<sup>+</sup>), whereas Tnon-fol contain Tconv (Foxp3<sup>-</sup>) and Treg (Foxp3<sup>+</sup>). (B) Percentage of Tfol among total CD4<sup>+</sup> T cells from mLNs (n = 8), spleens (n = 8) and tonsils (n = 8). (C) Top: percentage of effector populations Tconv and Tfh among CD4<sup>+</sup> T cells. Bottom: percentage of regulatory populations Tfr among CD4<sup>+</sup> T cells. \*\*P < 0.01; \*\*\*P < 0.001 by Mann-Whitney or paired Wilcoxon test.

< 0.01 compared to tonsils) while Tfr frequency was higher in mLN (P < 0.01 and P < 0.001 compared to spleens and tonsils, respectively) (**Fig. 15C**).

The phenotype of Tfol was strongly driven by the organ of origin (**Fig. 16**), since a core group of activation markers (CXCR5, PD-1, HLA-DR, CD25, ICOS) clustered samples accordingly. Notably, organ heterogeneity between samples was observed in CXCR5, ICOS, CD38, CTLA-4, Ki-67 and FoxP3 expression, which resulted in a closer phenotype of some tonsils (2, 3 and 4) and mLN (10, 11, 15) to spleens.

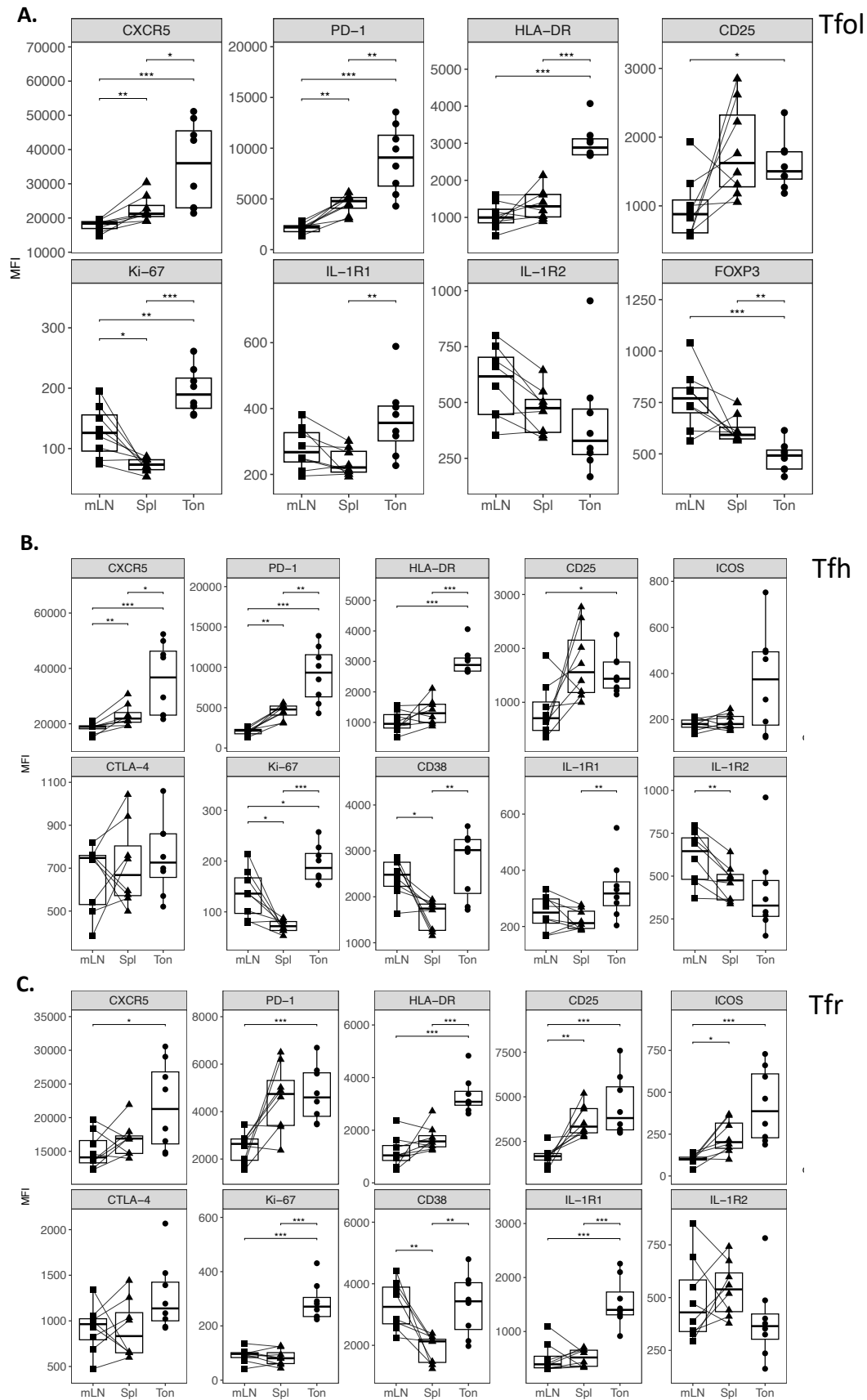


**Figure 16. Phenotype of Tfol across secondary lymphoid organs.**

Heatmap displaying relative marker expression in Tfol across samples from 3 lymphoid organs : mesenteric lymph node (mLN, n=8), spleen (Spl, n=8) and tonsil (Ton, n=8).

The quantification of marker expression highlighted an activation gradient from mLN to tonsils, since a significant increase in CXCR5, PD-1, HLA-DR and CD25 was observed, together with ICOS and CTLA-4 in a lesser extent (**Fig 17A**). Also, Foxp3 was significantly decreased according to this activation gradient, in agreement with the abovementioned higher frequency of Tfr in mLN. Interestingly, the expression of IL-1R1 was increased in tonsils compared to spleens and mLN, due to the contribution of both Tfh and Tfr to its expression (**Fig. 17B and C**), which suggests an association between SLO activation and IL-1R1 expression in follicular T cells. Unlike IL-1R1, IL-1R2 expression was decreased with SLO activation, mLN Tfh being significantly enriched in IL-1R2 compared to spleen while fewer variations of IL-1R2 in Tfr were observed between organs (**Fig. 17B and DC**). Cell proliferation, determined via Ki-67 expression, was significantly higher in tonsils thus confirming the augmented activity of Tfol compared to spleens and mLN. Of note, Tfh proliferation between

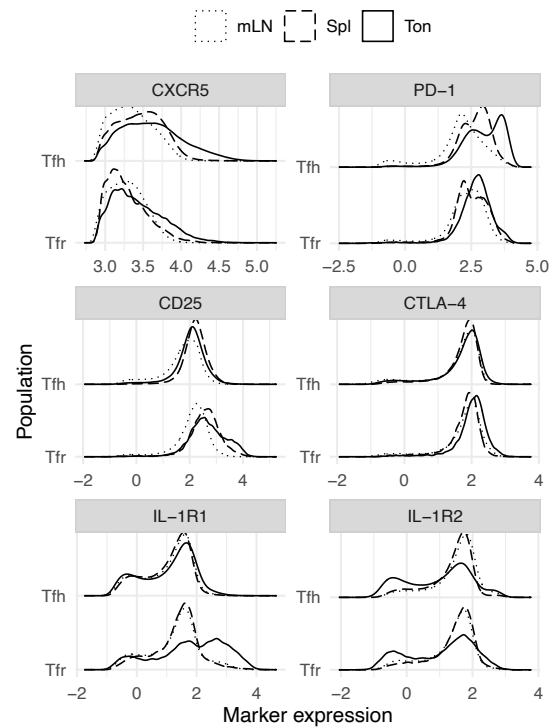
paired spleens and mLNs was statistically lower in spleens (**Fig. 17B and C**) similarly to CD38 expression, which indicates a particular resting phenotype of splenic Tfh.



**Figure 17. Marker expression in T<sub>fol</sub>, T<sub>fh</sub> and T<sub>fr</sub>.**

(A-C) Geometric mean fluorescence intensity (MFI) of selected markers across 3 lymphoid organs in T<sub>fol</sub> (A), T<sub>fh</sub> (B) and T<sub>fr</sub> (C). N = 8. \*P < 0.05; \*\*P < 0.01; \*\*\*P < 0.001 by Mann-Whitney or paired Wilcoxon test.

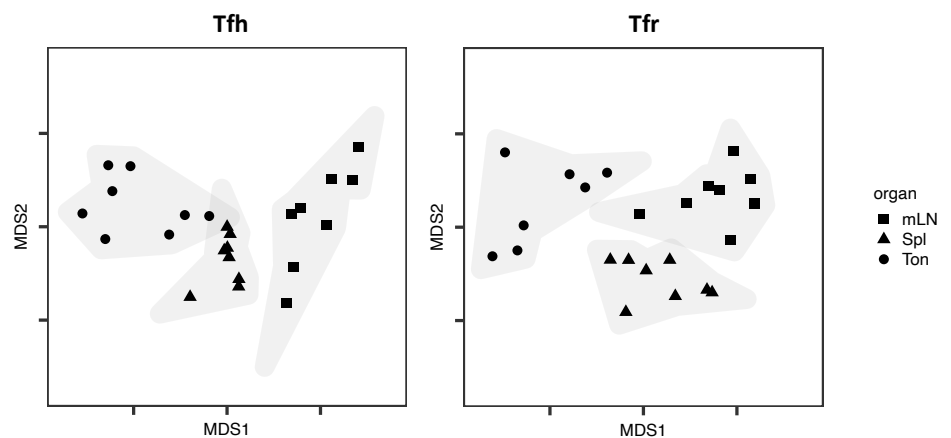
To address differences between Tfh and Tfr phenotype across lymphoid organs, we compared marker density in each of the populations (**Fig. 18**). As expected, Tfh displayed higher average expression of CXCR5 and PD-1 than Tfr, which was more pronounced in tonsils and spleens in agreement with the differential activation phenotype of lymphoid organs. On the contrary, CD25 and CTLA-4 were enriched in Tfr in all organs, especially tonsils. Regarding IL-1Rs, although IL-1R2 expression was similar between Tfh and Tfr cell populations, IL-1R1 was strikingly more expressed on Tfr, even more pronounced in the tonsils.



**Figure 18. Comparison of Tfh and Tfr marker density.** Density plots showing average marker expression in Tfh (top) and Tfr (bottom) across 3 lymphoid organs.

To integrate the expression of all the markers and evaluate Tfh and Tfr phenotypes across organs, we performed multidimensional scaling (MDS) on each population (**Fig. 19**). In such representation, each point corresponds to a sample and the distance between points is proportional to the MFI of integrated markers. Tfh and Tfr clustered separately according to the organ of origin, thus highlighting that Tfol phenotype is driven by their origin of lymphoid organs. In detail, Tfh formed a gradient along MDS1 dimension from tonsils to mLN, in line with the gradient of expression of activation markers and IL-1R1.

On the other hand, Tfr did not distribute according to this gradient, but tonsillar Tfr had a more heterogeneous phenotype than those from spleens and mLN.



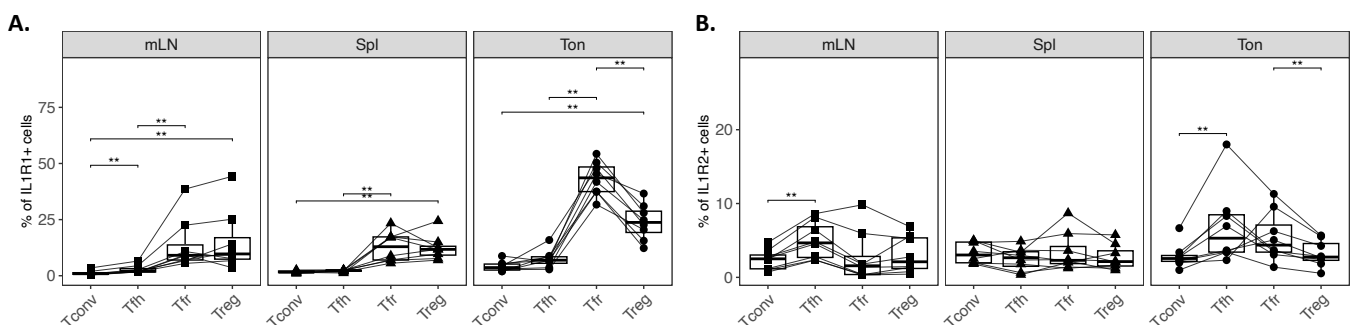
**Figure 19. Phenotypic heterogeneity of Tfh and Tfr across lymphoid organs.** Multidimensional scaling (MDS) plot of Tfh (left) and Tfr (right) per sample, shaped by organ.



In sum, the differences in Tfol abundance and phenotype across human lymphoid organs underline a distinct ongoing germinal center activity, that is higher in tonsils. IL-1R1 receptor expression by Tfr also seems to follow this activation gradient while only Tfh from tonsils seem to be distinguished by a higher expression of IL-1R2.

**B. The differential expression IL-1Rs in Tfh and Tfr captures distinct phenotypes**

To better understand the contribution of IL-1 receptors expression to Tfol phenotype *ex vivo*, we first quantified the proportion of IL-1R1-expressing and IL-1R2-expressing cells among CD4<sup>+</sup> T cells using flow cytometry. Remarkably, Tfr have the highest proportions of IL-1R1 expression in all lymphoid organs, with a gradient from mLNs (9.1 ± 3.8%) to spleens (12.93 ± 5.8%) and tonsils (43.6 ± 6.1%) (**Fig. 20A**). Compared to their Tfh counterparts, IL-1R1<sup>+</sup> Tfr are significantly more abundant in all organs (P < 0.01), as well as compared to tonsillar Treg (P < 0.01). Indeed, IL-1R1 expression seems enriched in regulatory T cells, while Tconv and Tfh have reduced IL-1R1<sup>+</sup> cells, only reaching 3.6 ± 1.5% and 6.9 ± 1.6% in tonsils, respectively. The proportion of IL-1R2<sup>+</sup> cells varies in a much lesser extent between organs and between CD4<sup>+</sup> T cell populations (**Fig. 20B**). Only IL-1R2<sup>+</sup> Tfh are significantly more abundant than their Tconv counterparts in mLN (4.7 ± 2.3%) and tonsil (5.3 ± 2.8%, P < 0.01), as well as tonsillar IL-1R2<sup>+</sup> Tfr (4.4 ± 2.1%, P < 0.01) compared to Treg.



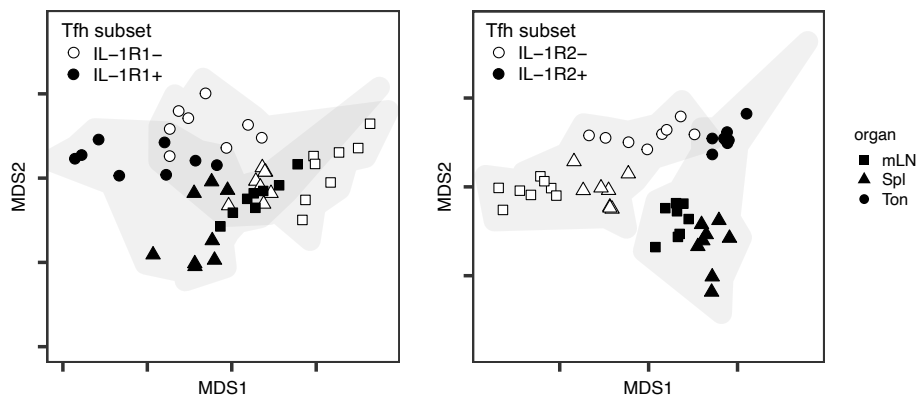
**Figure 20. Abundance of IL-1R1<sup>+</sup> and IL-1R2<sup>+</sup> cells among CD4<sup>+</sup> T cell subsets.**

(A-B) Percentage of IL-1R1<sup>+</sup> (A) and IL-1R2<sup>+</sup> cells (B) among Tconv, Tfh, Tfr and Treg, determined by manual flow cytometry gating. \*\*P < 0.01 by paired Wilcoxon test.

To decipher whether the enriched expression of IL-1R1 and IL-1R2 had an impact on Tfh and Tfr phenotypes, we performed separate MDS analyses on Tfh and Tfr. When

Tfh were clustered based on IL-1R1 expression, each Tfh subset tended to cluster separately, just as samples from the same organ did (**Fig 21A**, left). This observation shows that both the organ and the expression of IL-1R1 contribute to Tfh phenotype. IL-1R1<sup>+</sup> and IL-1R1<sup>-</sup> Tfh from spleens and mLNs exhibited some overlap that was reduced in Tfh from tonsills, which suggests that in highly activated organs, IL-1R1 highly contributes to Tfh heterogeneity. Regarding IL-1R2 expression and the organ of origin, both contributed equally to Tfh clustering without any overlap, thus indicating that IL-1R2<sup>+</sup> Tfh have a distinct phenotype that is also driven by the organ (**Fig. 21A**, right). Similarly, IL-1R1<sup>+</sup> and IL-1R1<sup>-</sup> Tfr subsets were segregated with some overlap between spleens and mLNs, but no overlap was observed between tonsillar Tfr (**Fig. 21B**, left). Tonsils being the tissues with most activation and proliferation, it appears that IL-1R1 expression on Tfr could be associated with a differential maturation phenotype. Finally, subsetting Tfr upon IL-1R2 expression led to a grouping of IL-1R2<sup>-</sup> Tfr with a low contribution of the organ of origin to the phenotype, while IL-1R2<sup>+</sup> Tfr were very heterogeneous and partially overlapped with tonsillar IL-1R2<sup>-</sup> Tfr (**Fig. 21B**, right). Therefore, IL-1R2 seems to moderately contribute to Tfr phenotype in an organ-specific fashion.

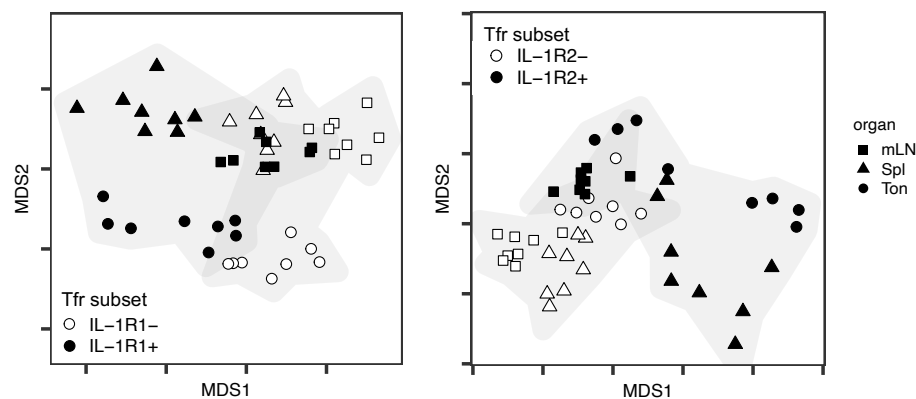
A.



**Figure 21. The expression of IL-1Rs shapes distinct Tfh and Tfr phenotypes.**

(A) MDS plot of IL-1R1<sup>+/−</sup> Tfh subsets (left) and IL-1R2<sup>+/−</sup> Tfh subsets (right), across 3 lymphoid organs. (B) MDS plot of IL-1R1<sup>+/−</sup> Tfr subsets (left) and IL-1R2<sup>+/−</sup> Tfr subsets (right), across 3 lymphoid organs.

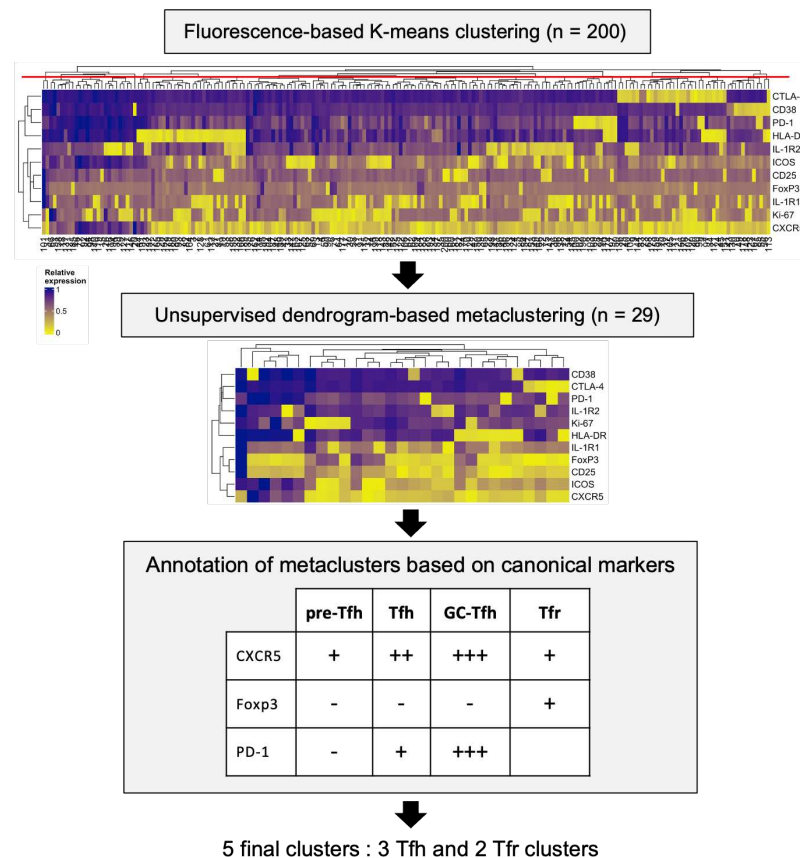
B.



Hence, the expression of IL-1R1 is strongly associated to Tfr and contributes to a different phenotypes of Tfol from activated lymphoid organs, whereas IL-1R2 expression has a preferential impact on Tfh phenotype in each organ.

C. IL-1R1 expression is associated with different stages of Tfh and Tfr maturation

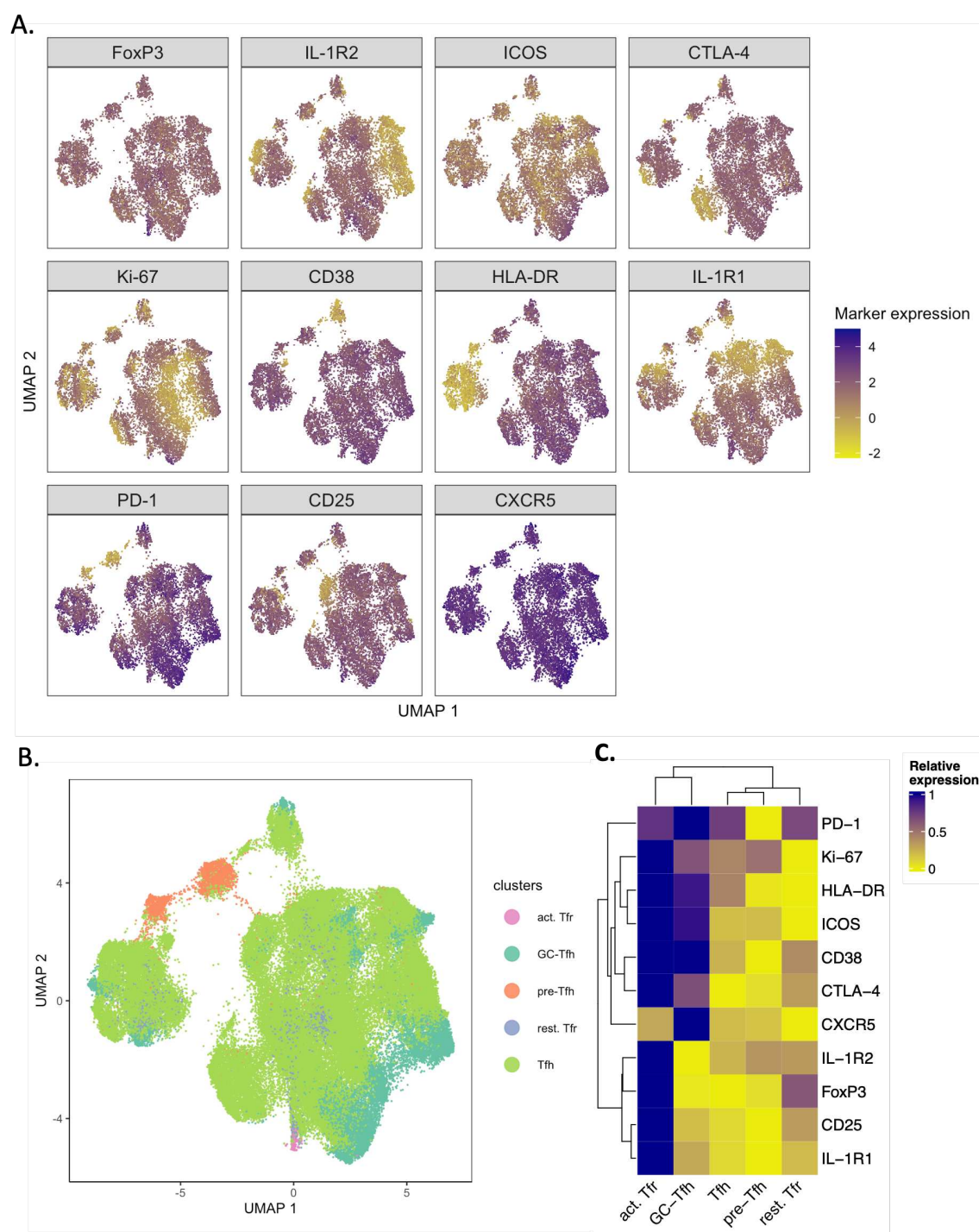
To determine whether IL-1Rs expression contributes to Tfh and Tfr heterogeneity, we explored their different stages of maturation through unsupervised methods. The strength of these computational approaches lies in their capacity to impartially dissect the complexity of cellular processes by simultaneously evaluating numerous parameters. Uncovering novel patterns in the data effectively requires the exploration of cellular phenotypes without relying solely on traditional, hypothesis-driven strategies.



**Figure 22.** Analytical strategy for the identification of Tfh and Tfr clusters among CD4<sup>+</sup> CXCR5<sup>+</sup> cells.

Using the *ex vivo* expression of 11 markers among CXCR5<sup>+</sup> Tfol from 3 lymphoid organs (n = 24), we chose an analysis strategy based on a first over-clustering step (n = 200) allowing the capture of all phenotypes including rare ones, followed by metaclustering upon cutting hierarchically-grouped clusters, and final annotation of the 29 metaclusters based on Tfh and Tfr canonical markers (**Fig. 22**). Since Tfr heterogeneity is not as well defined as the one in Tfh, we annotated Tfr clusters upon activation and proliferation markers.

We performed UMAP dimensionality reduction to visualize marker expression at the cell level (**Fig. 23A**). We identified 5 clusters that discriminated Tfh and Tfr according to their maturation status (**Fig. 23B**). Notably, 3 Foxp3<sup>-</sup> Tfh clusters had increasing PD-1, ICOS and HLA-DR expression (namely *pre-Tfh*, *Tfh* and *GC-Tfh*), and 2 Tfr clusters differed in the expression of Ki-67, HLA-DR, ICOS and CTLA-4 (namely *resting Tfr* and *activated Tfr*) (**Fig. 23C**). *Activated Tfr* phenotypically clustered together with *GC-Tfh* as a result of a shared expression of PD-1, Ki-67, HLA-DR, ICOS and CD38, while the expression of regulatory markers such as CD25, Foxp3, IL-1R2 and CTLA-4, was rather shared with *resting Tfr*.

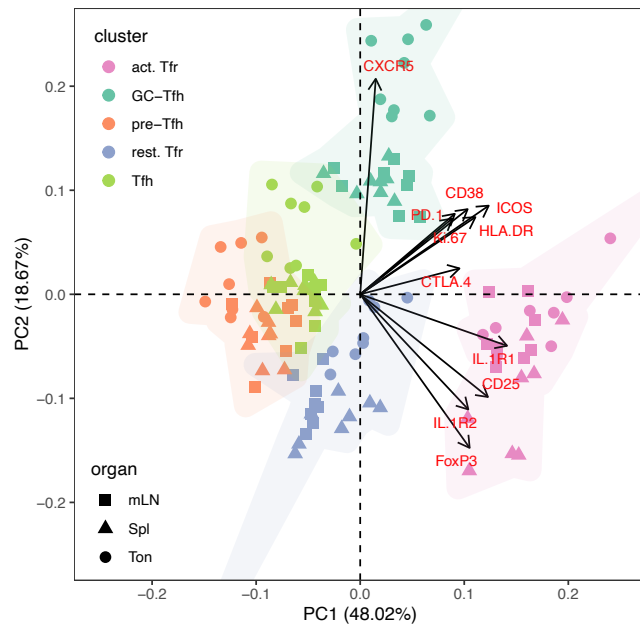


**Figure 23. Marker expression in Tfol defines 3 Tfh and 2 Tfr clusters.**

(A) Dimensionally-reduced CD4<sup>+</sup> CXCR5<sup>+</sup> Tfol from 3 lymphoid organs represented as a UMAP and colored upon marker expression as logicle-transformed MFI. (B) Tfh and Tfr clusters projection on the UMAP representation. (C) Heatmap showing relative marker expression of the identified clusters.

To apprehend the influence of markers on the phenotype of each cluster, we submitted our 5 clusters to a principal component analysis (PCA). The purpose of PCA is similar

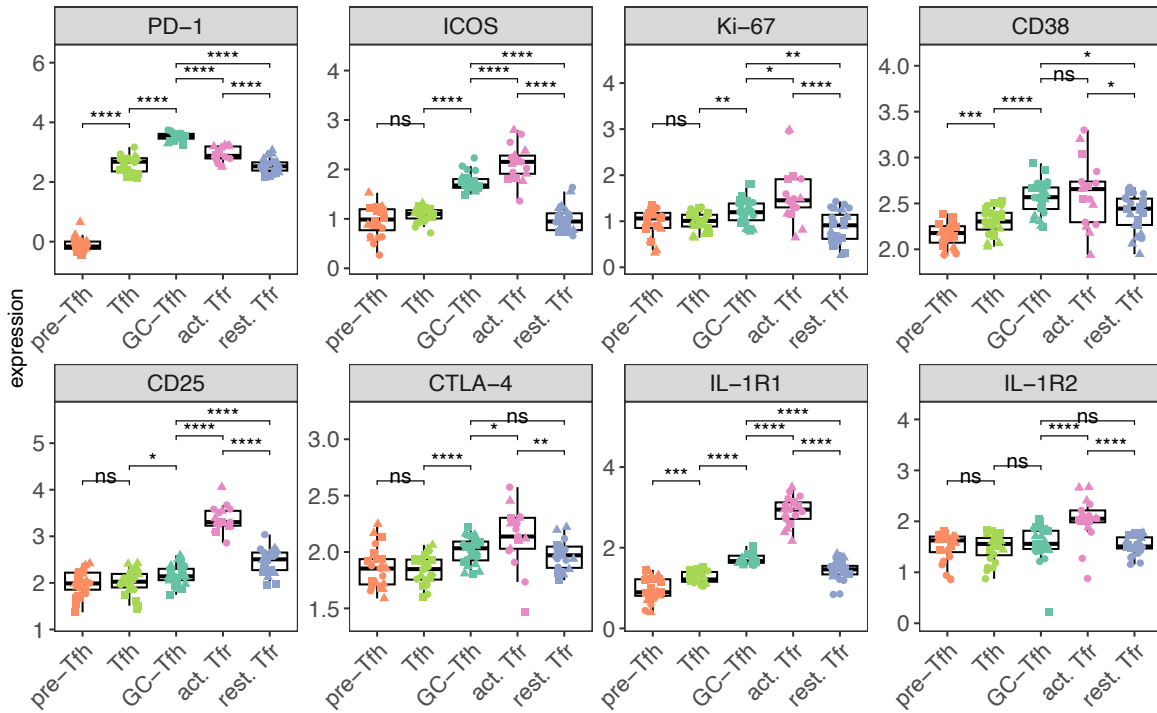
to MDS, but it also captures the contribution of each marker to the displayed samples. Results evidenced the continuum from *pre-Tfh* to *GC-Tfh* according to the expression of CXCR5, PD-1 and ICOS among others, with tonsillar samples being the most driven by this phenotype compared to spleens and mLN (Fig. 24). *Resting Tfr* were phenotypically closer to the least mature Tfh clusters, whereas *activated Tfr* segregated as a unique cluster strongly driven by markers associated to both regulation and co-stimulation. Interestingly, IL-1R1 is the marker that discriminated the most *activated Tfr* from other clusters, and its phenotype seemed common to all organs.



**Figure 24. PCA captures a unique Tfr cluster enriched in IL-1Rs.**

PCA displaying 5 clusters (colours) across 3 lymphoid organs (shape) and the contribution of each marker to principal components is indicated with vectors.

The quantification of marker expression confirmed that *GC-Tfh* and *activated Tfr* were the most mature and proliferative cells with a significant upregulation of PD-1, ICOS, Ki-67, CD38, CD25 and CTLA-4 (Fig. 25). Strikingly, each maturation step of Tfh maturation was associated with a significant increase of IL-1R1 ( $P < 0.001$  and  $P < 0.0001$ ), whereas median IL-1R2 expression did not vary. Similarly, IL-1R1 expression was significantly higher in *activated Tfr* than their resting counterparts ( $P < 0.0001$ ), and so was, in a lesser extent, IL-1R2 expression ( $P < 0.0001$ ). These observations indicate that IL-1R1 is associated with the maturation of both Tfh and Tfr within lymphoid organs, and IL-1R2 upregulation is specific to *activated Tfr*.

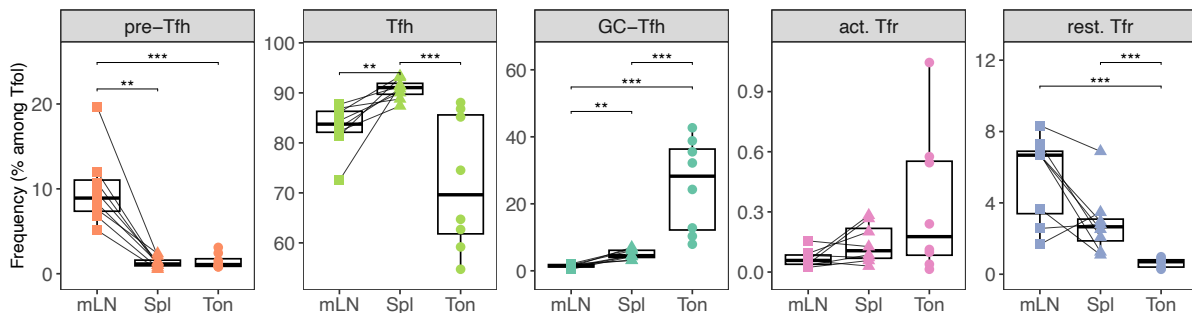


**Figure 25. Quantification of marker expression in Tfol clusters.**

Logicle-transformed MFI of selected markers across Tfol clusters is displayed in all 3 lymphoid organs (n = 24).

\*P < 0.05; \*\*P < 0.01; \*\*\*P < 0.001; \*\*\*\*P < 0.0001; ns: not significant by Mann-Whitney test.

We then calculated the frequency of all clusters in each lymphoid organ. In mLN, the frequencies of *pre-Tfh* and *resting Tfr* were significantly the highest (P < 0.001 compared to tonsils) while the ones of *GC-Tfh* and *activated Tfr* were the lowest (p < 0.001 and not significant, respectively compared to tonsils) (**Fig. 26**). The spleens were enriched in *Tfh* (P < 0.001 and P < 0.001 compared to mLN and tonsil respectively). As expected, tonsils had an enrichment of highly mature *GC-Tfh* and *activated Tfr* (P < 0.001 and not significant, respectively, compared to spleen).



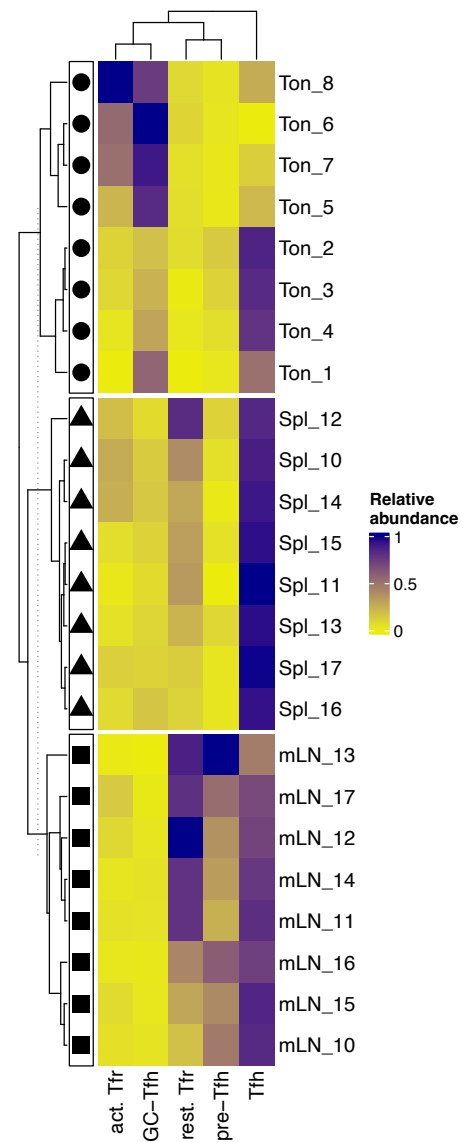
**Figure 26. Abundance of Tfol clusters across secondary lymphoid organs.**

Frequency of each cluster among CXCR5<sup>+</sup> Tfol, per organ (n = 8). \*P < 0.05; \*\*P < 0.01; \*\*\*P < 0.001; \*\*\*\*P < 0.0001; ns: not significant by Mann-Whitney test.

To estimate whether clusters co-varied at the sample level, we clustered them according to their relative abundance (**Fig. 27**). Samples with higher *GC-Tfh* abundance correlated with higher *activated Tfr* abundance, which was particularly noticeable in tonsils. When *GC-Tfh* lowered, *activated Tfr* did as well, unlike *Tfh* which were increased without affecting *resting Tfr*. On the other hand, *pre-Tfh* were more abundant in poorly activated mLNs, just like *resting Tfr* were. These results highlight a link between *pre-Tfh*, *Tfh*, *GC-Tfh* and *activated Tfr* while *resting Tfr* behave in more independent manner, preferentially represented in lymphoid organs with poor activation.

**Figure 27. Hierarchical clustering of the abundance of Tfol clusters across secondary lymphoid organs.**

Heatmap showing relative abundance of Tfol clusters in all samples, categorized according to the tissue of origin (shape). Square : mLN, triangle : spleen, cercle : tonsil.

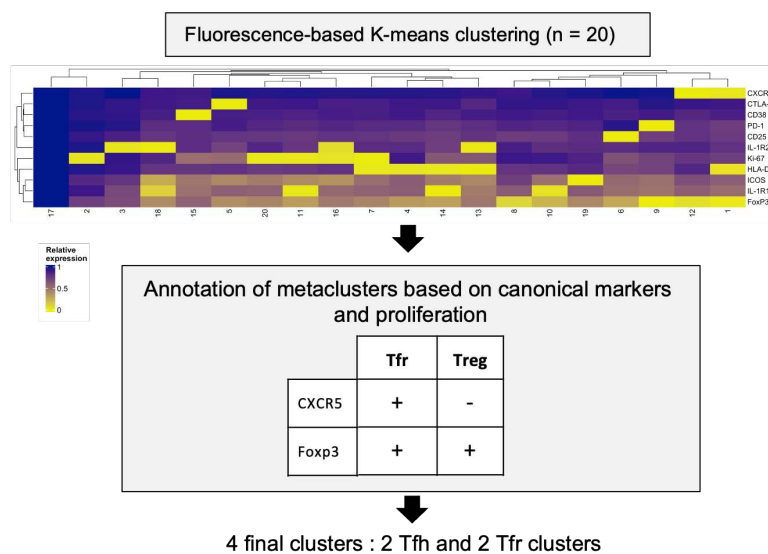


Taken together, our data underline the association between IL-1R1 expression and differential Tfol maturation across lymphoid organs, particularly linked to Tfr, indicating an important heterogeneity of this subset.



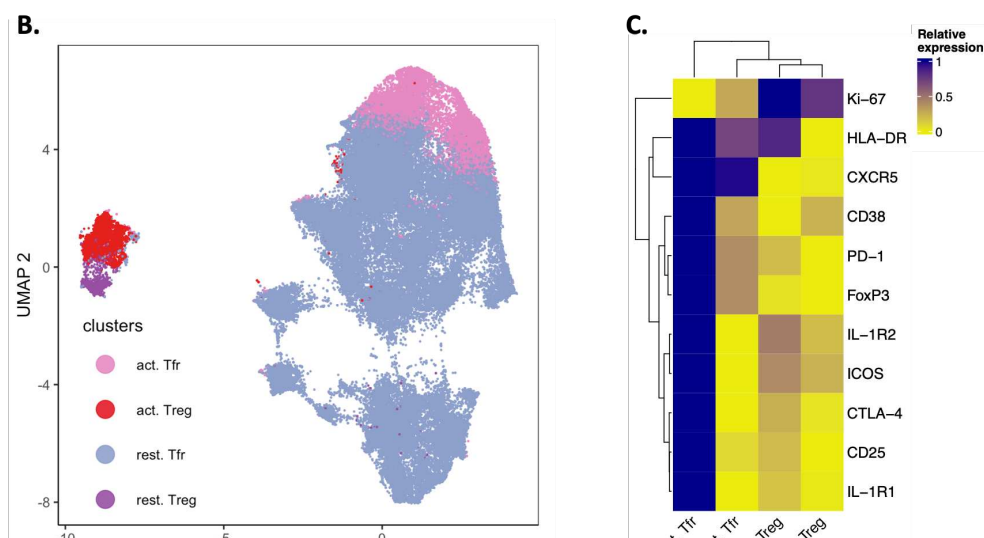
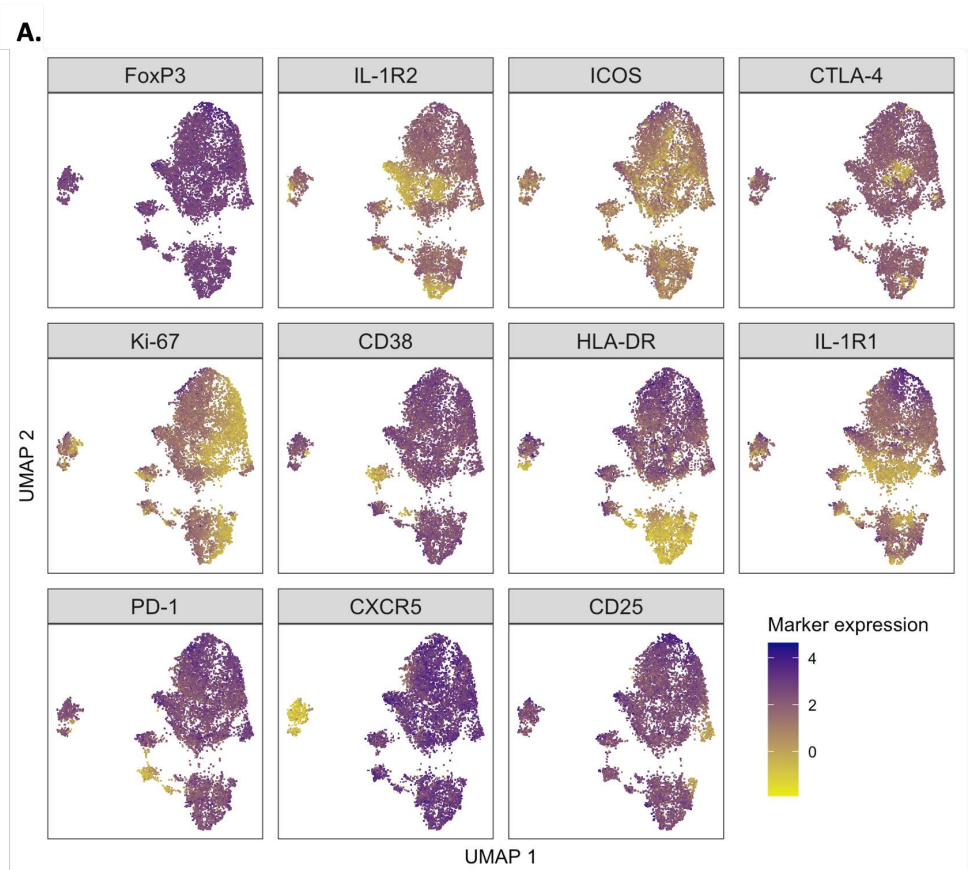
- D. IL-1Rs-expressing Tfr represent a distinct Tfr population with both suppressive and helper phenotype

To examine how Tfr subsets are phenotypically related to conventional Treg and how of IL-1Rs contribute to that phenotype, we explored regulatory T cell activation through unsupervised methods. Using the *ex vivo* expression of 11 markers among Foxp3<sup>+</sup> regulatory T cells from 3 lymphoid organs (n = 24), we chose an analysis strategy based on a first over-clustering step (n = 20) allowing the capture of all phenotypes including rare ones, followed by annotation of the 29 metaclusters based on Tfr and Treg canonical markers, as well as proliferation and activation markers (**Fig. 28**).



**Figure 28.** Analytical strategy for the identification of Tfh and Tfr clusters among CD4<sup>+</sup> Foxp3<sup>+</sup> cells.

We performed UMAP dimensionality reduction to visualize marker expression at the cell level (**Fig. 29A**). We identified 4 clusters, including Tfr and Treg subsets being heavily segregated upon CXCR5 differential expression (**Fig. 29B**). We found 2 Tfr clusters similar to those previously described: *resting Tfr* and *activated Tfr*, the latter displaying an increased expression of all markers, except CXCR5 and Ki-67 that were equally expressed (**Fig. 29C**). Also 2 Treg clusters were found and clustered together: *resting Treg* and *activated Treg*, which could notably be distinguished upon Ki-67, HLA-DR, PD-1, CTLA-4 and CD25 expression. While *resting Tfr* clustered in the same branch as Treg, *activated Tfr* branched apart.

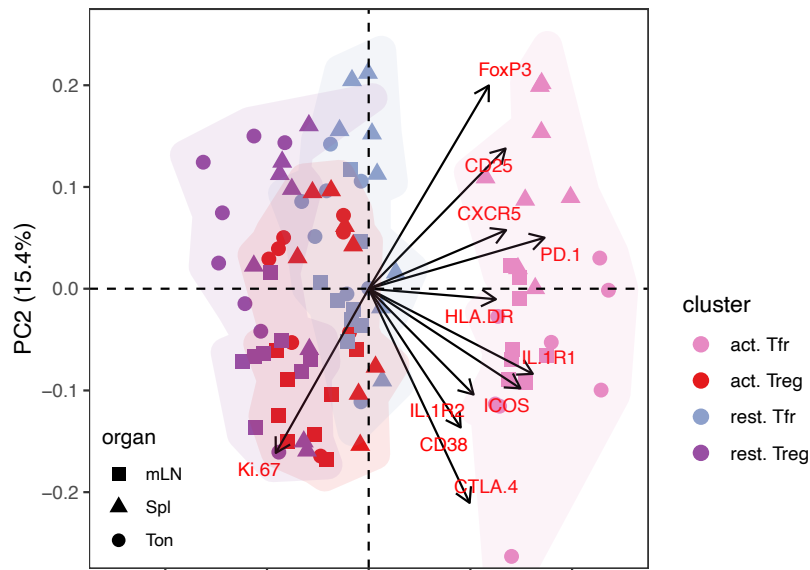


**Figure 29. Marker expression in regulatory  $CD4^+$   $Foxp3^+$  T cells defines 2 Treg and 2 Tfr clusters.**

(A) Dimensionally-reduced regulatory  $CD4^+$   $Foxp3^+$  T cells from 3 lymphoid organs represented as a UMAP and colored upon marker expression as logicle-transformed MFI. (B) Treg and Tfr clusters projection on the UMAP representation. (C) Heatmap showing relative marker expression of the identified clusters.

To evaluate the contribution of each marker to regulatory T cell heterogeneity, our 4 clusters underwent PCA. *Resting* and *activated Treg* partially overlapped, as expected (Fig. 30). Interestingly, *resting Tfr* showed an important overlap with *activated Treg*, underlining a related phenotype. However, *activated Tfr* strictly clustered separately

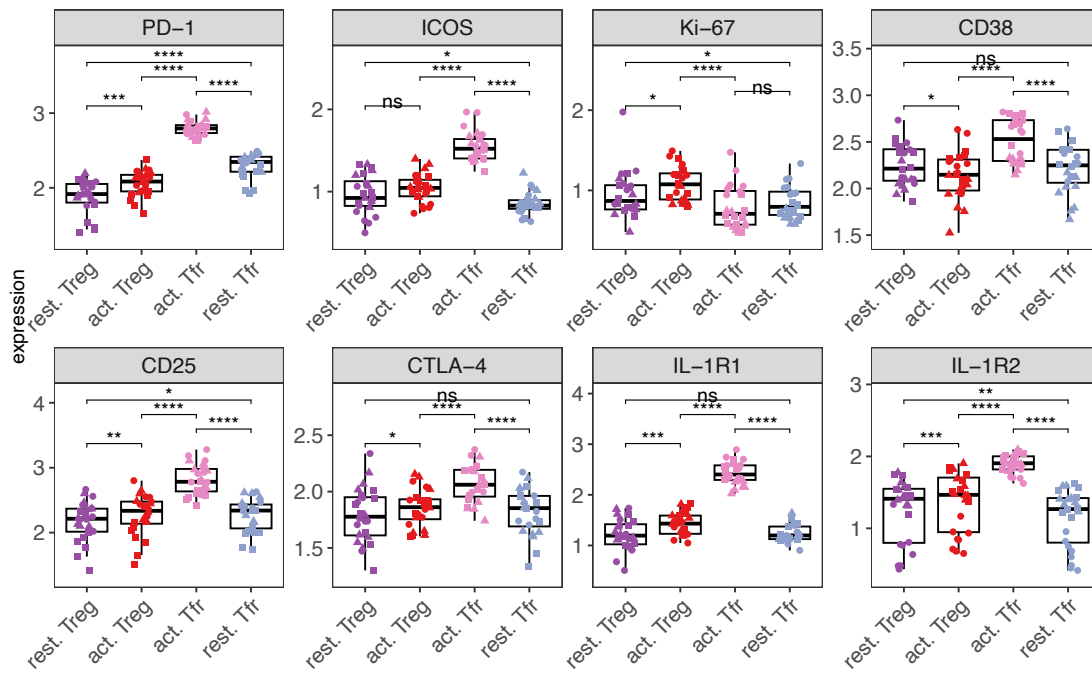
from others clusters, which was driven by all markers but Ki-67. Among those, IL-1R1, ICOS and PD-1 were the ones with higher contributions to this unique phenotype between regulation and activation.



**Figure 30. Activated Tfr phenotypically diverge from Treg and resting Tfr.**

PCA displaying 4 clusters (colours) across 3 lymphoid organs (shape) and the contribution of each marker to principal components is indicated with vectors.

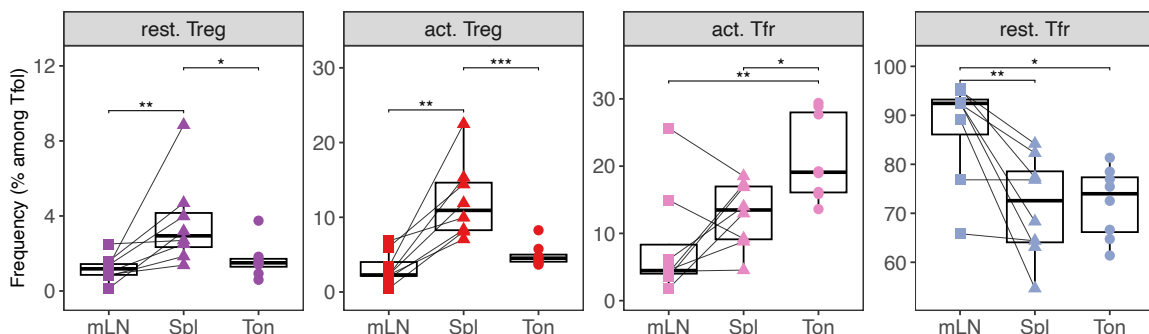
The quantification of marker expression confirmed the phenotype of *activated Treg* with significantly increased PD-1 ( $P < 0.001$ ), Ki-67 ( $P < 0.05$ ), CD25 ( $P < 0.01$ ) and CTLA-4 ( $P < 0.05$ ) compared to resting counterparts. Interestingly, activated Treg displayed increased IL-1R1 and IL-1R2 expression ( $P < 0.001$ ) compared to resting Treg (**Fig. 31**). Similarly, *activated Tfr* could be distinguished from resting counterparts by higher expression of PD-1, ICOS, CD38, CD25 and CTLA-4 ( $P < 0.0001$ ), accompanied by IL-1R1 and IL-1R2 expression ( $P < 0.0001$ ). Marker expression thus indicates that both Treg and Tfr activated phenotypes are associated with increased IL-1Rs expression. Of note, *resting Tfr* displayed fewer differences compared to *resting Treg*, notably regarding CD38, CTLA-4 and IL-1R1 expression (not significant). The lack of differences suggests that *resting Tfr* are phenotypically closer to conventional Treg.



**Figure 31. Quantification of marker expression in regulatory T cell clusters.**

Logicle-transformed MFI of selected markers across regulatory CD4<sup>+</sup> Foxp3<sup>+</sup> T cell clusters is displayed in all 3 lymphoid organs (n = 24).. \*P < 0.05; \*\*P < 0.01; \*\*\*P < 0.001; \*\*\*\*P < 0.0001; ns: not significant by Mann-Whitney test.

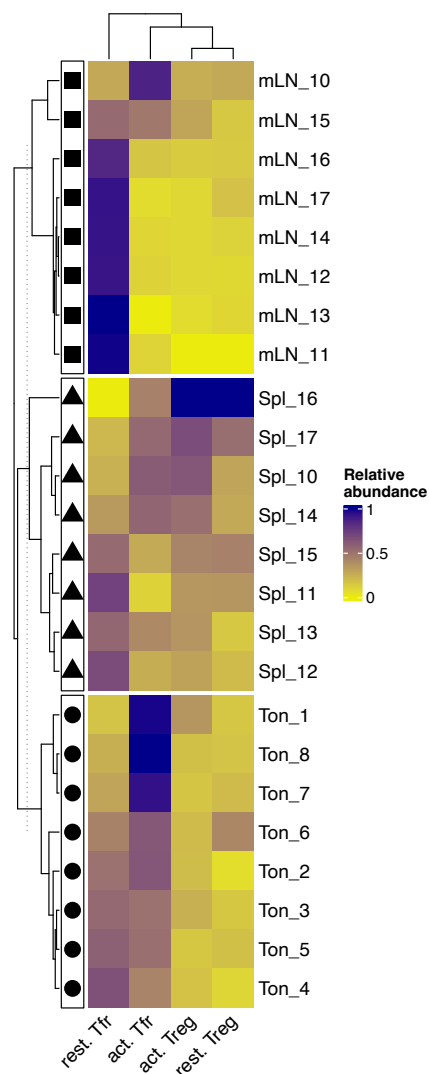
We then evaluated the abundance of each Foxp3<sup>+</sup> cell cluster in the different lymphoid organs (**Fig. 32**). Splens had a preferential abundance of both *resting Treg* (P < 0.01 and P < 0.05 compared to mLN and tonsil respectively) and *activated Treg* (P < 0.01 and P < 0.001 compared to mLN and tonsil respectively), while their frequency was low in tonsils and mLNs. On the other side, activated Tfr were significantly more abundant in tonsils compared to splens (P < 0.05) and mLNs (P < 0.01), following the same gradient of *GC-Tfh* activation shown in previously. On the contrary, *resting Tfr* were enriched in the lowly activated mLNs (P < 0.01 and P < 0.05 compared to splens and tonsils, respectively).



**Figure 32. Abundance of regulatory T cell clusters across secondary lymphoid organs.**

Frequency of each cluster among CXCR5<sup>+</sup> Tfol, per organ (n = 8). \*P < 0.05; \*\*P < 0.01; \*\*\*P < 0.001; \*\*\*\*P < 0.0001; ns: not significant by Mann-Whitney test.

To test whether clusters varied together at the sample level, we performed hierarchical clustering using their relative abundance (**Fig. 33**). The resulting heatmap highlighted that both Treg clusters co-varied with a preferential abundance in spleen, while *resting* and *activated Tfr* had very distinct patterns of abundance that did not cluster jointly. To sum up, our investigations emphasize the existence of a distinct Tfr population characterized by a higher expression of IL-1R1 and the co-expression of both suppressive markers (CTLA-4, PD-1) and co-stimulatory markers (ICOS, HLA-DR). This population is more abundant in lymphoid organs with highly active GC reaction and seems less related to conventional Treg, while the Tfr population displaying low expression of IL1Rs resembles Treg.

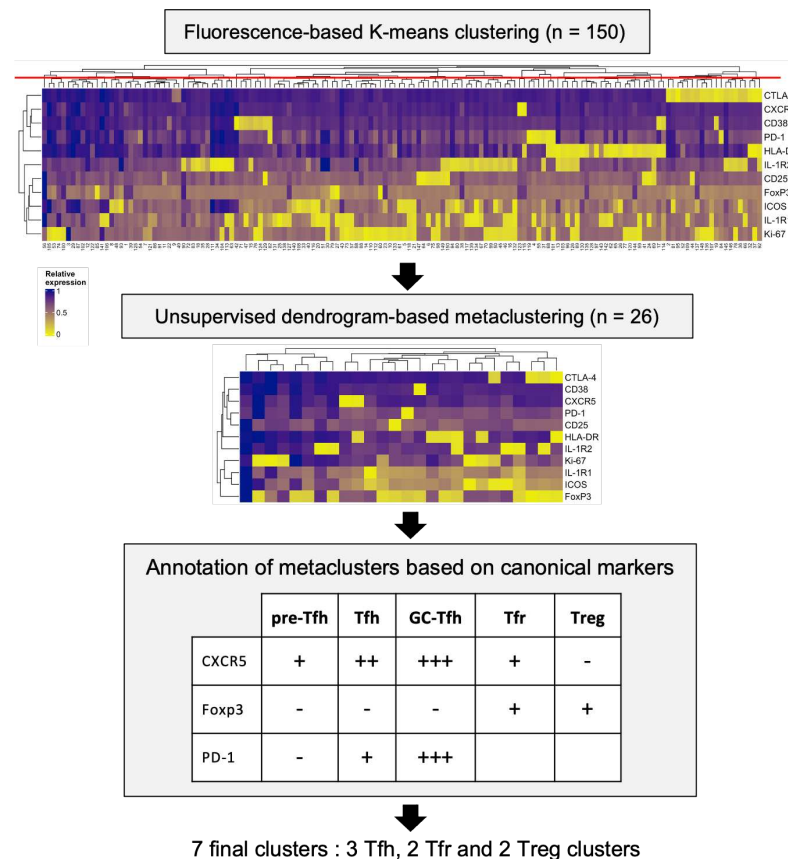


**Figure 33. Hierarchical clustering of the abundance of regulatory T cell clusters across lymphoid organs.**

Heatmap showing relative abundance of regulatory CD4<sup>+</sup> Foxp3<sup>+</sup> T cell clusters in all samples, categorized according to the tissue of origin (shape). Square : mLN, triangle : spleen, cercle : tonsil.

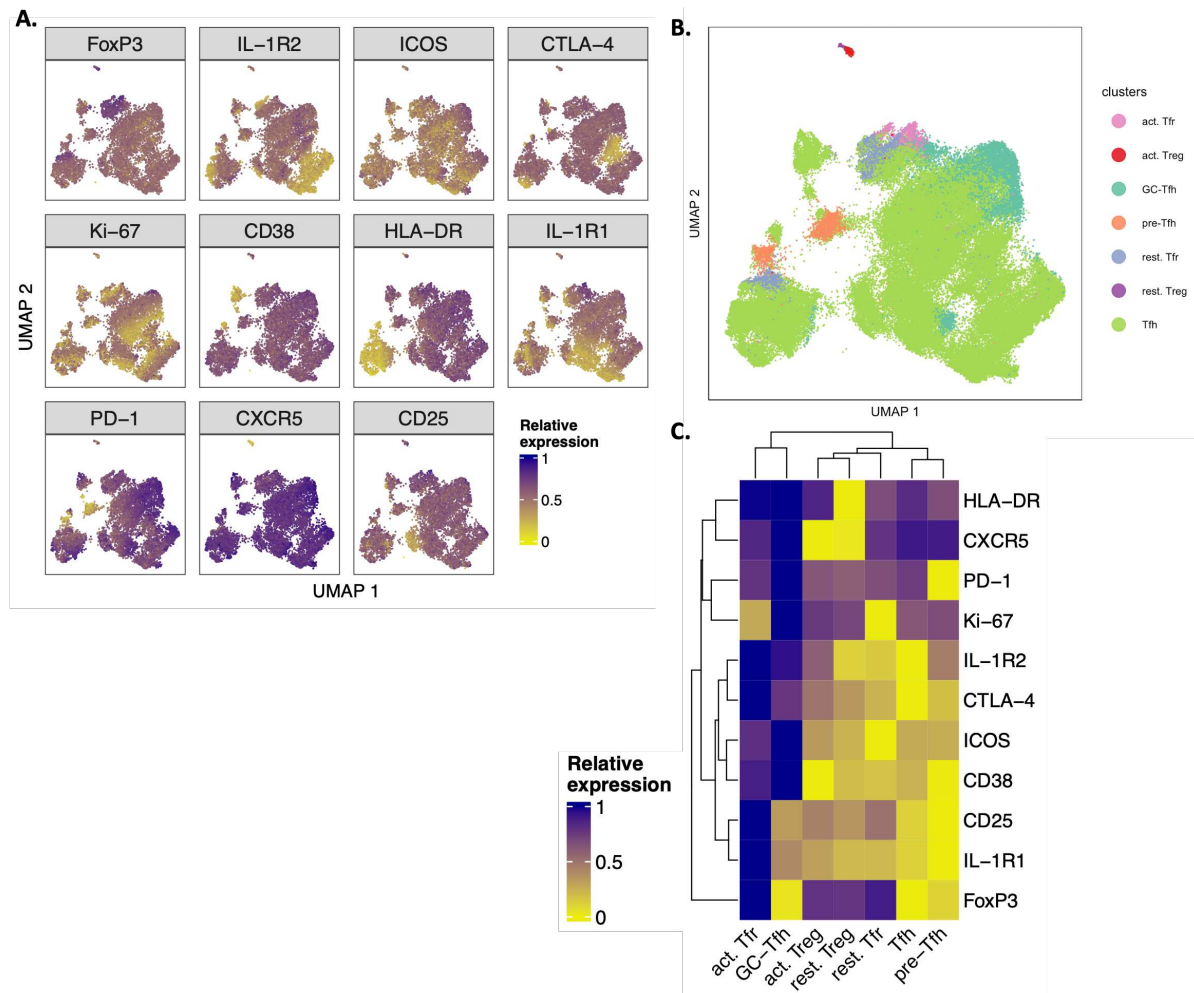
E. Trajectory inference predicts that Tfh transition to Tfr phenotype requires a predominant expression of IL-1R1 over IL-1R2

To explore the nature of the two distinct Tfr populations, we modeled *ex vivo* CD4<sup>+</sup> T cell trajectories from 3 lymphoid organs bearing distinct stages of Tfh and Tfr maturation. We built a metaorgan composed of CXCR5<sup>+</sup> cells (Tfh and Tfr), together with CXCR5<sup>-</sup> Foxp3<sup>+</sup> Treg, known to be a precursor of Tfr. Using the *ex vivo* expression of 11 markers among combined Tfh, Tfr and Treg from 3 lymphoid organs (n = 24), we chose an analysis strategy based on a first over-clustering step (n = 150) allowing the capture of all phenotypes including rare ones, followed by metaclustering upon cutting hierarchically-grouped clusters, and final annotation of the 26 metaclusters based on Tfh and Tfr canonical markers (**Fig. 34**).



**Figure 34.** Analytical strategy for the identification of Tfh, Tfr and Treg clusters among a joint metaorgan.

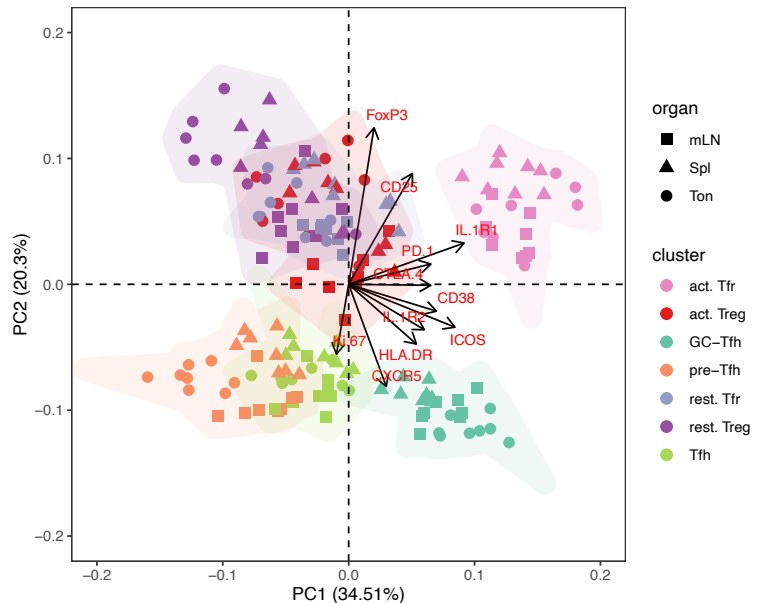
We performed UMAP dimensionality reduction to visualize marker expression at the cell level (**Fig. 35A**). We identified the same clusters as previously identified: *resting* and *activated Treg*, *resting* and *activated Tfr*, *pre-Tfh*, *Tfh* and *GC-Tfh* (**Fig. 35B**). In accordance with our previous results, *activated Tfr* phenotypically clustered with *GC-Tfh* while *resting Tfr* branched together with Treg clusters (**Fig. 35C**).



**Figure 35. Common identification and clustering of Tfol and regulatory CD4<sup>+</sup> T cells.**

(A) Dimensionally-reduced Tfol jointly with regulatory CD4<sup>+</sup> Foxp3<sup>+</sup> T cells from 3 lymphoid organs represented as a UMAP and colored upon marker expression as logicle-transformed MFI. (B) Tfh, Tfr and Treg clusters projection on the UMAP representation. (C) Heatmap showing relative marker expression of the identified clusters.

PCA of all clusters achieved the separation of Tfh clusters, which followed the *pre-Tfh* to *GC-Tfh* gradient, from Treg clusters, which presented a complete overlap with *resting Tfr*, suggesting a common origin (**Fig. 36**). *Activated Tfr* were placed apart, between regulatory clusters and *GC-Tfh*. IL-1R1 was confirmed as the most discriminant marker of *activated Tfr*, whereas IL-1R2 appeared to drive *GC-Tfh*, thus arguing for a differential phenotypic orientation linked to IL-1Rs. Of note, CD38 was situated in-between *act. Tfr* and *GC-Tfh*, indicating that these two subsets result from the differentiation of activated cells.



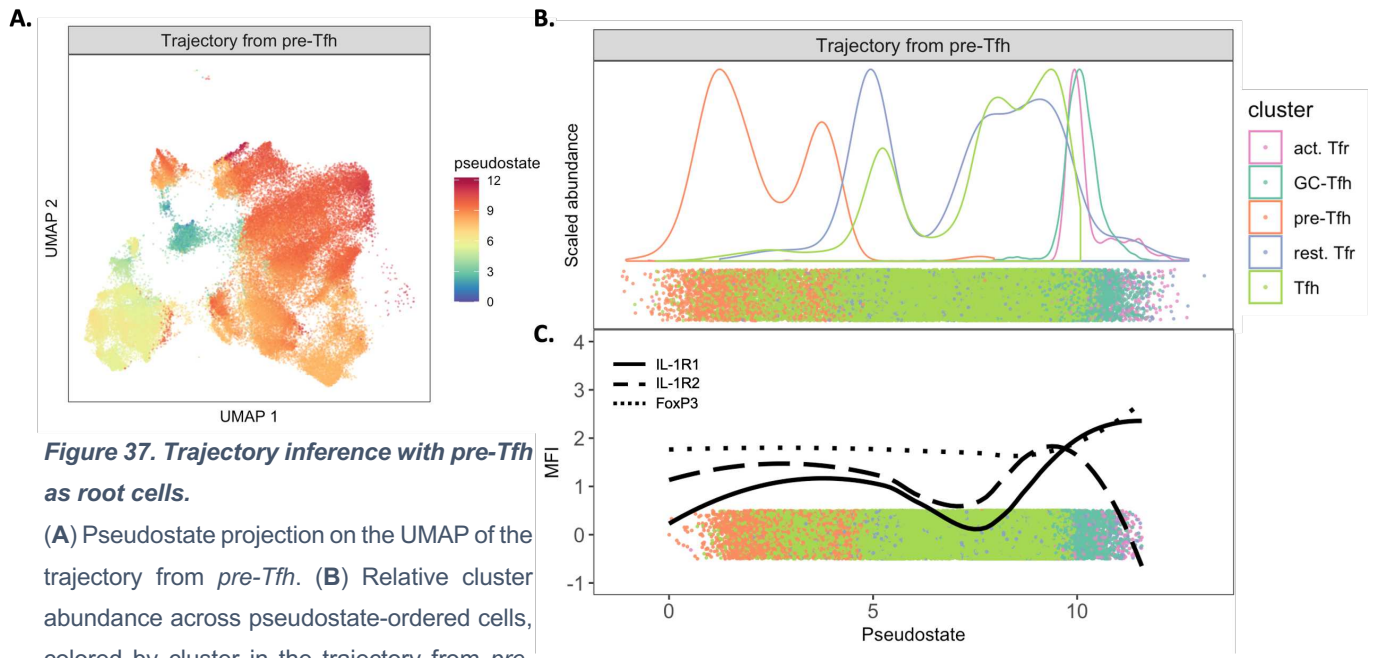
**Figure 36. Activated Tfr are phenotypically in-between GC-Tfh and Treg, while resting Tfr converge with Treg.**

PCA displaying 7 clusters (colours) across 3 lymphoid organs (shape) and the contribution of each marker to principal components is indicated with vectors.

Because Tfr can emerge from the differentiation of Tfh cells (Le Coz et al., 2023), we sought to determine how Tfh-to-Tfr transition was enabled in light of IL-1Rs expression. By defining *pre-Tfh* as root cells, we conducted trajectory inference and calculated a pseudostate value for each cell of the trajectory using the MFI of each marker (**Fig. 37A**). This strategy allowed us to order the cells and to project cluster abundance at each pseudostate point: *Tfh* were predicted to differentiate right after *pre-Tfh*, followed by *GC-Tfh* (**Fig. 37B**). *Resting Tfr* seemingly differentiated concomitantly with *Tfh*, whereas *activated Tfr* emerged together with *GC-Tfh* and completed their differentiation after them in a delayed fashion. This suggests that *GC-Tfh* may not be the terminal stage of Tfh maturation. Treg clusters were not involved in this trajectory. We then projected the average MFI of IL-1R1, IL-1R2 and FoxP3 on pseudostate-ordered cells (**Fig. 37C**). During *Tfh* to *GC-Tfh* transition, IL-1R1 and IL-1R2 expression is increased. Then, when *GC-Tfh* appear, IL-1R2 falls considerably, while IL-1R1 expression increases until the emergence of an *activated Tfr* population. Interestingly, the augmentation of Foxp3 expression, underlying the expansion of *activated Tfr*, began at a breakpoint where IL-1R1 expression ascended while IL-1R2

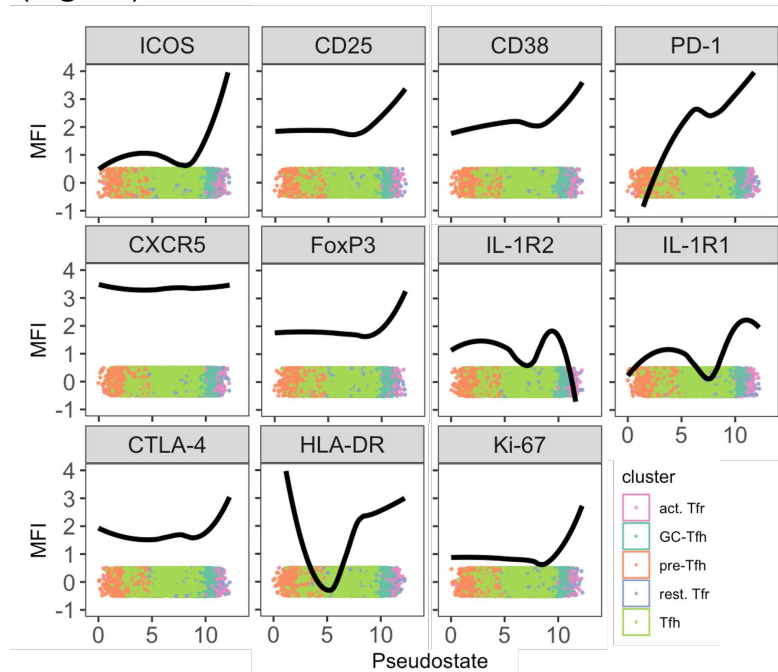


started decreasing. Thus, IL-1R1/IL-1R2 balance seems contribute to the transition to a Tfr phenotype.



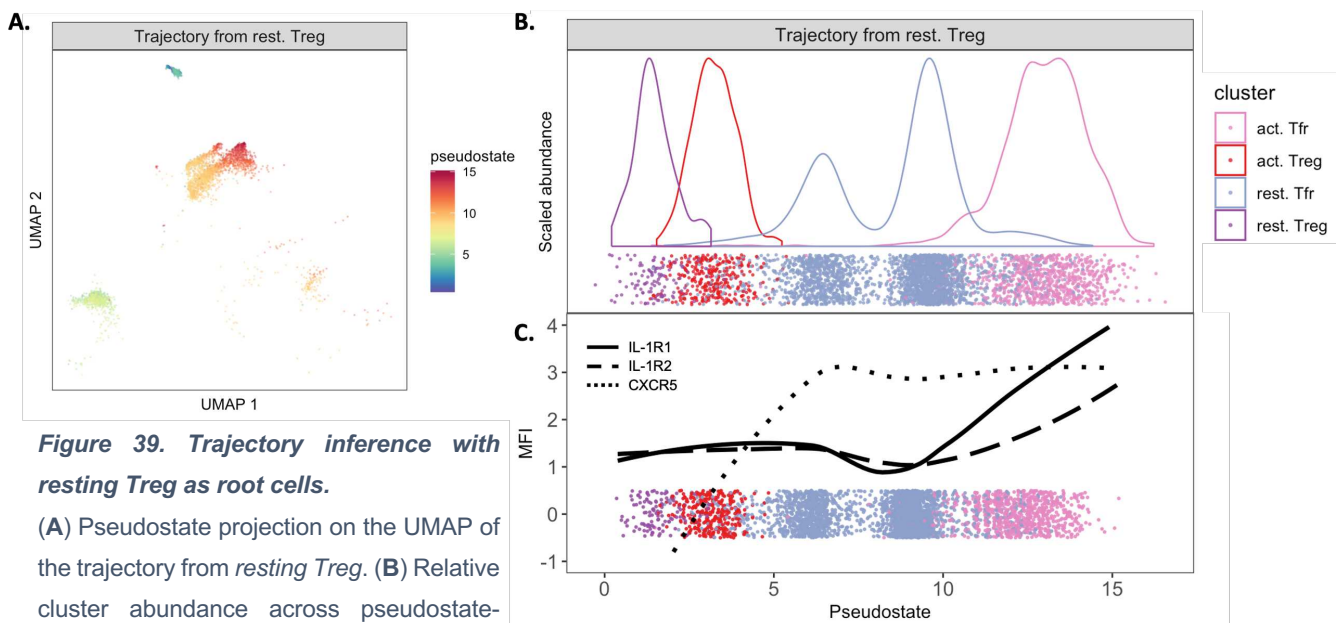
**Figure 37. Trajectory inference with pre-Tfh as root cells.** (A) Pseudostate projection on the UMAP of the trajectory from *pre-Tfh*. (B) Relative cluster abundance across pseudostate-ordered cells, colored by cluster in the trajectory from *pre-Tfh*. (C) Average IL-1R1, IL-1R2 and Foxp3 expression across pseudostate-ordered cells in the trajectory from *pre-Tfh*.

Interestingly, CD25, CD38 and CTLA-4 were also increased together with *activated Tfr* emergence (Fig. 38).



**Figure 38. Average marker expression along pseudostate-ordered cells in the trajectory from pre-Tfh.** Logicle-transformed MFI is projected as a plain curve.

Since Treg are classically considered as the main precursors of Tfr (Kumar et al., 2021; Wing et al., 2018) we also focus on Treg-to-Tfr transition and the role of IL-1Rs. When *resting Treg* were designated as root cells, trajectory inference passed through and ended with Tfr cells, without considering Tfh clusters (**Fig. 39A**). By ordering cells according to their pseudotime, *activated Treg* followed their resting counterparts, and resting Tfr seemed to emerge from *activated Treg* (**Fig. 39B**). However, constant coexpression of the IL-1R1 and IL-1R2 receptors appears to accompany the maintenance of the follicular phenotype with expression of CXCR5 (**Fig. 39C**). *Activated Tfr* appeared at the latest stage of Tfr maturation (**Fig. 39B**), concomitantly with a higher IL-1R1/IL-1R2 expression ratio (**Fig 39C**).



**Figure 39. Trajectory inference with resting Treg as root cells.**

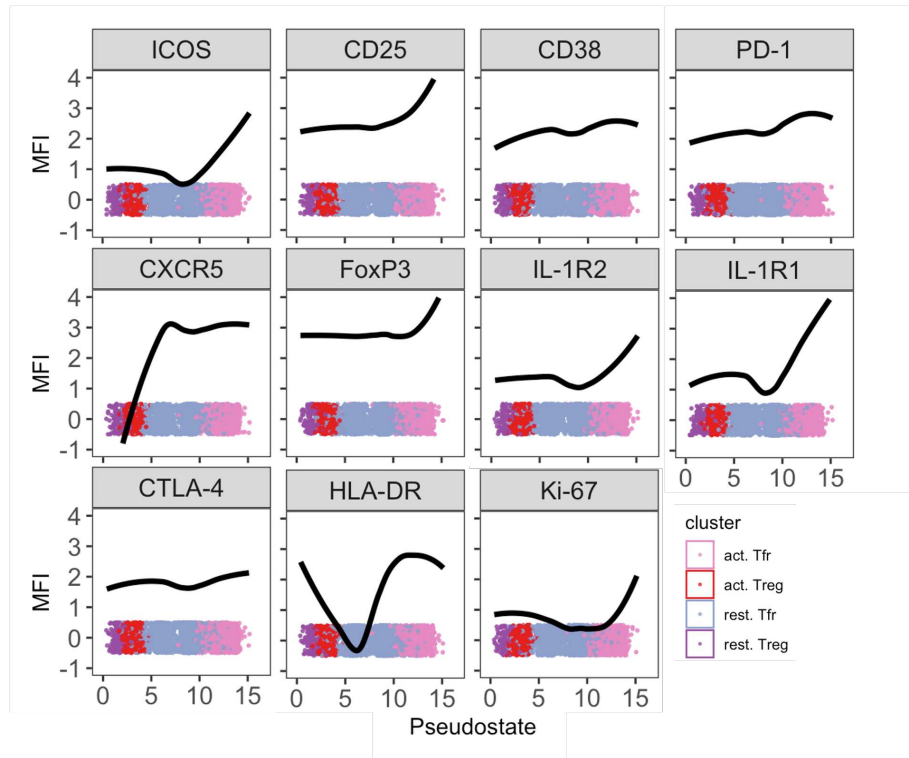
(A) Pseudotime projection on the UMAP of the trajectory from *resting Treg*. (B) Relative cluster abundance across pseudotime-ordered cells, colored by cluster in the trajectory from *resting Treg*. (C) Average IL-1R1, IL-1R2 and CXCR5 expression across pseudotime-ordered cells in the trajectory from *resting Treg*.

As well, higher expression of all other markers was observed in *activated Tfr* compared to *resting Tfr* (**Fig. 40**).

**Figure 40. Average marker expression along pseudostate-ordered cells in the trajectory from resting Treg.**

Logicle-transformed MFI is projected as a plain curve.

To conclude, *resting Tfr*, expressing low levels of IL-1R1 and IL-1R2, present a shared phenotype with Treg, with strong evidence for a

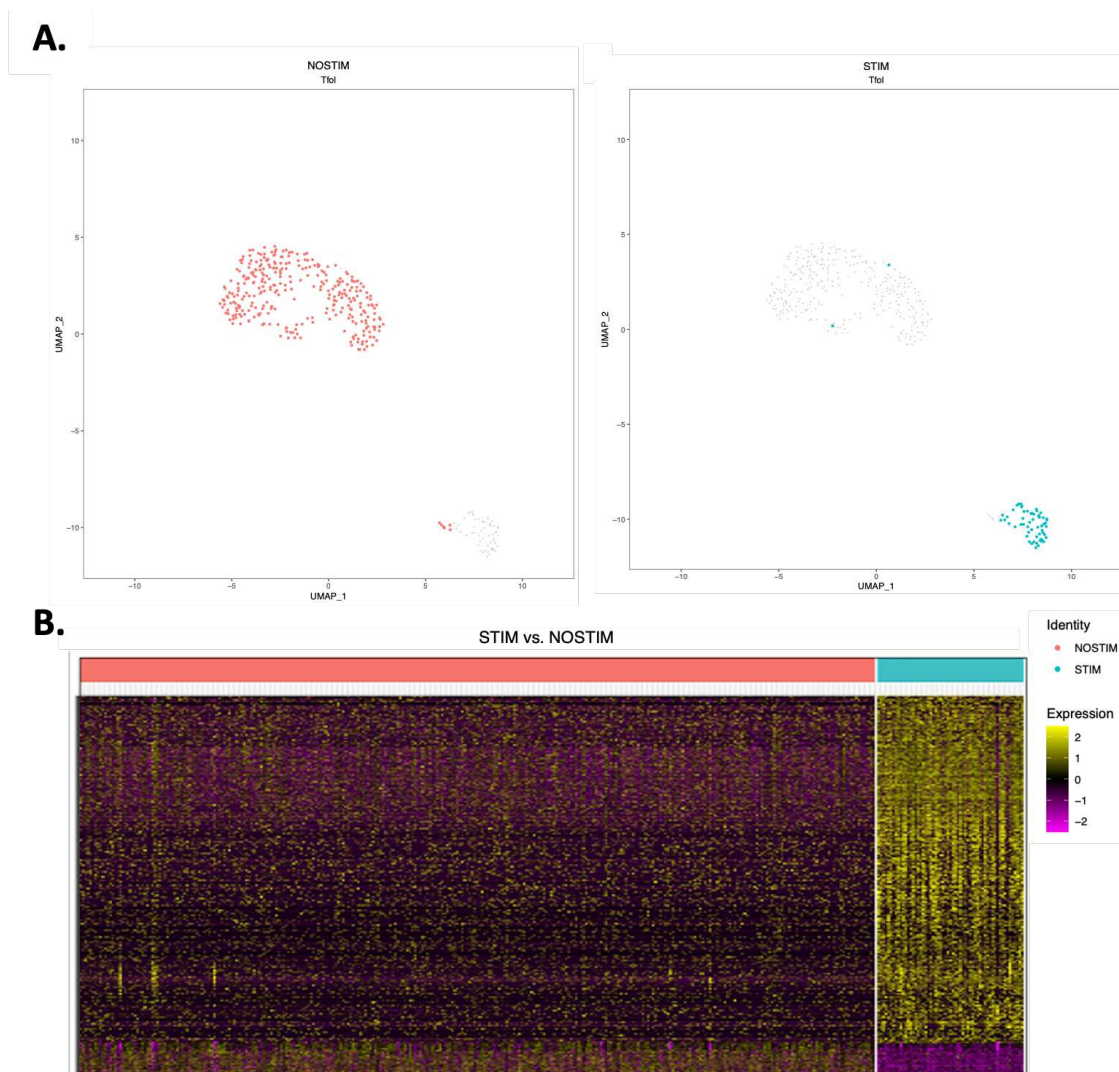


Treg-to-Tfr transition that is independent of IL-1Rs expression. However, IL-1R1<sup>hi</sup> *activated Tfr* display a phenotype that is closer to *GC-Tfh* while retaining suppressor markers' expression, and their emergence is compatible with a Tfh origin, without excluding a potential maturation from *resting Tfr*.

## PART II. TFOL ACTIVATION IS ENHANCED BY IL-1B IN VITRO UPON EXPRESSION OF IL-1Rs

- A. Tfol maturation is accompanied by the upregulation of IL-1R1 and its downstream signaling

To confirm that Tfol activation was indeed accompanied by IL-1 $\beta$  signaling, we performed *in vitro* cell activation with anti-CD3/CD28 beads. Because tonsils are SLO with the highest proportion of Tfol, we stimulated tonsillar cell suspensions from 2 donors. After 24 hours of *in vitro* T cell stimulation, live tonsillar lymphoid cells were sorted to perform single-cell RNA sequencing (scRNAseq, n = 2).



**Figure 41. Dimensionality reduction and differential analysis of Tfol from scRNA sequenced tonsillar cell cultures.**

(A) UMAP dimensionality reduction of CXCR5-expressing Tfol following 24-hour stimulation (STIM) or no stimulation (NOSTIM) from whole tonsillar lymphoid cultures, determined by scRNAseq (n = 2). (B) Heatmap showing marker expression among differentially expressed genes (DEG) between STIM and NOSTIM.

We selected CXCR5<sup>+</sup> Tfol to perform dimensionality reduction, which discriminated stimulated (STIM) from non-stimulated (NOSTIM) conditions (Fig. 41A). Differential analysis between the two conditions highlighted hundreds of upregulated genes during stimulation, whereas fewer genes were shown to be downregulated (Fig. 41B).

Volcano plot representation features each dot as a gene, which magnitude of expression is expressed as a fold change (log<sub>2</sub>-transformed) and statistically evaluated via the p-value (-log<sub>10</sub>-transformed). Among upregulated genes, we could find genes involved in follicular T cell proliferation and early activation such as *IL2RA* (CD25), *CD69*, *NME1*, *TNFRSF9* (4-1BB), *TNFRSF18* (GITR) (Szabo et al., 2019) (Fig. 42A). On the contrary, downregulated genes comprised *IL7R* (CD127) and *S100A4* which are associated to a resting T cell phenotype, as well as *ITM2B* known to be repressed by *BCL6* (Baron et al., 2015). Functional enrichment analysis using all upregulated genes revealed a significant enrichment of pathways linked to T cell proliferation and function: T cell receptor signaling and apoptosis, IL-2 and NF-κB pathways, as well as Myc and TNF-α inflammatory pathways (Fig. 42B).

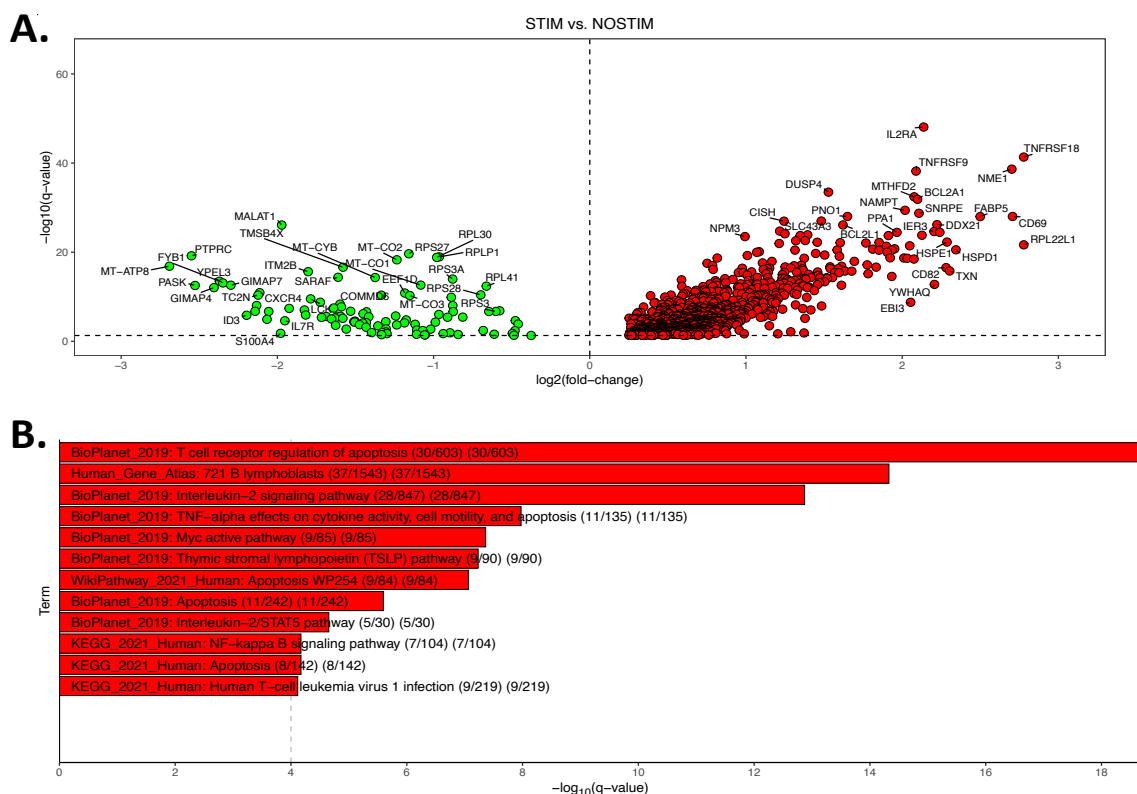
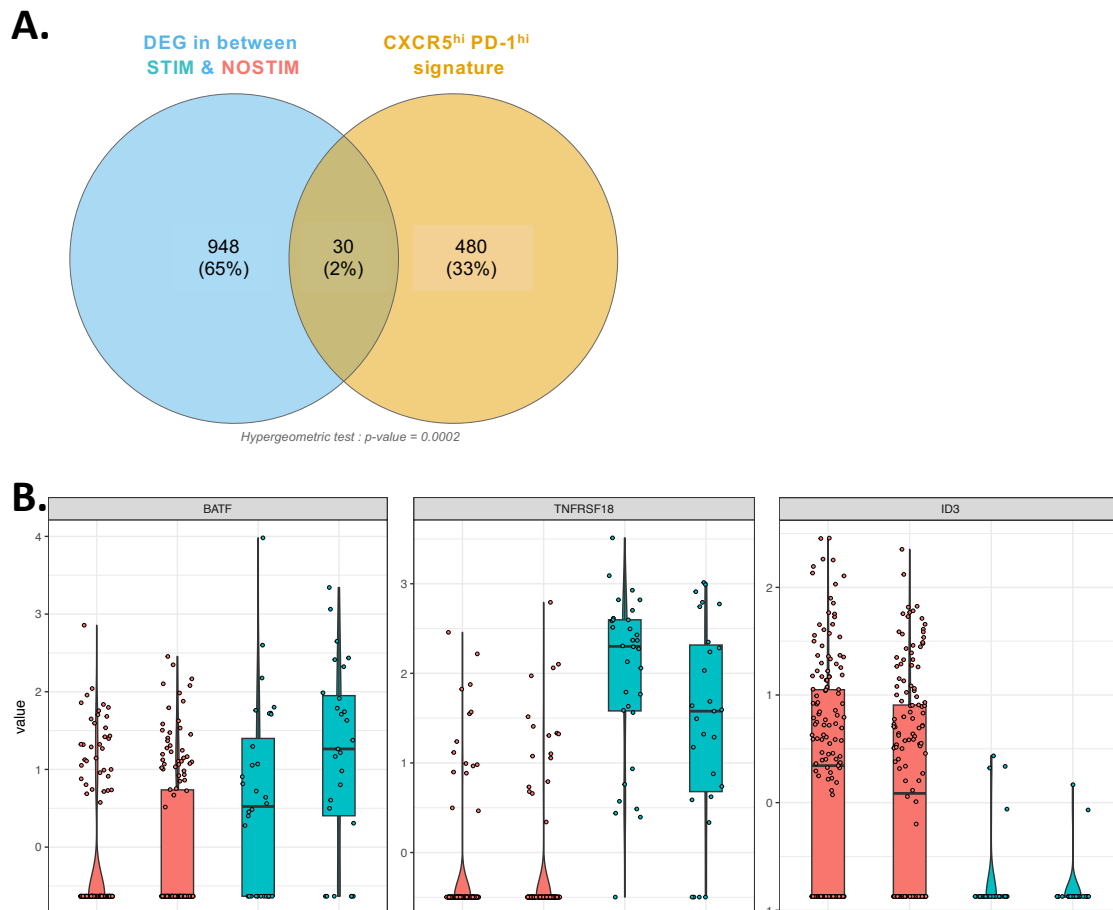


Figure 42. Most significant DEG in stimulated versus unstimulated Tfol and their functional enrichment.

(A) Volcano plot highlighting most significant upregulated genes in STIM (red) and in NOSTIM (green) conditions. (B) Functional enrichment plot displaying enriched pathways using upregulated genes in STIM condition.

To confirm that *in vitro* stimulation led to Tfol maturation towards a more differentiated stage such as GC-Tfh, we used differentially expressed genes (DEG) between STIM

and NOSTIM conditions and compared them to a GC-like CXCR5<sup>hi</sup> PD-1<sup>hi</sup> signature (Hill et al., 2021). Among the 510 genes forming the signature, 30 were significantly ( $P = 0.0002$ ) overlapping with DEG (Fig. 43A), 27 of them being upregulated (not shown). Notably, *TNFRSF18* (GITR) and *BATF*, known for promoting Tfh functions (Sahoo et al., 2015), were significantly increased in STIM while *ID3*, a repressor of Tfh differentiation (Liu et al., 2014), was diminished (Fig. 43B).

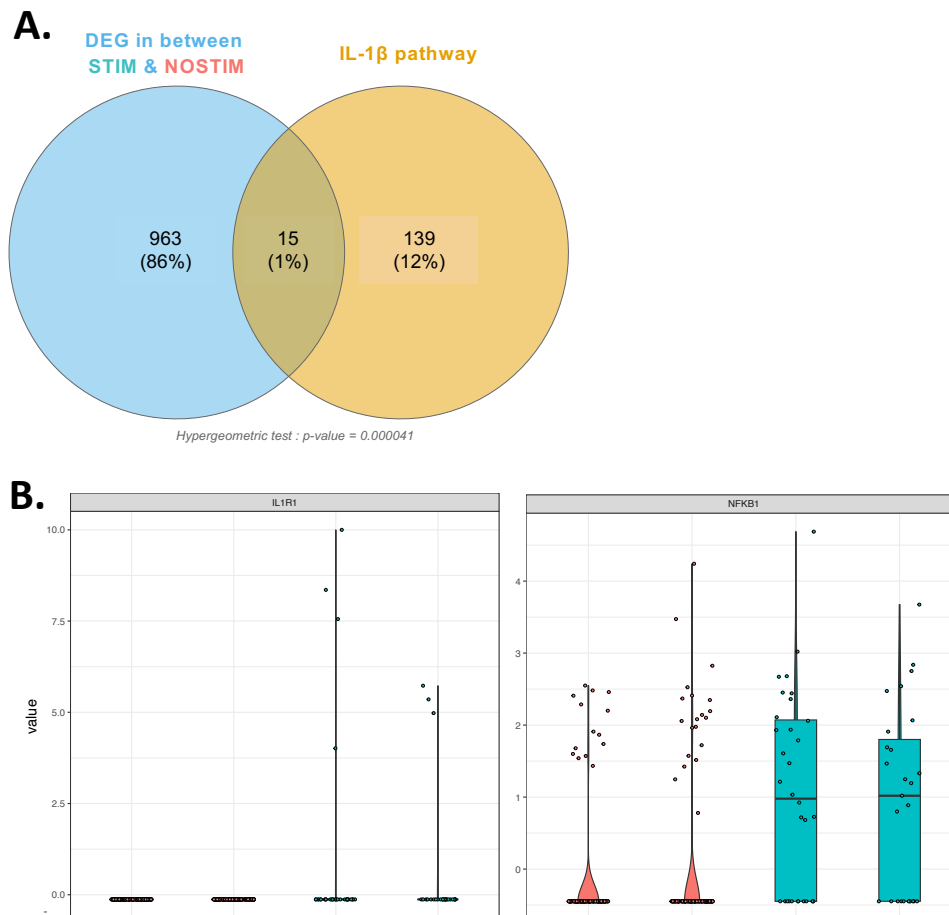


**Figure 43. Overlap between DEG and a GC-like signature during Tfol stimulation.**

(A) Statistical overlap between DEG and the genes from a CXCR5<sup>hi</sup> PD-1<sup>hi</sup> GC-like signature. The number of genes and their overall proportion is are indicated. (B) Boxplots showing gene expression of *BATF*, *TNFRSF18* and *ID3* between conditions, per cell.

Given the skewing of Tfol towards a GC-like phenotype induced by in vitro stimulation protocol, we tested whether DEG overlapped with IL-1 $\beta$  pathway. We constituted a 169-gene list of this pathway by searching public data bases (WikiPathways, Reactome, KEGG pathway) for all genes involved in sensing, processing, signaling and regulation of IL-1 $\beta$  and its direct targets. Among the 169 genes composing the pathway, 15 were significantly found ( $P = 0.000041$ ) among DEG (Fig. 44A), and all

of them were upregulated (not shown). Interestingly, *IL1R1* significantly augmented during stimulation together with *NFKB1* (NF- $\kappa$ B), a key downstream mediator of IL-1 $\beta$  signaling, asserting that IL-1 $\beta$  is directly involved in T<sub>fh</sub> maturation through NF- $\kappa$ B signaling following IL-1R1 agonism (**Fig. 44B**).



**Figure 44. Overlap between DEG and IL-1 $\beta$  pathway during T<sub>fh</sub> stimulation.**

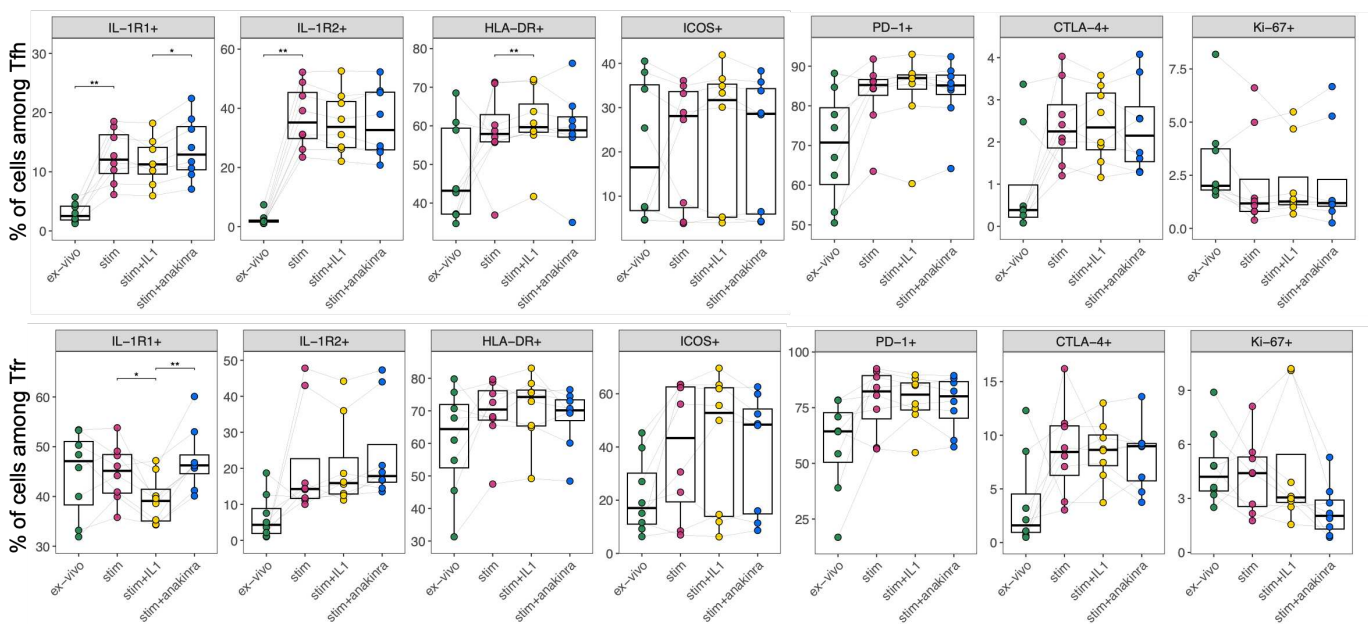
(A) Statistical overlap between DEG and the genes from IL-1 $\beta$  pathway. The number of genes and their overall proportion is indicated. (B) Boxplots showing gene expression of *IL1R1* and *NFKB1* between conditions, per cell.

**B. IL-1 $\beta$  potentiates T<sub>fh</sub> and T<sub>fr</sub> activation and induces negative feedback of surface IL-1R1 expression**

To assess the phenotypic impact of IL-1 $\beta$  on T<sub>fh</sub> and T<sub>fr</sub>, we cultured total tonsillar lymphoid cells (n = 8) for 24 hours and stimulated T cells with anti-CD3/CD28 beads alone or with IL-1 $\beta$  or Anakinra (IL-1 $\beta$  inhibitor). The phenotype of stimulated T<sub>fh</sub> and T<sub>fr</sub>, compared to *ex vivo* counterparts, was evaluated using multiparameter flow cytometry. First, the proportion of T<sub>fh</sub> expressing both IL-1R1 and IL-1R2 at the cell surface significantly increased with stimulation compared to *ex vivo*, in line with

transcriptomics results ( $P < 0.01$ ) (**Fig. 45**, top). Although the addition of IL-1 $\beta$  did not significantly change these proportions, its inhibition through Anakinra increased IL-1R1+ Tfh ( $P < 0.05$ ), which proved that IL-1 $\beta$  is produced *in vitro* during stimulation. Also, stimulation of Tfh with IL-1 $\beta$  led to an augmented proportion of HLA-DR-expressing cells ( $P < 0.01$ ) that was reversed by Anakinra. The same tendencies were observed with markers of activation and proliferation such as ICOS, PD-1, CTLA-4 and Ki-67, but did not reach significance threshold.

Conversely, Tfr did not exhibit significant IL-1Rs variations upon stimulation, potentially due to a high expression at the steady state (**Fig. 45**, bottom). IL-1R1+ Tfr were significantly diminished with IL-1 $\beta$  compared to control ( $P < 0.05$ ), and Anakinra restored normal levels ( $P < 0.01$ ). Activation and proliferation markers (HLA-DR, ICOS, CTLA-4 and Ki-67), although not significant, varied with a similar pattern as Tfh with IL-1 $\beta$  promoting their expression while Anakinra attenuated these variations.



**Figure 45. IL-1 $\beta$  potentiates tonsillar Tfh and Tfr activation from whole lymphoid culture.**

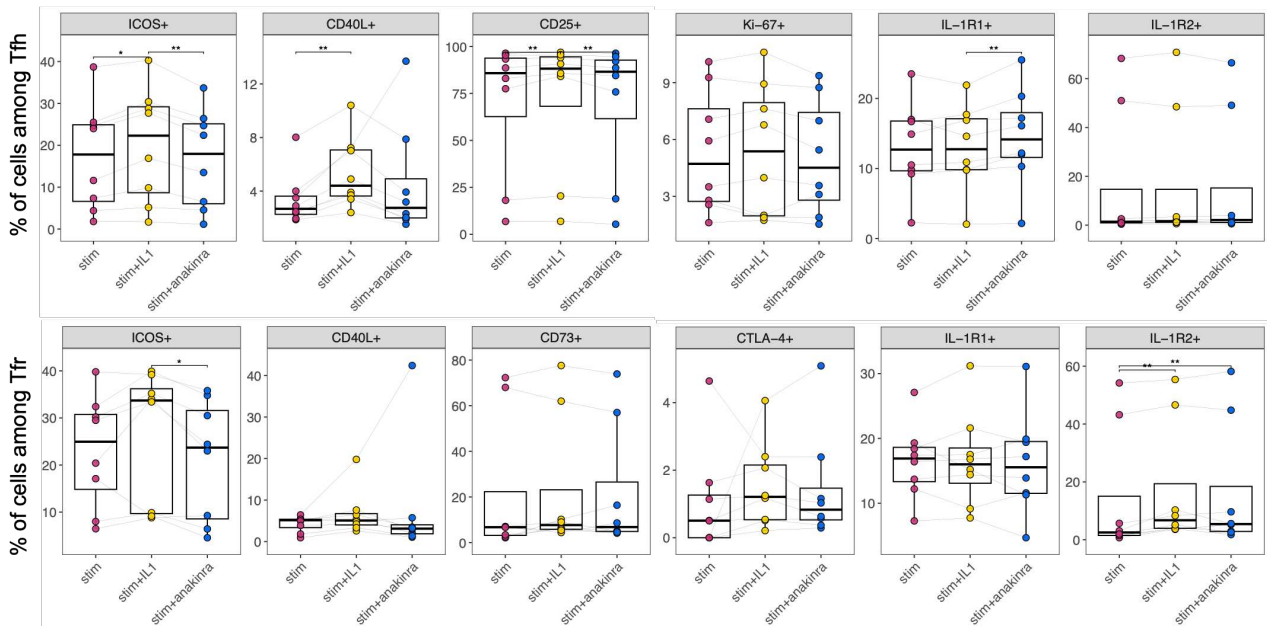
Percentage of positive cells for selected markers displayed as individual boxplots among Tfh (left) and Tfr (right) from while tonsillar lymphoid cultures compared to *ex vivo* ( $n = 8$ ). \* $P < 0.05$ ; \*\* $P < 0.01$  by paired Wilcoxon test.

Given the presence of several lymphoid and myeloid cell types in a whole cell culture of tonsils, we sought to determine whether the effects of IL-1 $\beta$  on Tfol were directly or indirectly mediated by other cells that are sensible to IL-1 $\beta$ . To achieve that, we sorted live CD4 $^+$  CXCR5 $^+$  Tfol and cultured them for 24 hours with stimulation alone or with IL-1 $\beta$  or Anakinra. Flow cytometry phenotyping of Tfh confirmed that IL-1 $\beta$  induces the expression ICOS ( $P < 0.05$ ), CD40L ( $P < 0.01$ ) and CD25 ( $P < 0.01$ ), while Anakinra



abolished this functional phenotype (ICOS and CD25 :  $P < 0.01$ ) (**Fig. 46**, top). IL-1R1 did not vary upon IL-1 $\beta$  condition, but Anakinra strikingly allowed a higher abundance of IL-1R1 $^+$  Tfh ( $P < 0.01$ ), which interrogates the ability of stimulated Tfol to produce IL-1 $\beta$ . Similarly, the proportion of functionally activated Tfr seemed increased with IL-1 $\beta$  regarding ICOS, CD40L and CTLA-4 patterns, whereas Anakinra re-established normal levels (ICOS :  $P < 0.05$ ) (**Fig. 46**, bottom). Despite no modifications linked to IL-1R1 $^+$  Tfr, IL-1R2 counterparts were augmented with IL-1 $\beta$  ( $P < 0.01$ ) and Anakinra partially blocked this effect, which appears as a retro control mechanism as a result of excessive IL-1 $\beta$ .

Taken together, our results underline the ability of IL-1 $\beta$  to directly support Tfh and Tfr



**Figure 46. IL-1 $\beta$  directly activates Tfh and Tfr in tonsillar Tfol-sorted culture.**

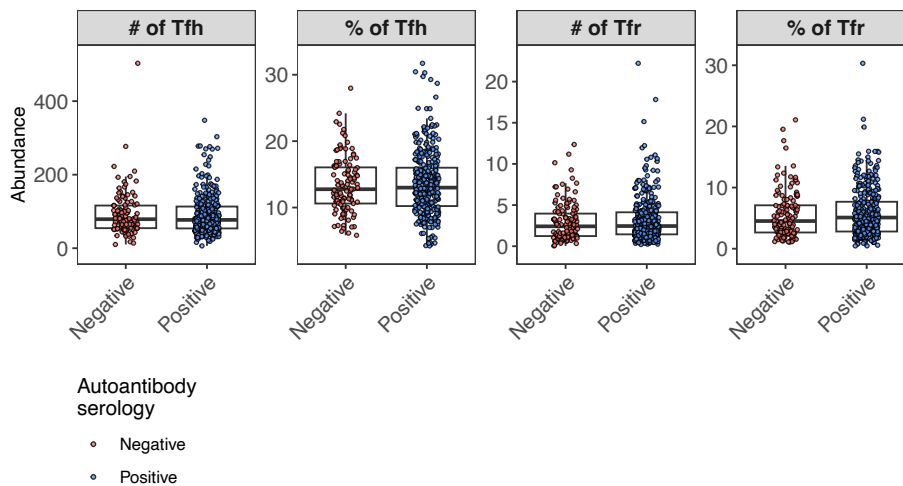
Percentage of positive cells for selected markers displayed as individual boxplots among Tfh (left) and Tfr (right) from sorted tonsillar Tfol-sorted culture ( $n = 8$ ). \* $P < 0.05$ ; \*\* $P < 0.01$  by paired Wilcoxon test.

maturation through an increased sensing mediated by IL-1R1. In response to IL-1 $\beta$  exposure, a negative feedback is noted via IL-1R1 downregulation, while Tfr specifically had the propensity to upregulate IL-1R2.

### PART III. ASSOCIATION BETWEEN IL-1 $\beta$ DYSREGULATION AND BOTH ABUNDANCE AND FUNCTION OF T<sub>fol</sub> IN AUTOIMMUNITY

#### A. Circulating T<sub>fol</sub> and their related cytokines associate with IL-1 $\beta$ increase during autoantibody production

Because IL-1 $\beta$  shapes the maturation and differentiation of T<sub>fol</sub> through differential expression of IL-1Rs, we wondered whether abnormal IL-1 $\beta$  conditions could lead to pathological antibody production mediated by T<sub>fh</sub> and T<sub>fr</sub>. To address this question, we made use of a cohort of patients with different autoimmune and autoinflammatory diseases, as well as healthy donors, for which blood samples were collected to perform flow cytometry immunophenotyping, cytokine dosage, autoantibody detection and bulk transcriptomics on sorted effector (T<sub>EFF</sub> : CD127<sup>+</sup> CD25<sup>-</sup>) and regulatory (T<sub>REG</sub> : CD127<sup>-</sup> CD25<sup>+</sup>) CD4<sup>+</sup> T cells (Lorenzon et al., 2018). Overall, autoantibody serology was distributed as follows: negative (n=152) and positive (n=327). Within the negative group were included: ankylosing spondylitis (n=30), antiphospholipid syndrome (n=3), Behcet (n=14), Crohn (n=2), GPA (n=8), osteoarthritis (n=17), RA (n=4), SLE (n=3), T1D (n=24), T2D (n=12), Takayasu arteritis (n=7), SjS (n=1) and healthy volunteers (n=28).

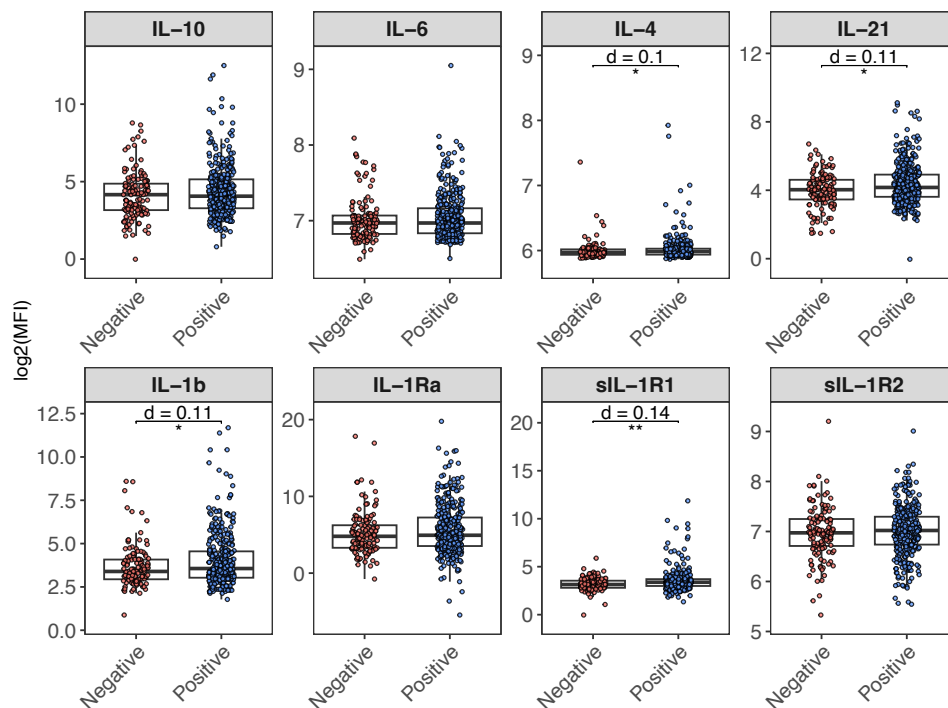


**Figure 47. Seropositive and seronegative individuals display similar cT<sub>fh</sub> and cT<sub>fr</sub> abundance overall.**

Flow cytometry quantification of the absolute number of circulating T<sub>fh</sub> and T<sub>fr</sub> and their frequencies (T<sub>fh</sub> : CXCR5<sup>+</sup> cells among CD3<sup>+</sup> CD4<sup>+</sup> T cells; T<sub>fr</sub> : CXCR5<sup>+</sup> cells among FoxP3<sup>+</sup> CD127<sup>-</sup> Treg), according to autoantibody serology of healthy and diseased individuals.

Within the positive group were included: ankylosing spondylitis (n=26), antiphospholipid syndrome (n=20), Behcet (n=24), Crohn (n=8), GPA (n=6), osteoarthritis (n=30), RA (n=86), SLE (n=30), T1D (n=35), T2D (n=18), Takayasu arteritis (n=15), SjS (n=16) and healthy volunteers (n=29). On a first disease-wide approach, we only segregated individuals upon their serology status to focus on common features involved in autoantibody production, which is a hallmark of ongoing GC activity. Hence, we calculated the abundance of circulating Tfh and Tfr (**Fig. 47**). No significant difference was observed between positive and negative conditions regarding the absolute number or percentage of Tfol populations.

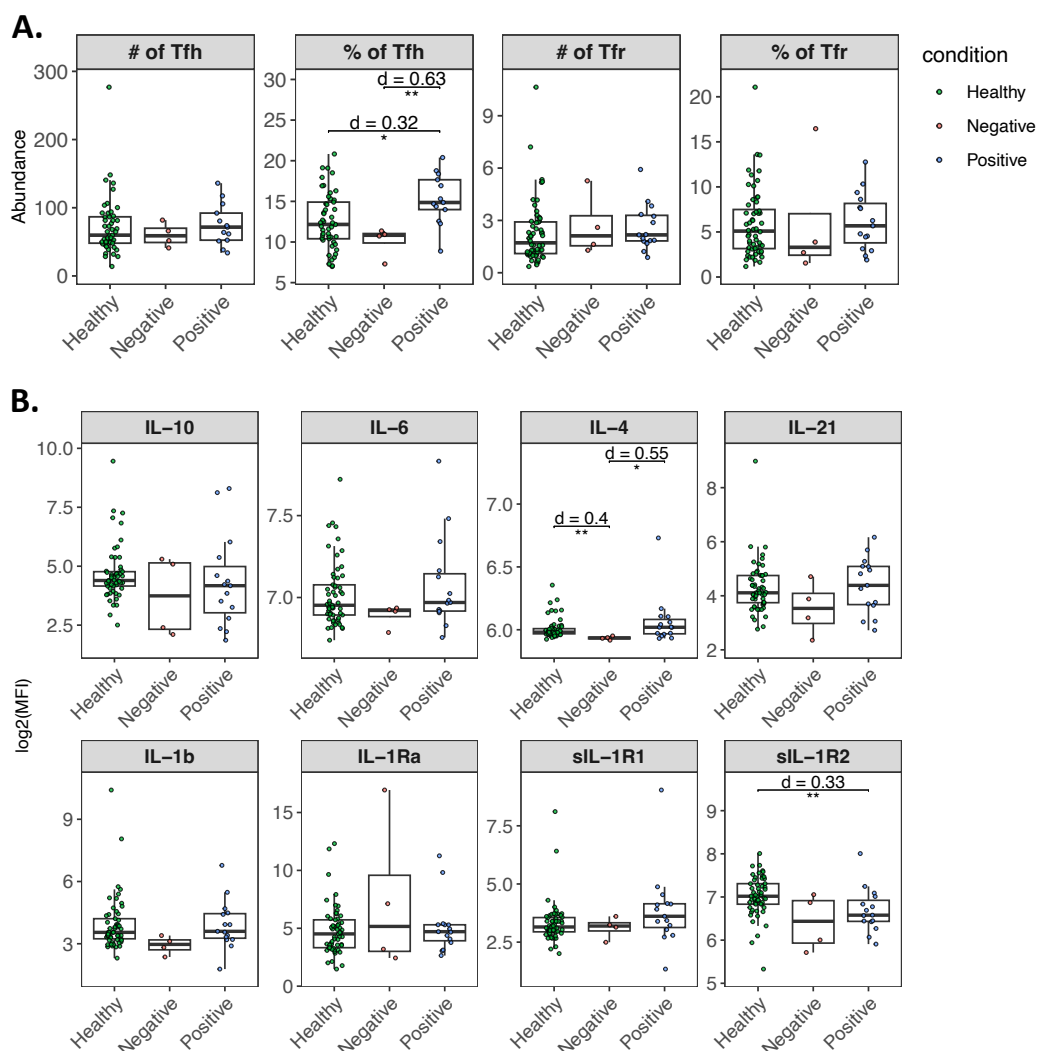
However, cytokine quantification evidenced a significant increase of circulating IL-4 and IL-21 in autoantibody-positive individuals ( $P < 0.05$ ;  $d = 0.1$  and  $d = 0.11$ , respectively), which are indicators of ongoing Tfh activity (**Fig. 48**). In a similar fashion, IL-1 $\beta$  was increased in the autoantibody-positive individuals ( $P < 0.05$ ;  $d = 0.11$ ), so did soluble IL-1R1 (sIL-1R1) ( $P < 0.01$ ;  $d = 0.14$ ). These observations indicate that, despite no variations in the abundance of circulating Tfh and Tfr, positive autoantibody serology is associated with an augmented activity of these cells in association with IL-1 $\beta$  and sIL-1R1 levels.



**Figure 48. Positive autoantibody serology is associated to increased Tfh and IL-1-related cytokines overall.**

Cytokine dosage using  $\log_2(\text{MFI})$  according to autoantibody serology of healthy and diseased individuals.

To dig deeper into our hypothesis, we adopted a disease-specific strategy to compare positive and negative serology for autoantibodies, with healthy volunteers as control. This strategy highlighted Sjögren's syndrome (SjS), whose autoantibody-positive patients displayed higher frequency of circulating Tfh compared to negative and control ( $P < 0.01$  and  $P < 0.05$ ;  $d = 0.63$  and  $d = 0.32$ , respectively) (**Fig. 49A**). Despite few variations in IL-10 (associated to regulatory T cell function) and IL-6 (associated to T cell function) quantification, IL-4 was significantly increased in the positive condition compared to negative ( $P < 0.05$ ;  $d = 0.55$ ) and IL-21 had a similar pattern (not significant) (**Fig. 49B**).



**Figure 49. In Sjögren's syndrome, positive autoantibody serology associates with increased Tfh features and IL-1 $\beta$  upregulation.**

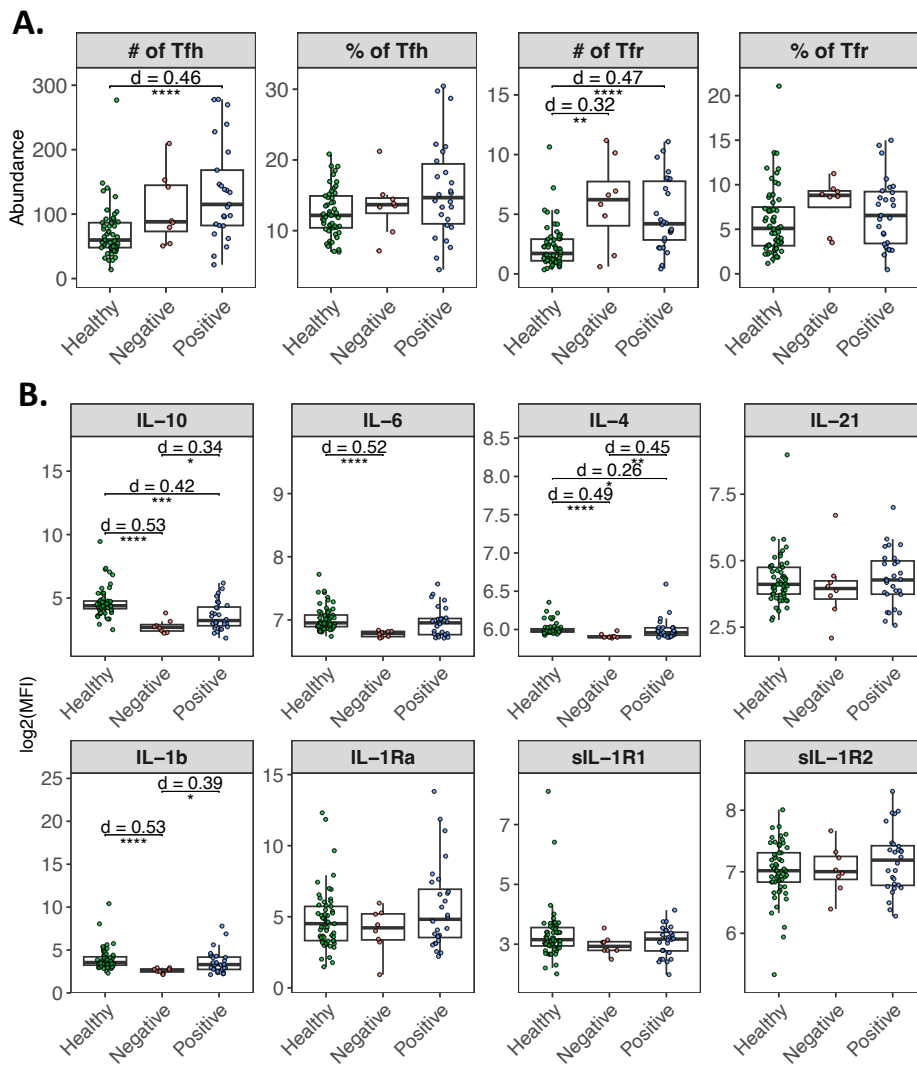
(A) Flow cytometry quantification of the absolute number of cTfh and cTfr and their frequencies, according to autoantibody serology of Sjögren's syndrome patients compared to healthy controls. (B) Cytokine dosage using  $\log_2(\text{MFI})$  according to autoantibody serology of Sjögren's syndrome patients compared to healthy controls. \* $P < 0.05$ ; \*\* $P < 0.01$  by paired Wilcoxon test. d: Cliff's delta effect size. sIL-1R1 : soluble IL-1R1; sIL-1R2 : soluble IL-1R2.

Similarly, IL-1 $\beta$  and sIL-1R1 appeared upregulated in positive versus negative comparisons, while negative condition tended to display lower levels of these cytokines. sIL-1R2 did not differ in both diseased conditions, but was significantly lower than in healthy ( $P < 0.01$ ;  $d = 0.33$ ), suggesting a disease-specific loss of regulation.

Another disease emerged as having great interest, namely vasculitis, which displayed higher numbers of both cTfh and cTfr compared to control ( $P < 0.0001$ ;  $d = 0.46$  and  $0.47$  respectively), though no significant difference was observed between positive and negative conditions (**Fig. 50A**). In addition, positive patients had higher levels of IL-10 than negative ones ( $P < 0.05$ ;  $d = 0.34$ ), but both were surprisingly underneath median healthy levels ( $P < 0.001$ ;  $d = 0.42$ ) (**Fig. 50B**). Likewise, IL-4 was significantly augmented in positive versus negative patients ( $P < 0.01$ ;  $d = 0.45$ ), so did IL-21 (not significant). In addition to Tfh-related cytokines, autoantibody-positive vasculitis patients carried increased levels of IL-1 $\beta$  ( $P < 0.05$ ;  $d = 0.39$ ) and sIL-1R1 (not significant) compared to negative.

**Figure 50. In vasculitis, positive autoantibody serology associates with increased Tfh and Tfr features and IL-1 $\beta$  upregulation.**

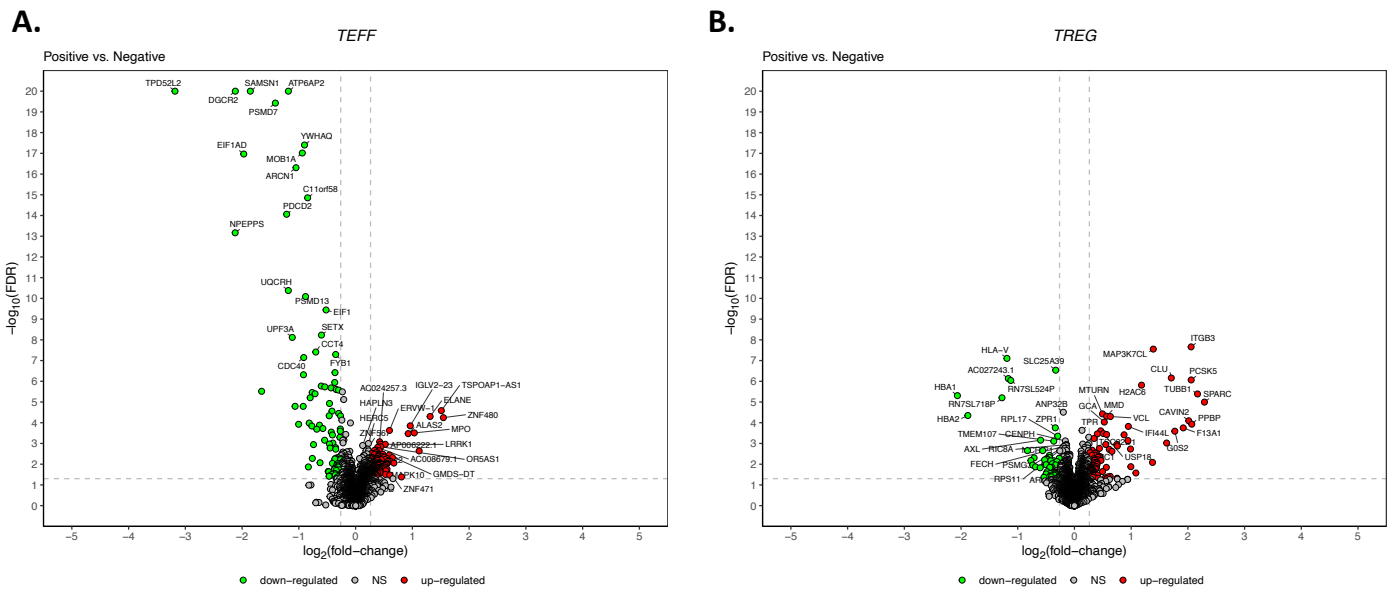
(A) Flow cytometry quantification of the absolute number of cTfh and cTfr and their frequencies, according to autoantibody serology of vasculitis patients compared to healthy controls. (B) Cytokine dosage using log<sub>2</sub>(MFI) according to autoantibody serology of vasculitis patients compared to healthy controls. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; \*\*\*\* $P < 0.0001$  by paired Wilcoxon test.  $d$ : Cliff's delta effect size. sIL-1R1 : soluble IL-1R1; sIL-1R2 : soluble IL-1R2.



**B. Transcriptomics analysis of circulating effector and regulatory CD4<sup>+</sup> T cells reveals poor signal of both IL-1 $\beta$  signaling and Tfol signatures**

Finally, we took advantage of transcriptomics data of circulating T<sub>EFF</sub> and T<sub>REG</sub> from positive and negative individuals; the sorting strategy based on CD127 and CD25 expression was expected to place Tfh and Tfr among the T<sub>EFF</sub> and T<sub>REG</sub>, respectively. Differential expression analysis in T<sub>EFF</sub> revealed both an up- and downregulation of genes in positive individuals (**Fig. 51A**). However, no significant pathway enrichment was detected ( $-\log_{10}(q\text{-value}) < 4$ ; not shown), since only few genes overlapped with intracellular metabolic pathways. In T<sub>REG</sub>, the same analysis also revealed up- and downmodulation of genes, including *ITGB3* (integrin  $\beta$ 3) which increased expression in T cells has been associated with inflammatory and autoimmune conditions (Du et al., 2016; Lacy-Hulbert et al., 2007) (**Fig. 51B**). Functional enrichment of upregulated

genes only showed limited and unrelated pathway implications (platelet, neutrophil and complement pathways; not shown).

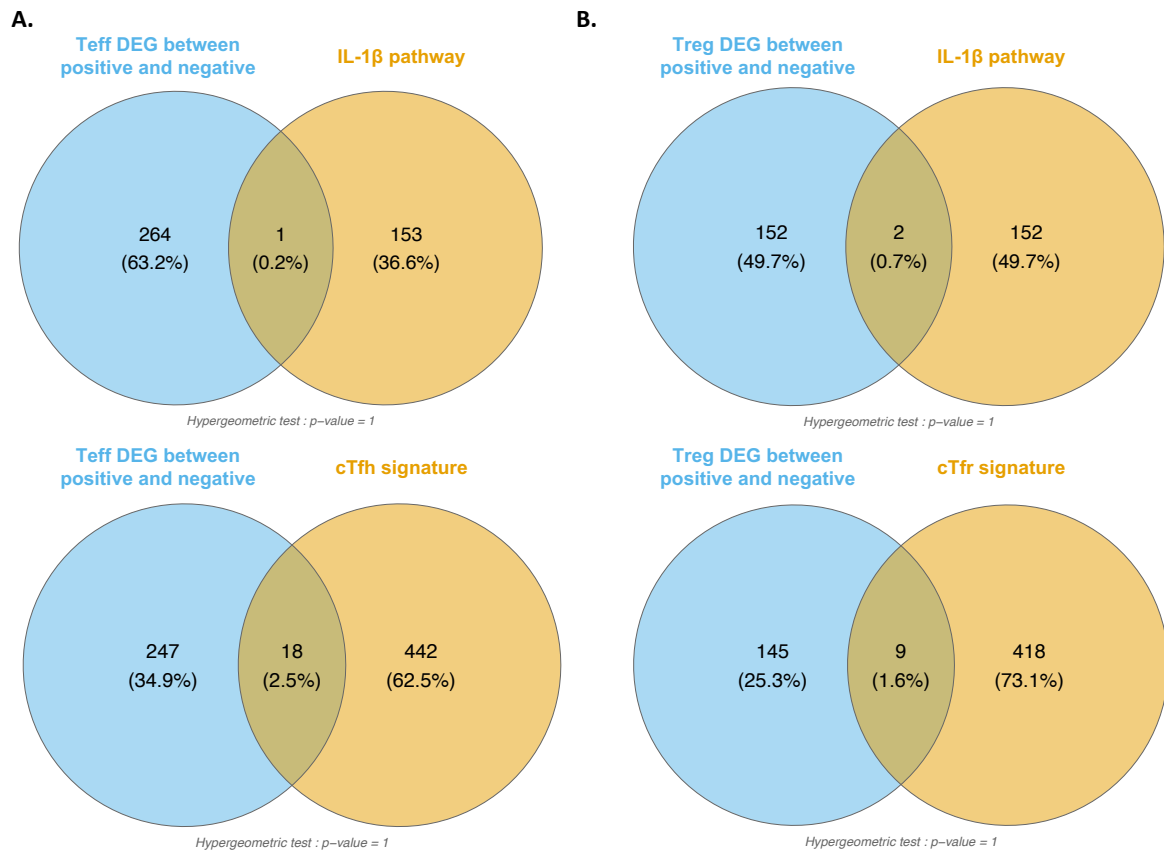


**Figure 51. DEG in circulating  $T_{EFF}$  and  $T_{REG}$  from individuals of the Transimmunom cohort.**

(A-B) Volcano plot showing DEG between autoantibody-positive and -negative individuals in  $T_{EFF}$  (A) and  $T_{REG}$  (B).

FDR: false discovery rate; NS: not significant.

To verify if  $T_{EFF}$  and  $T_{REG}$  DEG included genes of IL-1 $\beta$  pathway and genes linked to Tfh and Tfr, we used the previously described IL-1 $\beta$  gene list, together with 2 publicly available cTfh and cTfr signatures (Kumar et al., 2021). Neither  $T_{EFF}$  nor  $T_{REG}$  displayed significant overlap with IL-1 pathway (Fig. 52A & B, top). Likewise, both cTfh and cTfr signatures had limited and not significant sharing with DEG in  $T_{EFF}$  and  $T_{REG}$ , respectively, thus indicating the lack of IL-1 $\beta$  and Tfol variations in circulating CD4<sup>+</sup> T cells during autoantibody onset (Fig. 52A & B, bottom).



**Figure 52. Circulating T<sub>EFF</sub> and T<sub>REG</sub> lack signatures relative to Tfh, Tfr and IL-1 $\beta$ .**

(A) Statistical overlap between T<sub>EFF</sub> DEG and IL-1 $\beta$  pathway (top) or circulating Tfh (cTfh) gene signature (bottom).  
 (B) Statistical overlap between T<sub>REG</sub> DEG and IL-1 $\beta$  pathway (top) or circulating Tfr (cTfr) gene signature (bottom).  
 The number of genes and their overall proportion is are indicated.

Altogether, our findings underscore the potential involvement of IL-1 $\beta$ -related molecules in the emergence of autoantibodies in association with Tfh-related molecules, thus witnessing ongoing Tfh activity. The variations of circulating Tfh and Tfr cells seem influenced by the differential disease activity and chronology of the disease, which pinpoints disease-specific mechanisms having IL-1 $\beta$  dysregulation in common.





## Discussion

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In this study, we aimed to elucidate how the key modulators of the GC reaction, namely Tfh and Tfr, are impacted by IL-1 $\beta$  in the context of physiological and pathological antibody production. Here, we review our main findings and discuss their relevance in light of current scientific literature. Based on our contribution to the subject, we also explore new opportunities and challenges in the field of physiopathological GC reaction.

## **PART I. IL-1B IN THE REGULATION OF TFH AND TFR DIFFERENTIATION AND FUNCTIONS**

### Conclusions of the study (I)

Here, we first sought to capture Tfh and Tfr heterogeneity across distinct human secondary lymphoid organs, in association their expression of IL-1 receptors, IL-1R1 and IL-1R2. We found that the composition of the follicular T cell compartment is highly dependent on the organ of origin, as well as their activation status and the expression of IL-1Rs. The latter distinguished Tfh and Tfr phenotype, suggesting a contribution of IL-1Rs to their function. Our approach led us to build a meta-organ, representative of the whole maturation range of Tfh and Tfr. We found that the maturation of Tfh and Tfr is linked to higher expression of IL-1Rs, notably of IL-1R1, and that this expression defined a distinct Tfr subset carrying an intermediary phenotype between Tfh and Treg. By conducting trajectory inference, we uncovered 2 unique trajectories likely leading to the emergence of 2 Tfr subsets with heterogeneous properties : a GC-Tfh-derived Tfr subset and another derived from Treg. Using scRNAseq, we found that follicular T cell maturation is accompanied by the expression of IL-1R1 and the induction of IL-1 $\beta$  signaling. *In vitro* stimulation of Tfh and Tfr in different settings confirmed that IL-1 $\beta$  is able to potentiate their activation, while it induces a negative feedback of surface IL-1R1 expression. This effect of IL-1 $\beta$  on Tfh and Tfr was direct and reveals a finely tuned regulation mechanism of the germinal center reaction following inflammation.

### Follicular T cells : heterogeneity between human secondary lymphoid organs

Examining diverse lymphoid organs such as tonsils, mLN, and spleens is essential, as they each present unique characteristics tied to their respective functions and localizations. Our findings highlight a discernable variance in Tfh abundance, predominantly driven by the organ's origin, coupled with their activation phenotype.

Notably, tonsils, given their mucosal association, are frequently confronted with an array of antigens, including commensals, pathogens, and aero-digestive antigens. This necessitates a robust IgA production, among other isotypes, in response to such encounters. This is underscored by studies demonstrating abnormal Tfh activation in recurrent pediatric tonsillitis (Dan et al., 2019). Contrarily, mLNs, being profound organs, tend to maintain a more stable state, while the spleen offers an intermediate setting as observed in a study with a lower number of GC foci in LNs compared to spleens in monkeys (Moriyama et al., 2011). Interestingly, the spleens display increased proportions of non-follicular T cells (Tconv and Treg), which may reflect their particular capacity of sampling blood-borne antigens, in particular bacteria, and thus a propensity to feed cellular adaptive immunity. The differential antigenic exposure inherent to these organs insinuates a distinctive lymphoid microenvironment in each. Further nuances in these environments might arise from varying contributions of macrophages within the GCs, the role of fibroblastic cells and their complex networks, and the distinct patterns of DC-mediated antigen presentation.

#### Expression of IL-1Rs and divergence with murine studies

The expression of IL-1R1 and IL-1R2, in addition to being divergent across organs, was particularly heterogeneous among T cell subsets. IL-1R1 is seemingly enriched in regulatory cells, mostly Tfr, while IL-1R2 has a preferential expression in Tfol, notably tonsillar and mesenteric Tfh. These results strongly contradict a previous study in mice that observed preferential IL-1R1 expression in Tfh and IL-1R2 expression in Tfr (Ritvo et al., 2017b), which highlights the differences that can be found between murine and human models. Such differences imply potentially divergent mechanisms of GC regulation and antibody production, which is supported by previous work showing dissimilarities in antigen presentation, costimulation, delay of response, helper T cell proportions and autoimmunity onset (Mestas and Hughes, 2004). Others have also found IL-1R1 and IL-1R2 expression supporting our findings using scRNAseq on Tfh and Tfr (Kumar et al., 2021; Human Protein Atlas, not shown). In addition, murine studies showed that CD25 downregulation was associated to improved Tfr activation and localization in the GC; here we confirm other human studies sustaining that activated Tfr have higher CD25 expression which correlates with GC markers like ICOS, CD40L and PD-1 (Le Coz et al., 2023).

### Contribution of IL-1 $\beta$ to Tfh maturation

The expression of IL-1Rs was strikingly very discriminant in Tfh and Tfr, having a strong contribution to their differential phenotypes. On the one hand Tfh maturation, classically defined by the increase of CXCR5, PD-1 and ICOS, was gradually associated with an increase in IL-1R1 expression at the gene level (scRNAseq) and at the protein level (flow cytometry). This observation resonates with the increased IL-1 pathway genes observed following 24 hours of Tfol stimulation, indicating a link between Tfh maturation and IL-1 signaling. Such results are in line with previous studies stating that IL-1 $\beta$  contributes to helper T cell polarization (Ben-Sasson et al., 2009) and that Tfh are particularly sensible to this signaling (Kothari et al., 2021). Trajectory inference having *pre-Tfh* as root cells shows that, at the cell level, IL-1R1 and IL-1R2 expression peak at the emergence of GC-Tfh; prior to that peak is observed a contraction of their expression, suggesting a regulatory mechanism.

Indeed, *in vitro* culture of stimulated tonsillar T cells (either total cells or only sorted Tfol) manifests a removal of surface IL-1R1 when IL-1 $\beta$  is added, which may occur in case of excessive IL-1 agonism. As described in the introduction, this mechanism can be achieved by IL-1R1 internalization (Bourke et al., 2003), which allows Tfh to effectively get the agonistic signal while avoiding the receptor to receive more signals. Also, the receptor can be cleaved and secreted as a soluble form, thus sequestering extracellular IL-1 $\beta$  when present in high concentrations (Elzinga et al., 2009). On the contrary, culture with Anakinra reverses this effect and even increases IL-1R1 surface expression, by reducing internalization or shedding of the receptor. To exactly determine what the regulation mechanism is, combining extracellular and intracellular flow cytometry staining of Tfh and Tfr could be performed, in addition to measuring sIL-1R1 (and sIL-1R2) in the culture supernatant via enzyme-linked immunosorbent assay (ELISA).

Culture with IL-1 $\beta$  exhibited an increased activation of Tfh regarding HLA-DR, ICOS, CD40L, CTLA-4, CD25 and PD-1 with a certain variability, in both total and sorted Tfol cultures. Sorted Tfol, comprising Tfh and Tfr, displayed a clearer IL-1 $\beta$ -mediated activation, probably due to the presence of other cells in the total culture. Indeed, these cells could compete with Tfh and Tfr for IL-1 $\beta$  consumption; B cells being one of the major populations present in our culture, we preliminarily observed IL-1R1 expression

among them (not shown), suggesting that they can also be sensible to IL-1 $\beta$  signaling. The reverse effects of Anakinra indicate that IL-1 $\beta$  is endogenously expressed in whole-cell cultures; B cells have been shown to produce their own IL-1 $\beta$  (Ritvo et al., 2017b), among other subsets like DCs and macrophages. However, the effects of Anakinra in sorted Tfol were more surprising, since they suggest Tfh and Tfr could produce their own IL-1 $\beta$  too. In fact, T cells have been shown to synthesize precursor pro-IL-1 $\beta$  that is cleaved and secreted under the mature form following activation (Pulugulla et al., 2018), and public single cell databases indicate that small amounts of this cytokine are found in CD4<sup>+</sup> T cells (Human Protein Atlas, not shown). Therefore, stimulating Tfol can induce secretion of the cytokine; measuring its concentration in the culture supernatants would provide further evidence of this endogenous production in whole-cell and Tfol-sorted settings. Similarly, to confirm that Tfh maturation is positively impacted by IL-1 $\beta$ , IL-4 and IL-21 production could be measured as an indication of Tfh functionality. The ultimate confirmation assay would be estimating the functional capacity of IL-1 $\beta$ -primed Tfh to help B cells become plasmablasts (flow cytometry) and antibody-secreting cells (isotype quantification by ELISA) *in vitro*.

The selection of our culture settings underwent rigorous optimization. We settled on the tonsil as the organ of choice, based on a balance between the Tfol phenotype and their prevalence. Our objective was to ensure adequate IL-1R1/IL-1R2 expression in our cells to explore its role in modulating IL-1 $\beta$  signaling *in vitro*. Hence, while both tonsils and spleens were considered, we aimed for moderate Tfol activation, neither under nor over-activated. This criterion pointed us towards spleens and mLNs. Yet, the necessity for abundant Tfol, crucial for cell sorting and diverse culture conditions, discarded spleens and mLNs due to their limited Tfh and Tfr numbers. Hence, the tonsil was chosen. The most conventional method to trigger non-specific activation, anti-CD3/CD28 beads, was used for T cell stimulation. Subsequent testing involved varying concentrations of IL-1 $\beta$  (0.1, 0.5, 1, and 5  $\mu$ g/ml) and Anakinra (0.1, 0.5, 1, 5, and 10  $\mu$ g/ml). We evaluated Tfol phenotype markers, namely CXCR5, PD-1, ICOS, and Ki-67, to identify the most effective doses in comparison to stimulation alone. Furthermore, given that cytokines quickly respond post-agonist binding, we centered our attention on initial activation timepoints, choosing between 12h, 24h, 36h, and 48 hours. Concentrations of 0.5  $\mu$ g/ml for IL-1 $\beta$  and 1  $\mu$ g/ml for Anakinra emerged as the most revealing. The IL-1 $\beta$  dose, although higher than typical physiological

concentrations (50-500 ng/ml), was chosen to evaluate effects under saturating conditions (Ben-Sasson et al., 2009). Similarly, the elevated Anakinra concentration aimed to neutralize all endogenous IL-1 $\beta$ .

#### IL-1 $\beta$ : driver of Tfr differentiation derived from GC-Tfh ?

On the other hand, Tfr could be separated into 2 subpopulations : *resting Tfr* and *activated Tfr*. The latter is enriched in IL-1R1 and IL-1R2, and shares markers with GC-Tfh like PD-1, ICOS, HLA-DR, CD38 and CXCR5, while possessing other markers rather in common with *resting Tfr* (Foxp3, CD25, CTLA-4). Unsupervised methods like PCA were able to capture this unique IL-1R1-driven phenotype organ-wide, which underlines its relevance across human lymphoid tissues. *Resting Tfr* had an important phenotypic overlap with Treg, and trajectory inference predicted a transition from Treg to *resting Tfr* without modifications of IL-1Rs expression, but rather an increased expression of CXCR5 and PD-1. In contrast, *in silico* predictions suggest that *activated Tfr* emerge from GC-Tfh via the augmentation of IL-1R1 and IL-1R2 together with CD25 and CD38. Of note, marker expression across Tfh and Tfr pseudostates also indicates an increase of PD-1 and ICOS when *activated Tfr* arise, which may actually be attributed to GC-Tfh differentiation a not solely to Tfr lineage. Overall, phenotypic data comfort the existence of 2 distinct Tfr populations likely emerging from either Tfh or Treg lineages and being distinguished by their sensibility to IL-1 $\beta$ . The emergence of Treg-derived *resting Tfr* seemed concomitant to the maturation of *pre-Tfh* into *Tfh*, which sustains the idea of 2 distinct lineages, with *resting Tfr* possibly controlling Tfh differentiation.

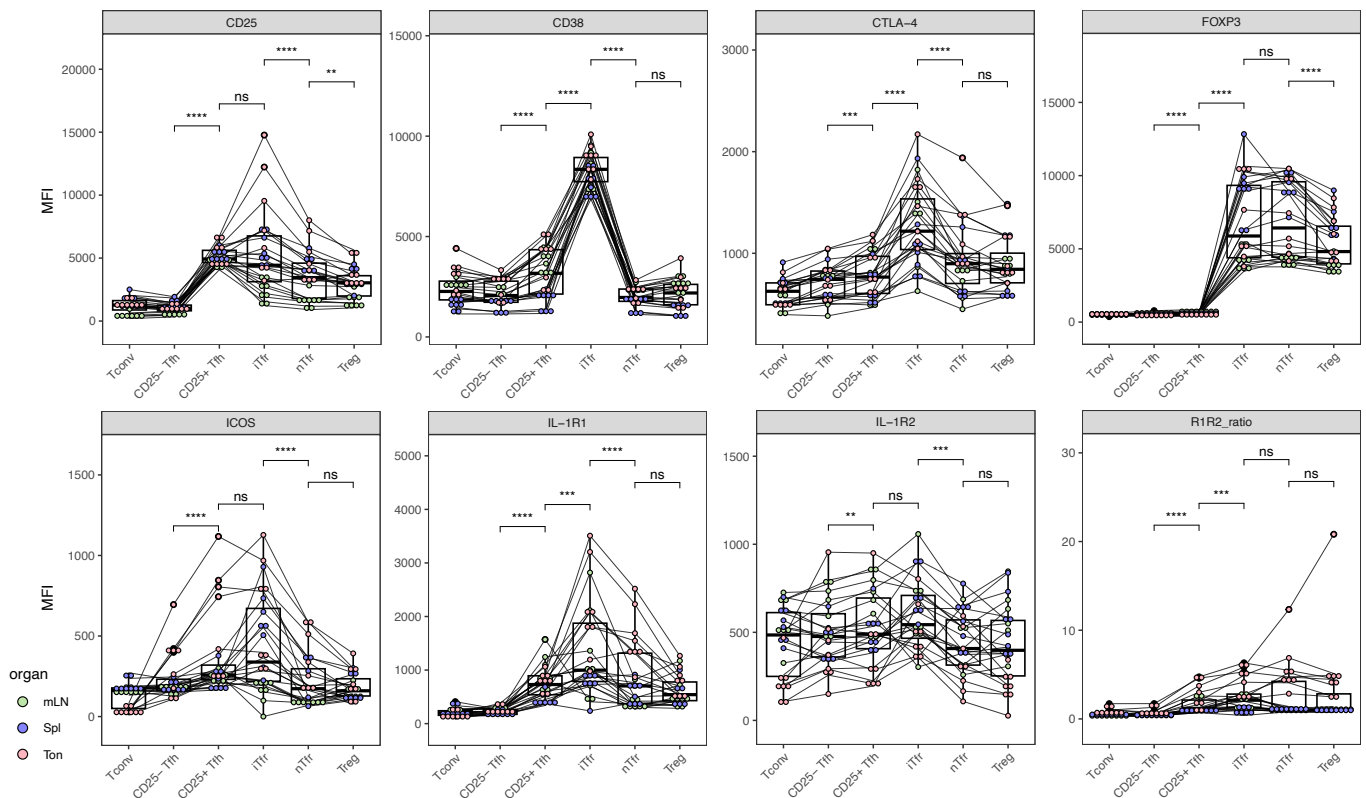
The existence of 2 Tfr populations has recently been documented by Carole Le Coz and colleagues in a groundbreaking study that gathered phenotypic, functional, positional and TCR sharing evidence (Le Coz et al., 2023). Indeed, "natural" nTfr are derived from Treg, sharing multiple suppressive markers with them, and are preferentially localized close to the T-B border of B follicles; "induced" iTfr share both Tfh and Treg phenotype, they differentiate from highly activated Tfh and they are mainly found within the GC (LZ > DZ). These iTfr, distinguished by the unique expression of CD38, had the capacity to promote the production of different antibody isotypes by helping B cells through IL-21 and IL-10 secretion, while nTfr could not. Instead, nTfr were able to suppress effector T cell proliferation more than iTfr.



Interestingly, the transition from Tfh to iTfr passed through a CD25<sup>hi</sup> CTLA-4<sup>hi</sup> IL-10<sup>hi</sup> ICOS<sup>hi</sup> Tfh intermediate.

Our findings also indicate that Tfh-to-Tfr transition required increased expression of CD25, ICOS, CD38 and CTLA-4, in addition to IL-1R1. This strongly suggests that our *activated Tfr* population corresponds to iTfr, whereas *resting Tfr* are thought to be nTfr. More studies support this dual Tfr origin : one study pointed out Foxp3 upregulation in the most differentiated Tfh prior to the termination of the GC reaction (Jacobsen et al., 2021); other studies highlighted the emergence of an IL-10-producing Tfh subset resembling Tfr in chronic inflammation conditions (Almanan et al., 2020) (Xin et al., 2018) (Kumar et al., 2021). Such findings break with the until then idea of GC-Tfh being the end-stage of Tfh differentiation.

To confirm that our results are in line with recent discoveries, we reproduced Tfr differential gating upon CD38 expression (iTfr : CD38<sup>hi</sup>; nTfr : CD38<sup>lo</sup>) in flow cytometry, as well as Tfh differential gating using CD25, in addition to Tconv and Treg as a comparison (**Fig. 53**). Accordingly, CD25 expression is similar between CD25<sup>+</sup> Tfh and our iTfr, whereas nTfr had lower CD25 expression. Plus, CD38 was upregulated in CD25<sup>hi</sup> Tfh, just like Foxp3, ICOS and CTLA-4 did. nTfr, however, did not show any difference with Treg regarding CD38, CTLA-4 and ICOS. Of utmost interest, IL-1R1 and IL-1R2 were also gradually increased from CD25<sup>-</sup> to CD25<sup>+</sup> Tfh and iTfr, while no difference between nTfr and Treg was observed. These results strongly sustain that *activated Tfr* expressing higher levels of IL-1R1 and IL-1R2 are iTfr, whereas *resting Tfr* display lower expression of these and are considered nTfr. Upon visualization of IL-1R1/IL-1R2 expression ratio in the aforementioned subsets, we discerned a remarkable pattern. The ratio escalated sequentially in CD25<sup>+</sup> Tfh and even more in iTfr, but stayed constant between Treg and nTfr. This suggests that Tfh-to-Tfr differentiation may result from IL-1R1 overtaking IL-1R2 expression in Tfh, culminating in intensified IL-1 $\beta$  signaling.



**Figure 53. The transition from Tfh to Tfr is associated to increased IL-1R1/IL-1R2 expression ratio, but not in Treg-to-Tfr transition.**

We manually gated Tconv, CD25<sup>-</sup> Tfh, CD25<sup>+</sup> Tfh, iTfr (CXCR5<sup>+</sup> Foxp3<sup>+</sup> CD38<sup>+</sup>), nTfr (CXCR5<sup>+</sup>, Foxp3<sup>+</sup>, CD38<sup>-</sup>) and Treg from our *ex vivo* immunophenotyping of secondary lymphoid organs. MFI of selected markers and ratio of IL-1R1/IL-1R2 MFIs are displayed as boxplots, each dot coloured according to the organ (n = 24). \*\*P < 0.01; \*\*\*P < 0.001; \*\*\*\*P < 0.0001; ns: not significant by paired Wilcoxon test.

*In vitro* analysis demonstrated that, like Tfh, Tfr respond to IL-1 $\beta$  in both total tonsillar and sorted Tfol cultures. Notably, IL-1R1, which did not show an upregulation during stimulation — possibly due to its inherently high expression in Tfr — did exhibit a significant decline upon the addition of IL-1 $\beta$ . This emphasizes the previously mentioned negative feedback mechanism. In isolated Tfol cultures, however, IL-1R1's response in Tfr remained unaffected, which might be attributed to the reduced IL-1 $\beta$  concentration compared to total culture. This pattern points towards a dose-response effect where IL-1 $\beta$  initially activates the cells and subsequently prompts either the regulatory shedding or internalization of the receptor. In contrast, IL-1R2 expression pattern diverged from that observed in Tfh. Introduction of IL-1 $\beta$  markedly elevated its expression, while Anakinra reverted it to baseline levels. This suggests a suppressive role of Tfr in mitigating inflammatory signals. As for activation and functional markers,

they exhibited an upregulation similar to Tfh, underscoring IL-1 $\beta$ 's common role in amplifying Tfol activation.

Examining the differential abundance of Tfr populations across the investigated lymphoid organs, we noted a pronounced enrichment of *activated Tfr* in tonsils. Their abundance in there was correlated with *GC-Tfh* levels, reinforcing their shared origin. In contrast, spleens and mLNs exhibited fewer *activated Tfr*, aligning with their more quiescent phenotype. These organs were enriched in *pre-Tfh*, *Tfh*, and notably, *resting Tfr*. This distribution suggests that while the most suppressive Tfr are predominant at baseline, active GC reactions shift the balance in favor of *activated Tfr*.

#### Role of IL-1 $\beta$ in the molecular regulation of follicular T cell activation

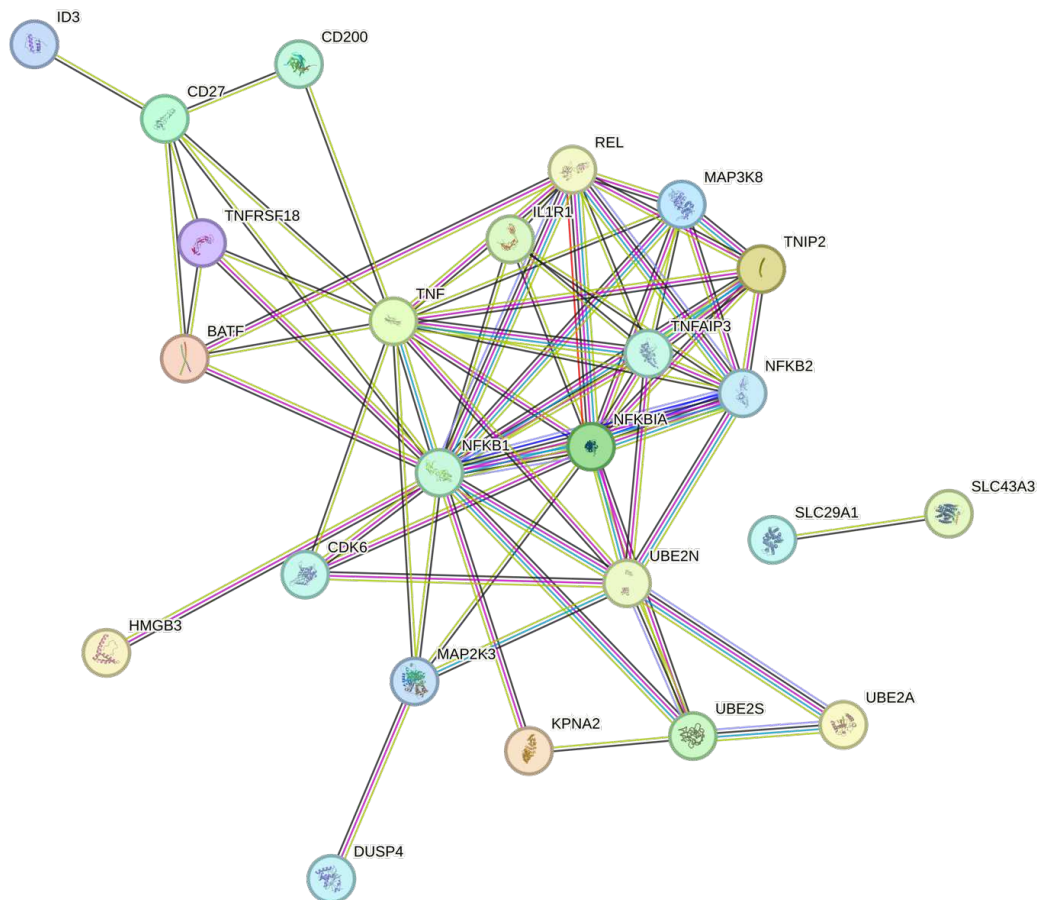
Our scRNAseq strategy didn't allow for a clear identification of Tfr due to the sequencing of live lymphoid cells, which encompassed Tfol, Tnon-fol, B cells, CD8 T cells, NK cells, among others. As a result, *FOXP3*<sup>+</sup> cells were scarce. Initially uncertain about the role of IL-1 $\beta$  signaling in Tfol, we opted to encompass all cells in the microenvironment that could be sensitive to IL-1 $\beta$  and thereby indirectly influence Tfh and Tfr. Consequently, only around 100 cells matched the Tfr definition, limiting their comprehensive study. While our initial sequencing included IL-1 $\beta$ -primed tonsillar cultures to unravel the cytokine's specific impact, the lack of discernible difference from the control led us to merge the conditions for enhanced statistical robustness. Now with a broader understanding of IL-1 $\beta$ 's role in Tfol activation, we believe that sequencing exclusively sorted live Tfol, along with Treg, could provide valuable insights.

The UMAP results of scRNA-sequenced Tfol show that STIM Tfol were more numerous than NOSTIM Tfol despite the same number of cells was sequenced initially. We think that selecting a cell population solely on 3 genes (*CD3*, *CD4*, *CXCR5*) may be limited, since in vitro stimulation can change the known phenotype of the cells. Another approach to capture more Tfh would be to base their identification upon a "Tfh signature" from public data (Kumar et al., 2021), instead of only core genes.

Using this strategy, we show that T cell stimulation in tonsillar lymphoid culture results in an enhanced GC-like phenotype of Tfol, associated with IL-1 $\beta$ /NF- $\kappa$ B signaling as well as IL-2 signaling. In fact, among the upregulated genes could be found the high affinity IL-2 receptor chain *IL2RA* (CD25) : while IL-2 is known for its T cell stimulation effects, it also inhibits follicular T cell phenotype and thus carries a regulatory function during Tfh maturation (Wing et al., 2018). Noteworthy, *CXCR4* was downregulated during Tfol activation; its loss of expression is associated with a preferential localization of Tfh in the GC LZ and distinguishes a Tfh phenotype with improved B cell help functions (Elsner et al., 2012). This downregulation among GC-Tfh was also observed in vitro following splenic T cell stimulation (Jeger-Madiot et al., 2022).

Furthermore, we identified 30 of DEG between STIM and NOSTIM that aligned with the GC-like signature, and 15 of these intersecting the IL-1 $\beta$  pathway. We visualized the gene interrelations using the STRING platform (**Fig. 54**). Several GC signature genes, such as *BATF*, *TNFRSF18* (GITR), *ID3*, *CD200*, and *DUSP4*, exhibited interconnections with IL-1 $\beta$  pathway genes. GITR, known to enhance IL-21 and IL-10 production in Tfh and Tfr and thus essential for GC-B cell antibody class switching (Oja et al., 2020), had associations suggesting IL-1 $\beta$  's role in Tfol functions. Similarly, *BATF* plays a pivotal role in IL-4 secretion by Tfh (Sahoo et al., 2015). Conversely, *ID3*, a transcriptional suppressor tied to the Th1 phenotype orientation (Anderson, 2022), appeared downregulated in our dataset. To gain deeper insights into the regulation dynamics, we turned to the TFLink database (Liska et al., 2022), a repository of empirically derived transcription factor targets. It revealed that *ID3* binds and inhibits the *IL1R1* promoter, elucidating the upregulation of IL-1R1 upon *ID3* downregulation. In parallel, *BATF* is posited to activate the transcription of both *IL1R1* and *IL1R2*. The *REL* gene, associated with the IL-1 $\beta$  pathway and observed to be upregulated upon stimulation, can augment *FOXP3* transcription. Intriguingly, *FOXP3* can bind to both *REL* and *IL1B* promoters, even though *FOXP3* was not detected in our data. Lastly, the IL-2/CD25 signaling, known to augment *FOXP3* expression (Zorn et al., 2006), might foster a positive feedback loop, amplifying IL-1 $\beta$  signaling and the Tfr phenotype. This is sustained by recent findings demonstrating an increased NF- $\kappa$ B and IL-2/STAT5 signaling in Tfr compared to Tfh in LNs following severe acute

respiratory syndrome-coronavirus 2 (SARS-CoV2) vaccination (pre-print : (Mudd et al., 2023).



**Figure 54. Network of the DEG between STIM and NOSTIM Tfol overlapping with GC-like and IL-1 signatures.**

We used the STRING platform to create a gene network based on the DEG identified in scRNAseq between STIM and NOSTIM Tfol. The 30 DEG overlapping with GC-like signature and the 15 DEG overlapping with IL-1 pathway were taken as input, and only those displaying connections are shown. Connections are of the following types: known interactions (curated databases, experimentally determined), predicted interactions (gene fusions, gene co-occurrence) and other (co-expression, textmining, protein homology).

### A new model of GC regulation by heterogeneous Tfh and Tfr

In light of our findings and recent literature, we propose a novel mechanism regulating the GC reaction by Tfh and Tfr under IL-1 $\beta$ 's influence (**Fig. 55**). Moving beyond the traditional view that frames Tfh as helper and Tfr as suppressive, we see these populations as heterogeneous, each contributing distinctively to B cell antibody production control.

Upon antigen presentation by APCs in lymphoid organs' T cell zones, naive CD4<sup>+</sup> T cells, influenced by the Bcl-6 transcription factor, undergo Tfh differentiation with

concurrent Blimp-1 downregulation. In parallel, Treg can differentiate into *resting Tfr* in response to self-antigen recognition or a bystander effect. The emergence of *resting Tfr* is marked by the expression of both Foxp3 and Bcl-6, as well as regulatory molecules like CTLA-4 and CD39, and immunomodulatory cytokines such as IL-10 and TGF- $\beta$ . These *resting Tfr*, expressing CXCR5, can move to the B follicle and, given their reduced ICOS and PD-1 expression, remain at the T-B border. Here, they suppress self-reactive Tfh undergoing differentiation, ensuring that only Tfh specific for non-self-antigens can interact with B cells (Crotty, 2019).

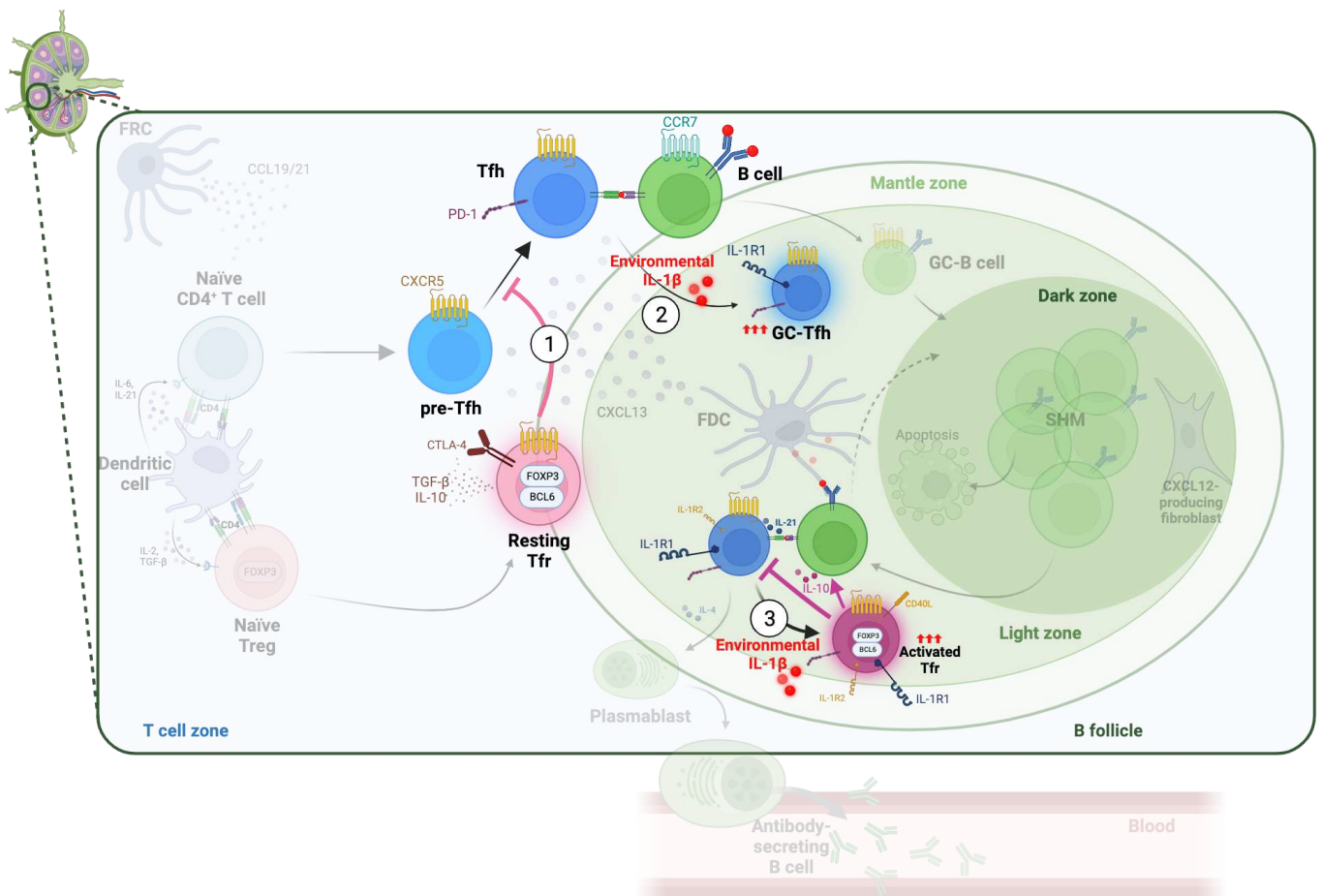
Following this interaction, either an extrafollicular B cell antibody production arises, or both Tfh and B cells initiate the GC formation within B follicles. As this migration progresses, largely mediated by ICOS and restricted by PD-1, the GC expands, forming the DZ and LZ, with GC-Tfh predominantly in the LZ. We posit that macrophage-produced IL-1 $\beta$  within the GC facilitates Tfh maturation via their expression of IL-1R1.

Within the established GC, GC-Tfh promote GC-B cell proliferation and SHM in the DZ. These centroblasts expressing CXCR4, attracted by CXCL12-producing fibroblasts, attempt to enhance their BCR affinity. Upon entering the LZ, they become centrocytes, interacting with antigen-presenting FDCs. Depending on affinity strength and interaction with GC-Tfh, various outcomes ensue, ranging from apoptosis to becoming memory B cells or early plasmablasts. Weak BCR affinity leads to apoptosis, while intermediate affinity without GC-Tfh help promotes memory B cell differentiation and GC exit via CXCR3 expression (Arroyo-Díaz et al., 2023). If intermediate BCR affinity is accompanied by GC-Tfh help, then the latter promote provide signals for CSR and reentry into the DZ mediated by CD40L, IL-21 and IL-10. Finally, if BCR affinity is strong, GC-Tfh help provides signals for early plasmablast differentiation and migration towards outside the GC. In their way out, B cells also encounter GC-Tfh cells rather specialized in the promotion of CSR via IL-4 and exit of the GC (Gonzalez et al., 2018).

The repeated reentry of GC-B cells into the DZ for BCR affinity maturation increases the risk of self-reactive receptor formation. Moreover, increasing apoptotic cells, due to non-productive rearrangements, elevate macrophage activity and IL-1 $\beta$  secretion

(Rahman et al., 2010) (Grootveld et al., 2023) (Chabaud et al., 2001). This continual interaction loop between GC-Tfh and GC-B cells chronically stimulates PD-1 (Aloulou et al., 2016) and necessitates control, prompting the emergence of activated Tfr. GC-Tfh, influenced by excessive IL-1 $\beta$  and IL-2 signaling, begin their Foxp3 expression-mediated transition to a Tfr phenotype, passing by an intermediate CD25<sup>hi</sup> CD38<sup>hi</sup> stage with increased IL-1R1/IL-1R2 ratio.

These activated Tfr, while recognizing the same non-self-antigens as Tfh, offer more nuanced functionalities. They not only restrict GC-Tfh activities but also assist GC-B cells. Through CD40L and IL-10 expression, they significantly favor GC-B cell re-entry into the DZ, forcing SHM and improving antibody quality. Moreover, they elevate the GC-B cell activation threshold to interact with GC-Tfh, ensuring only high-affinity BCRs progress. Consequently, activated Tfr aid in concluding the GC reaction, producing high-affinity antibodies while diminishing GC-Tfh activation.



**Figure 55. A new model of regulation of GC dynamics by IL-1 $\beta$ .**

Within secondary lymphoid organs, following antigen presentation and CXCR5<sup>+</sup> pre-Tfh polarization, the latter pursue their migration to the T-B border to meet their cognate B cell and become a CXCR5<sup>+</sup> PD-1<sup>+</sup> Tfh. (1) This interaction is thought to be negatively regulated at the T-B border by resting Tfr, a Treg-derived subset specialised in T cell suppression through cytokine secretion (IL-10, TGF- $\beta$ ), co-inhibitory molecules (CTLA-4) and surface inhibitory molecules (CD39, CD73). While this impedes self-reactive Tfh to engage interactions with B cells, those specific for non-self-antigens manage to get fully activated and enter the GC to become GC-Tfh. (2) This maturation step is enhanced by environmental IL-1 $\beta$  through their increased expression of IL-1R1. IL-1 $\beta$  can be provided by macrophages, B cells, stromal cells and even T cells themselves. Once B cells have completed their affinity maturation cycle, they require GC-Tfh help in the LZ either to exit the GC as mature plasma cells or reenter the DZ for CSR and further maturation. (3) Repeated stimulation of GC-Tfh and increased IL-1R1/IL-1R2 ratio in the presence of IL-1 $\beta$  can lead to activated Tfr differentiation. This GC-resident subset can dampen GC-Tfh activation via IL-10 and CTLA-4, while it can promote GC-B cell affinity maturation by producing IL-10 and expressing CD40L and ICOS. The emergence of this unique Tfr subset could enable the termination of GC reaction while promoting the maturation of B cell clones with most affinity.

**Future directions to further characterize GC regulation by IL-1 $\beta$**

The proposed model necessitates additional characterization to confirm the role of IL-1 $\beta$  in the GC reaction; in this context, we outline several experimental approaches to address its key aspects.

First, to confirm the differential lineages contributing to the heterogeneous Tfr pool, TCR repertoire sequencing should be analyzed. In our settings, we actually performed such a sequencing, but the lack of Tfr in our results makes TCR sharing analysis irrelevant. Another possibility is fate mapping : by sorting Tfh and Treg from different lymphoid organs, it can be possible to stain the cells and put them back into culture under stimulating conditions with or without IL-1 $\beta$ . After a few days, the contribution of each stained population to Tfr emergence be measured. We initially tested this approach by sorting Tfh (CD4<sup>+</sup> CXCR5<sup>+</sup> CD127<sup>+</sup> CD25<sup>-</sup>) and Treg (CD4<sup>+</sup> CXCR5<sup>-</sup> CD127<sup>-</sup> CD25<sup>+</sup>) according to other studies (Kumar et al., 2021), but the need for huge amounts of cells faced the limited number of samples. We tested another strategy based CD25 and CD39 differential expression among Tfh and Tfr, but this strategy only allowed a Tfr subset with 60% purity at maximum (Sayin et al., 2018). Plus, in tonsils where Tfh abundance is important, the majority of the cells were CD25<sup>+</sup> given their activation, which questioned the relevance of the sorting if only a fraction of total Tfh are studied. Also, studying the differentiation in an organ with an already advanced stage of Tfh maturation is not the solution.



*In vitro* culture of lymphoid cells represents a challenge. Our study, like many studies so far, has relied on 2-dimensional culture with lymphoid cells modeling the physiological composition of germinal centers. However, this can only be achieved using 3-dimensional models. Indeed, the GC reaction depends on a very precise structure that is hardly mimicked *in vitro*. Mark M. Davis and colleagues published a fascinating article in which they form tonsil organoids from lymphoid cell suspension (Wagar et al., 2021). These were used as platforms to generate GC-like structures with DZ and LZ, with different B cell maturation and antibody production under the supervision of Tfh in a vaccinology context. This kind of approach would be of utmost interest in our project by introducing IL-1 $\beta$ . Indeed, the use of organoids not only would help decipher the functional regulation of Tfh and Tfr by the cytokine, but also would unravel positional features. The following questions could then be answered : where are situated IL-1Rs-expressing Tfh and Tfr ? In what proportions are activated Tfr and resting Tfr present in and around the GCs ? What happens in the context of antigenic stimulation (using influenza or SARS-CoV2 antigens for instance)?

Imaging is a field in constant progress; the utilization of intravital imaging in murine models has helped uncover important features in the context of the GC reaction (Pae et al., 2022). Confocal microscopy using human cell cultures or organoids is also an interesting promise for future studies in the field (Vanderleyden and Linterman, 2017). In fact, understanding Tfh and Tfr localization in light of IL-1Rs cannot be performed without taking into account the dynamics of these cells, namely the cellular contacts they engage with each other or with B cells, the duration of immunological synapses, the speed of the cells and their migration between GC of the same lymphoid tissue. The use of 3-dimensional slices from *ex vivo* lymphoid organs is also interesting for such purposes, though the samples are rare compared to tumor slices (Espie et al., 2023).

One aspect that these new approaches do not encompass is the fluidics component : mechanobiology has proven that physical forces exerted by fibroblasts can shape T cell migration (Chang et al., 2019). This is particularly relevant since these fibroblasts govern GC formation and antibody production. New technologies like organ-on-chips integrate both the 3-dimensional scaffold with collagen matrices and the constant flux

of antigen with microfluidic perfusion (Shanti et al., 2021). This approach is currently being implemented by the team and represents a relevant alternative to murine models that drastically differ from human findings and that are being challenged by ethics concerns.

Lastly, the antigenic stimulation that we used is unspecific and aimed to stimulate all T cells in our culture conditions. However, the existence of different Tfr lineages with distinct TCR specificities would require antigen-specific stimulation, either with self-antigens or with non-self-antigens. The addition of IL-1 $\beta$  in such settings would bring further knowledge about the precise function of each subset. In addition to spleens and mLNs from healthy donors, we also had blood samples for some donors, which we used for SARS-CoV2 serology. As well, we also had donor information concerning their serology for different viruses like cytomegalovirus (CMV) and EBV, which were very common. Utilizing these information would ensure the presence of specific Tfh and Tfr in higher quantities, and thus perform functional experiments. Among those, verifying whether resting Tfr and activated Tfr have distinct helper and suppressive capacities upon antigen presentation and stimulation, with or without IL-1 $\beta$ , is a key interrogation that will have to be addressed.

## PART II.     DISRUPTION OF IL-1 $\beta$ IN AUTOIMMUNITY AND TFH/TFR               IMBALANCE

### Conclusions of the study (II)

We also wanted to examine whether the modulation of Tfh and Tfr by IL-1 $\beta$  was relevant in pathogenic autoantibody production, which results in autoimmunity. For that purpose, we segregated individuals from an observational cohort — comprising healthy donors, autoimmune and autoinflammatory patients — upon their autoantibody serology. While overall cTfh and cTfr did not seem impacted, key cytokines regulating the GC reaction were upregulated in accordance with dysregulated IL-1 $\beta$  in autoantibody-positive individuals. In particular, Sjögren's syndrome patients and vasculitis patients were among the most impacted by these disruptions, including cTfh and cTfr equilibrium. The transcriptomic analysis of circulating effector and regulatory cells overall did not provide significant input about cTfh/cTfr/IL-1 $\beta$  interplay, indicating that the circulatory T cell compartment may be limited to fully understand functional properties of the cells *in situ*.

### Rationale of investigating defective Tfh and Tfr to characterize autoantibody production

The Transimmunom observational cohort comprises 18 conditions, from healthy volunteers to patients with diverse autoimmune and autoinflammatory diseases. All individuals had their blood samples quantified for dozens of cytokines, soluble receptors, autoantibodies and immune cell populations. As well, bulk transcriptomic data were obtained from circulating effector and regulatory T cells. In the context of the GC reaction and its modulation by IL-1 $\beta$ , we wondered what the best way was to evaluate an ongoing pathological antibody production, given the variety of clinical manifestations and patients profiles found in the study. We postulated that the common pattern between all antibody-mediated autoimmune disease was a dysfunctional GC reaction, possibly associated to abnormal Tfh and Tfr responses. We therefore decided to separate individuals upon their autoantibody serology : positive and negative. We included healthy individuals indistinctly of the diagnosis. Indeed, autoantibodies are constantly produced in a physiological context, without leading to clinical manifestations thanks to the tolerance mechanisms in place (Shome et al., 2022). But here, since we precisely assess the presence of pathology-specific

antibodies, we think that autoantibody detection disease-wide may reveal defects in the GC. Hence, such a presence in healthy individuals could indicate a future disease onset, or in a patient it could predict the rise of a secondary disease. Different autoinflammatory diseases typically not presenting autoantibodies were included for that purpose: the identification of GC defects could occur following chronic inflammation.

By conducting this approach, we observed cytokine concentration of Tfr-related, Tfh-related and IL-1 $\beta$ -related molecules, namely IL-10, IL-6, IL-4, IL-21, IL-1 $\beta$ , IL-1Ra, sIL-1R1 and sIL-1R2. We decided not to focus on other cytokines, which may complexify our understanding and may not be related to our mechanisms of interest. We notably identified an increase in IL-1 $\beta$  concentration in positive autoantibody individuals, thus revealing an ongoing inflammatory, as expected. Interestingly, sIL-1R1 was similarly and significantly increased in the blood of these individuals, suggesting that a regulatory mechanism could be ongoing when excessive IL-1 $\beta$  is detected by the cells, just like our *in vitro* data showed. Also, IL-4 and IL-21 were found upregulated in the same fashion. These cytokines are secreted in tissues, mainly by Tfh, and are a strong evidence of an ongoing GC activity. However, finding these cytokines in the blood was not expected. Indeed, when comparing to Tfh and Tfr abundance from cytometry data, we found no variations between positive and negative individuals. This indicates that there is no common pattern between autoantibody positive conditions regarding circulating Tfol. Thus, the increased Tfh-related cytokines observed in the blood of these individuals reveal that the ongoing GC activity may take place within peripheral tissues, and the presence of the cells in the blood could be a mere transient event. Finding these cytokines appears as a biomarker of GC activity *in situ*, similarly as described with CXCL13 in the literature (Havenar-Daughton et al., 2016).

Studying autoantibody production across multiple pathologies has some limitations. Indeed, each pathology can display a specific pattern of Tfh, Tfr and cytokine quantifications, which may be masked by opposite observations in another disease. That is why we also performed a similar analysis by taking into account the individual diagnosis compared to healthy controls. By taking into account all autoantibodies, each being specific of a pathological condition, we hoped to find

common patterns of GC dysregulation. Indeed, having a disease does not prevent from getting another one characterized by another autoantibody.

Among the 2 diseases with significant upregulation of either Tfh, Tfr or cytokine levels between negative and positive serology, we found Sjögren's syndrome. Cytometry quantifications highlighted that Tfh were specifically upregulated in positive patients compared to negative and healthy controls, as described in the literature (Chen et al., 2021). Accordingly, IL-4, IL-6, IL-21 and IL-1 $\beta$  were upregulated in the positive patients, showing an association between Tfh abundance in the blood, Tfh function in situ through cytokines, and augmented IL-1 $\beta$  signaling which is in line with our experimental results discussed earlier. Of note, no difference was observed between negative and positive patients regarding sIL-1R2 titers. However, it was significantly decreased in patients compared to control, suggesting that a sIL-1R2 acts in a protective way by blocking IL-1 $\beta$ . Thereby, excess of IL-1 $\beta$  could be a driver of disease onset.

Finally, vasculitis also displayed a modulation of multiple parameters between negative and positive patients. This group of diseases comprises GPA and Takayasu arteritis, which are pathologies that can notably display ANCA autoantibodies, but not mandatorily. In cytometry, a common upregulation of both Tfh and Tfr amounts was found in all patients compared to control, but no difference between negative and positive : this again underscores that autoantibody positivity does not necessarily involve circulating subsets, but may rather require tissue Tfol dysregulation. Interestingly, the cytokines IL-10, IL-4 and IL-1 $\beta$  were upregulated in positive patients compared to negative, but all negative and positive had lower levels of these cytokines than healthy controls. Such observations manifest that there may be a dose-dependent effect. Too few of these cytokines could impede normal function of the immune system, while intermediate levels ensure a normal behaviour of the cells by maintaining a suppressive activity on the GC, and higher levels precipitate an autoantibody response due to an exacerbated B cell help (Laidlaw et al., 2017). Also, the increased presence of the cytokines could be associated to an excessive inflammation of the affected tissues, resulting in their release in the bloodstream. Our results link IL-1 $\beta$  production to Tfh (IL-4) and Tfr (IL-10) function during pathological antibody release in vasculitis. In accordance, it has been documented that GPA

pathogenesis relies on both Tfh and Tfr involvement (Kim et al., 2022) (Long et al., 2021). More generally, ANCA-associated vasculitis have been associated to an imbalance of cTfh and cTfr, which we observe in favor of cTfh, although not significant (data not shown).

#### Limitations of leaning on cTfh and cTfr to decipher antibody-mediated autoimmunity

The results discussed so far evidence that variations of Tfh and Tfr in the blood may not reveal the ongoing (auto)antibody production. These cells are effectively functional within the GC in lymphoid organs or in ectopic structures forming when chronic inflammation persists.

Our transcriptomic results reflect the hurdles of studying cTfh and cTfr in the context of autoimmunity. We unfortunately were not able to find a Tfh or Tfr signature among effector and regulatory T cells, respectively. Multiple reasons can explain these results. First, cTfh and cTfr are relatively rare : only 10-15% of CXCR5<sup>+</sup> cells among CD4<sup>+</sup> T cells and 2-7% of CXCR5<sup>+</sup> cells among Foxp3<sup>+</sup> CD127<sup>-</sup> regulatory T cells. Bulk RNA sequencing may not be sensible enough to detect signatures represented by a minority of transcripts. Also, cTfr are rather CD25<sup>lo/mid</sup> (Fonseca et al., 2017), while the gating of Treg prior to sequencing is based on high expression of CD25, which would exclude our cTfr. Plus, the lack of functions of Tfol in the blood render their transcriptional activity poor and therefore almost no genes are found between negative and positive individuals. In addition to digging deeper at the disease level, it would be more interesting to study Tfh and Tfr within the affected tissues directly. Multiple studies have focused on ELS, such as in the synovial joints of rheumatoid arthritis patients in which Tfh phenotype is associated to autoantibodies production (Rao et al., 2017) (Pan et al., 2023).

#### Challenges and opportunities for the study of defective GC reaction and its link to IL-1 $\beta$ in autoimmunity

In the scope of our study, the identification of disease-specific patterns associated to autoantibody onset and IL-1 $\beta$ /Tfh/Tfr dysregulation is complicated by the strong heterogeneity between patients and between diseases. Indeed, all patients had their own treatments, with distinct disease stages and different age/sex proportions. Plus, patients could also have multiple pathologies, rendering the disease-specific

observations tricky. The presence of autoantibodies in healthy donors or in inflammatory strict disease raises the question of the relevance of this classification. Are they really healthy or inflammatory only ? Will they develop an autoimmune disease despite the current absence of symptoms ? Alternatively, the absence of autoantibodies in some patients for diseases classically defined by the presence of the former makes us wonder whether the results can carry false negatives or errors in diagnosis.

We also looked at the treatments received by patients in order to find an IL-1 $\beta$ -targeting therapy like Anakinra. However, no patient was treated with that kind of medicine, which could have been of great interest to compare treated and untreated patients to estimate the effect of IL-1 $\beta$  on Tfh and Tfr in autoimmunity.

As discussed earlier, the heterogeneous Tfh and Tfr populations point out the necessity to investigate deeper and not sticking to cTfh and cTfr to explain autoimmunity mediated by antibodies. The populations of activated Tfr and resting Tfr could have very distinct functions in the periphery given their different antigen specificities, thus calling for a more precise characterization in the blood and ELS of patients. Tfh have also been described as heterogeneous, with a subset that could acquire cytotoxic properties with the production of granzyme K in ectopic tissues, for instance (Aoyagi et al., 2023). This indicates that the quantitative analysis of cell subsets does not suffice: we need to know the phenotypic landscape of the cells (both circulating and *in situ*) as highlighted by our cytokinic results. Reasoning upon a strict "Tfh/Tfr balance" from a quantitative point of view is limited and we propose a more nuance, functional balance taking into account cell heterogeneity.

However, working with patient-derived lymphoid or peripheral tissues is not an easy task given the scarcity of the samples and the relatively rare cTfh and cTfr. Original approaches have been undertaken for functional assays or for characterization of Tfh/Tfr *in situ*. Some have described a fine needle aspiration approach to harvest Tfh and Tfr directly from patient-derived lymphoid tissues, which represents a limited source of cells but minimally invasive and interesting for *ex vivo* characterization (Mudd et al., 2022). To go beyond single cell lymphoid culture and organoids, which reproduce a limited model of the true lymphoid complexity, a new technique has been

proposed : 3-dimensional tissue explants directly culture ex vivo to follow Tfh and Tfr in their native environment, with the possibility of modulating multiple parameters, like IL-1 $\beta$  (Schmidt and Baumjohann, 2022). Finally, original studies try to implement *in silico* models of the GC reaction and manage to reproduce the most likely dynamics of the cells : it represents an attractive first step to identify or rule out molecular and cellular candidates prior to experimental validation (Binder and Meyer-Hermann, 2016).

As a perspective, the burgeoning field of bioinformatics offers invaluable tools to maximize insights gleaned from limited sample availability, harnessing the power of high-dimensional data acquired from complex experiments. To decipher the intricate roles of Tfh and Tfr in pathological GC reactions, an integrative approach is required. This requires analyzing data from SLOs, leveraging blood-derived biomarkers, and incorporating information from ELS. A holistic perspective, which considers cell population abundance, their phenotype and specificity, their gene expression and metabolic activity, as well as the interactions they engage with both cells and microbes, is crucial for elucidating the nuanced influence of IL-1 $\beta$  on immunological balance.

#### Tolerance breakdown in the new model of IL-1 $\beta$ -mediated regulation of the GC reaction by heterogeneous Tfh and Tfr

There is increasing evidence of an involvement of IL-1 $\beta$  in the regulation of Tfh and Tfr in the context of antibody production, notably in autoimmunity. As an example, mutation of the *NFKB1* gene, a direct target of IL-1R1 signaling, leads to Tfh deficiency and therefore pinpoints the necessity of an equilibrated IL-1 $\beta$  to avoid both lack and excess of antibody response (Li et al., 2023). In allergy, as well, pathogenic antibody production has been linked to an exacerbated IL-1 $\beta$ /MyD88 signaling (Immormino et al., 2023).

In light of the newly described model, where the emergence of 2 distinct Tfr populations may control the GC reaction at its initiation or at its termination stage, we propose a potential tolerance breakdown mechanisms that may lead to autoimmunity. Regarding early steps in the GC reaction, the *resting Tfr* population is thought to play a key role : when decreased abundance and function of these cells is observed, a lack



of negative selection of differentiating Tfh could elicit self-reactive clones to help B cells produce autoantibodies, leading to autoimmunity.

In addition, the enhanced Tfh phenotype resulting from IL-1 $\beta$  signaling may also generate overactivated GC-Tfh that will help B cells with poor BCR affinity, therefore increasing the risk of unspecific antibody response. While IL-1 $\beta$  potentiates GC-Tfh phenotype, it can also promote an important *activated Tfr* differentiation from the Tfh lineage. Although *activated Tfr* are thought to be involved the termination of the GC reaction, similarly as iTfr they could promote B cell maturation through DZ reentry signals like CD40L and IL-10. Excess of IL-1 $\beta$  could result in profusion of *activated Tfr* and thus excessive B cells. Indeed, by forcing B cells to reenter the cycle of SHM, they induce a high mutational rate in the cells, potentially leading to self-reactive clones and to non-productive ones that end up dying. Apoptotic bodies can accumulate and produce inflammation due to increased activity of IL-1 $\beta$ -producing macrophages, which has a deleterious effect in infiltrating lymphoid aggregates at ectopic sites (Chabaud et al., 2001). Excessive apoptosis also augments the availability of self-antigens in the GC, which could help select GC-B cell clones for plasma cell or memory B cell differentiation. High antigen availability is effectively associated to less competition between B cell clones for acquiring Tfh help (Glaros et al., 2021). Thereby, they can exit the GC more easily without proper help from Tfh. Lastly, over-abundance of *activated Tfr* can impede GC-B cells to reach and receive help from GC-Tfh (Schips et al., 2023). This highlights the need for a balanced Tfh and Tfr activation, between help and suppression, and therefore a balanced IL-1 $\beta$  signaling. IL-1 $\beta$  could act similarly as IL-10 in Transimmunom patients : low concentration seems deleterious, but high concentration seems also pathogenic through another mechanism.

Our study points out the key role of cytokines in modulating the dynamics of the GC reaction. Notably, IL-1 $\beta$  and IL-2 seem to dictate the fate of Tfh and Tfr in a finely tuned balance. IL-2 fosters Treg differentiation at low doses as demonstrated by multiple clinical trials in autoimmunity (Collison, 2019), potentially expanding the pool of Treg-originated *resting Tfr* cells. Concurrently, IL-2 suppresses GC-Tfh phenotype, pushing the transition towards an *activated Tfr* phenotype (Le Coz et al., 2023). Contrarily, at higher concentrations, IL-2 activates Tconv and inhibits Bcl-6, impeding the differentiation of activated Tfr. This nuanced cytokine interplay highlights therapeutic

potential by precisely controlling the balance. In conditions like autoimmunity, where the manipulation of IL-2 has been tested, promising results indicate a restored Treg/Tfh balance (Wang et al., 2023).

Moreover, our findings suggest that IL-1 $\beta$  positively influences Tfh maturation, fostering the differentiation of *activated Tfr* and thereby enhancing the quality and resolution of the GC reaction. However, in autoimmune patients exhibiting elevated IL-1 $\beta$  levels as shown in our results, an excess could perpetuate a detrimental cycle of excessive Tfh and Tfr support to B cells. This overstimulation can lead to unchecked B cell reentry into the DZ, Tfh exhaustion, and, ultimately, cellular apoptosis and inflammation. Therefore, maintaining a delicate equilibrium of Tfr relative to Tfh is critical. Therapeutically, IL-1 $\beta$  represents a double-edged sword. While low doses in tissue may augment *activated Tfr* differentiation, in autoimmune conditions characterized by an IL-1 $\beta$  surplus, a fine-tuned balance between IL-2 and IL-1 $\beta$  may be imperative. Alternatively, direct inhibition of IL-1 $\beta$  may be beneficial at precise doses. These insights propose a synergistic modulation of IL-1 $\beta$  and IL-2 as a strategy to recalibrate the delicate balance between immunological help and suppression, thereby dampening autoimmune pathologies.



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## Appendix 1 : published article as second author

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### Naive and memory CD4<sup>+</sup> T cell subsets can contribute to the generation of human Tfh cells

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This article is the result of the work of Raphaël Jeger-Madiot, the previous PhD student of the team. During the first year of my PhD I was involved in the analysis of mass cytometry data that he acquired. I used bioinformatics tools to characterize Tfh heterogeneity *ex vivo* and after 3 days of stimulation in an original 2-dimensional culture favoring the differentiation of functional Tfh. Through the inference of trajectories potentially leading to Tfh generation, I confirmed that memory Tfh can arise from both naïve and memory non-Tfh T cells subsets.





## Appendix 2 : submitted article as first author

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### Differential expression of IL-1 receptors guides Tfh maturation and predicts a transition to Tfr cells

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