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# IL-33/ST2 and tissue Treg/AREG pathways in the pathophysiology of HIV infection

Mubashira Tariq

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**IL-33/ST2 and Tissue Treg/Amphiregulin  
pathways in the pathophysiology of HIV  
infection**

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# RESUME

Depuis les thérapies antirétrovirales (ART), les principales causes de mortalité et de morbidité sont liées à des pathologies non liées aux SIDA qui sont dûes à un niveau d'inflammation chronique de bas bruit. Cette inflammation est liée entre autres à la persistance de la perte d'homéostasie intestinale qui conduit à des translocations microbiennes dans la circulation systémique. En effet, les sujets traités et infectés par le VIH présentent une déplétion sévère et massive des lymphocytes T CD4<sup>+</sup>, en particulier dans le tissu lymphoïde associé à l'intestin (GALT) une altération de la barrière épithéliale et une fibrose au niveau des ganglions intestinaux.

Le récepteur sST2, un récepteur leurre de l'alarmine IL-33, constitue un facteur prédictif important de la mortalité toutes causes confondues, chez les patients séropositifs sous traitement antirétroviral. L'IL-33, précédemment connue comme un moteur des réponses immunitaires Th2, est désormais reconnue comme un adjuvant cytokinique de type "*switch-hitting*". Libérée par les cellules endommagées, elle favorise l'homéostasie tissulaire; notamment IL-33 permet de restaurer l'intégrité de la muqueuse intestinale après des lésions épithéliales d'origine virale. De plus, IL-33 renforce les réponses immunitaires Th1 pour éliminer les agents pathogènes, elle agit sur les lymphocytes T CD8<sup>+</sup> qui expriment ST2. Les Tregs tissulaires induites par l'IL-33 médient la réparation par la libération d'amphiréguline (AREG), un facteur de croissance semblable à l'EGF.

Dans cette thèse, nous sommes intéressés à l'axe IL-33/ST2 dans la réparation tissulaire et sur son rôle sur les lymphocytes CD8<sup>+</sup> chez des patients traités et infectés par le VIH.

Dans une première étude, nous avons analysé si la persistance des lésions intestinales pouvait s'expliquer par la dérégulation de la réparation tissulaire. Dans une deuxième étude, nous avons cherché à caractériser les cellules T CD8 exprimant ST2 et à évaluer le rôle de l'IL-33 sur la réponse T CD8 spécifique du VIH. Les taux plasmatiques de sST2 étaient élevés. Les expressions de l'ARNm et de la protéine IL-33 sont élevées dans la muqueuse intestinale des patients VIH, alors qu'elles étaient indétectables dans leur plasma. L'IL-33 était associée à une augmentation de la fibrose et de l'activation immunitaire tandis que la restauration des T CD4<sup>+</sup> a diminué. La caractérisation des Tregs tissulaires a révélé deux populations. Au sein des lymphocytes de la lamina propria (LPL), la fréquence des Tregs ST2<sup>+</sup> était augmentés chez les patients HIV traités et cette population de Treg est celle qui produit majoritairement de l'AREG. Nous avons observé une absence des Tregs qui produisent de l'AREG parmi les LPL des patients HIV traités.



Dans une deuxième étude, la caractérisation des cellules T CD8<sup>+</sup> exprimant ST2 dans les cellules mononucléées sanguines périphériques chez les patients infectés par le VIH a permis de déterminer que cette population est cytotoxique avec un phénotype effecteur (CD45RA<sup>+</sup> CCR7<sup>-</sup>) et une forte capacité à proliférer après stimulation du TCR. Nous avons montré que ces cellules conservaient une forte expression de ST2 par rapport aux donneurs sains après 5 jours de culture. Ensuite, nous avons observé une augmentation des réponses cytokines et cytotoxiques spécifiques de GAG et de CEF chez les patients VIH traités après culture avec l'IL-33 comme l'attestait l'augmentation de la concentration de l'IFN $\gamma$ , du Granzyme A, du Granzyme B et du sFAS-L dans les surnageants de culture.

En résumé, nos résultats mettent en évidence un rôle double de l'IL-33 dans l'infection chronique par le VIH : i) un rôle délétère contribuant à la fibrose dans l'intestin de l'infection et ii) un rôle positif renforçant les réponses spécifiques aux peptides GAG et CEF chez les patients infectés par le VIH sous traitement antirétroviral, indiquant son potentiel en tant qu'immunoadjuvant pour améliorer les réponses vaccinales.

**Mots clés :** Amphireguline, IL-33/ST2, Tregs tissulaires



# Summary

HIV has transformed into a chronic disease, since the advent of ART. There is persistence of immune activation and inflammation. It leads to a severe and massive CD4+T cell depletion, particularly in the gut associated lymphoid tissue (GALT). In addition, persistent inflammation exacerbates tissue damage, particularly in the GI tract. Epithelial barrier damage is a prerequisite for leaky gut and microbial translocation, contributing to persistent immune activation in individuals with chronic HIV infection. This is a potential mechanism of impaired CD4 reconstitution by contributing to fibrosis. Moreover, persistent antigen exposure, negative co-stimulation and chronic inflammation despite ART induced viral suppression, leads to CD8 T cell dysfunction.

sST2, a decoy receptor of the alarmin, IL-33, is reported to be a significant predictor of all-cause mortality in HIV patients on HAART. IL-33, previously known as a driver of Th2 immune responses, is now recognized as a switch-hitting cytokine adjuvant. Released from damaged cells, it promotes tissue homeostasis and repair. IL-33 functions to restore gut mucosal integrity following viral- or commensals- induced epithelial damage. It enhances Th1 immune responses attempting to eliminate the pathogens, followed by ILCs- and tissue Tregs- induced repair. IL-33 induces protective immunity against viral infections by boosting CD8+ T cell response. IL-33 induced tissue Tregs play a role in tissue repair mediated by the release of Amphiregulin, an EGF-like growth factor.

In this thesis, we assessed the involvement of the IL-33/ST2 axis in epithelial tissue repair and its role on CD8+ T cell function. In a first study, we analyzed whether the persistence of gut damage might be explained by the dysregulated tissue repair involving IL-33/ST2 and tissue Treg/Amphiregulin pathways. In a second study, we aimed to characterize CD8 T cells expressing ST2 and to assess the role of IL-33 on HIV specific CD8 T response.

Investigations were carried out on mucosal and blood samples from HIV infected patients on c-ART and seronegative healthy controls. Plasma sST2 levels were elevated. IL-33 mRNA and protein expressions revealed elevated expression in the gut mucosa of HIV patients, whereas it was undetectable in the plasma. IL-33 was associated with increased fibrosis and immune activation while decreased CD4 restoration. Phenotypic and functional characterization of tissue Tregs revealed two distinct subsets. ST2+ Tregs were upregulated in the LPL of HIV infected patients and identified as the source of AREG-producing Tregs. However, we observed a functional defect of these cells with a decrease of AREG-producing Tregs in the HIV LPL.

Overall, these results suggest that the profound defect of AREG production by Tregs may contribute to the persistence of gut barrier dysfunction despite ART in HIV infected patients. Phenotypic and functional characterization of ST2 expressing CD8 T cells in the PBMCs, deciphered this subset to be a cytotoxic population of effector (RA+ CCR7-) CD8 T cells, with a high capacity to proliferate with TCR stimulation. Their characterization did not differ between HIV infected and healthy controls. CD8 T cells from blood of HIV infected patients on c-ART were shown to maintain a high expression of ST2 compared to healthy donors. These cells were negatively associated with sST2 levels in the plasma. We observed that, after 5 days of culture with IL-33, GAG- and CEF specific CD8 T cells displayed more cytolytic and non-cytolytic responses with an increased concentration of IFN $\gamma$ , Granzyme A, Granzyme B and sFAS-Lin the culture supernatant.

To summarize, our results highlight the dual role of IL-33 in chronic HIV infection: i) a deleterious one contributing to fibrosis in the gut of HIV infection and ii) a positive one enhancing GAG- and CEF specific responses in HIV infected patients on c-ART, indicating its potential as an immunoadjuvant for enhancing vaccine responses.

**Keywords:** Amphiregulin, IL-33/ST2, Tissue Tregs



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# LIST OF ABBREVIATIONS

AIDS	Acquired immune deficiency syndrome
alpha-SMA	alpha-smooth muscle actin
AREG	Amphiregulin
Bcl-6	B cell lymphoma
BLIMP	B lymphocyte-induced maturation protein-1
cART	Combined antiretroviral treatment
CCL2	chemokine ligand 2
CCR	C-C Motif Chemokine Receptor
CNS	Central nervous system
CTLA-4	Cytotoxic T-lymphocyte antigen 4
DSS	Dextran Sodium Sulfate
ECM	Extracellular matrix
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EM	Effector memory
Foxp3	Forkhead box P3
FRC	Fibroblastic Reticular Cell
GALT	Gut associated lymphoid tissue
GI	Gastrointestinal
GITR	Glucocorticoid-Induced TNFR-Related
HAART	Highly active antiretroviral therapy
HIV	Human immunodeficiency virus



HSC	primary human hepatic stellate cells
IBD	Inflammatory bowel disease
ICOS	Inducible T cell co-stimulator
IDO	Indoleamine 2,3-Dioxygenase
IELs	intraepithelial lymphocytes
IgA	Immunoglobulin A
IgE	Immunoglobulin E
IL-17	Interleukin 17
IL-33	Interleukin 33
ILC2	Innate Lymphoid Cells 2
IPEX	Immunodysregulation polyendocrinopathy enteropathy X-linked
KLRG1	Killer Cell Lectin Like Receptor G1
LAG3	Lymphocyte-activation protein 3
LAMP	Lysosome-associated membrane glycoproteins
LN	Lymph node
LP	Lamina Propria
LPL	lamina propria lymphocytes
LTNPs	Long-term non progressors
LTs	Lymphoid tissues
MMP2	matrix metalloproteinases 2
mRNA	messenger RNA
Nrp	Neuropilin
PD1	Programmed cell death protein 1
PLWH	People living with HIV

pTreg	Peripheral T regulatory cell
ROR	RAR-related orphan receptor
RT-PCR	real-time polymerase chain reaction
SEMFs	Subepithelial Myofibroblasts
sST2	soluble interleukin 1 receptor-like 1
ST2	Interleukin 1 receptor-like 1
ST2L	ST2 long chain (IL-33 receptor)
STAT	Signal transducer and activator of transcription
T-bet	T-box expressed in T cells
TCM	T central memory
Tconv	T conventional cell
Teffs	T effector cells
TEMRA	T effector memory RA positive cells
TGF- $\beta$	Transforming Growth Factor-beta
TIGIT	T cell immunoreceptor with Ig and ITIM domains
TIMP1	tissue inhibitor of metalloproteinases 1
Tregs	Regulatory T cells
tTreg	Tissue T regulatory cell
UC	Ulcerative colitis

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# INTRODUCTION

The Gastrointestinal mucosa serves as an important structural and immunological barrier against the trillions of microbial cells within the GI tract and also provides a microenvironment for their survival and their largely beneficial interactions with the host (Vancamelbeke and Vermeire, 2017). Commensal bacteria of the gut are separated from the host by a single layer of epithelial cells, forming a barrier that is both physical and chemical in nature. Gut barrier integrity is further maintained by specialized lymphocyte subsets that reside within GI tract tissues (Mudd and Brenchley, 2016a; Vancamelbeke and Vermeire, 2017). Thus, intestinal homeostasis is maintained.

Gastrointestinal tract is the main target organ affected by HIV infection, with loss of gut homeostasis and impairment of the epithelial barrier, which is a prerequisite for microbial translocation (Brenchley et al., 2006a). Microbial translocation has been described as a mechanism for immune activation and an underlying cause of T-cell activation in HIV infection which persists in ART treated patients at significant higher levels as compared to HIV-uninfected controls (Brenchley et al., 2006a). Both inflammation and immunological abnormalities that occur in the gut during the HIV-1 or SIV disease course are tightly linked to structural damage to the GI tract. Damage to the gastrointestinal (GI) tract occurs early and irreversibly in progressive HIV-1 and SIV infections. Gut mucosal injury, CD4<sup>+</sup> T-cell depletion and microbial translocation are persistent even in long-term ART-treated individuals (Mudd and Brenchley, 2016a).

GI tract also serves as a HIV reservoir, promoting persistent antigenic burden during HIV infection even with c-ART. The loss of integrity of gut mucosa along with the viral reservoir, are responsible for a high antigenic burden, driving immune dysfunction including CTL activation and exhaustion (Perdomo-Celis et al., 2019).

The alarmin IL-33, is rapidly released from damaged cells following tissue damage (Martin and Martin, 2016). It promotes tissue homeostasis and repair by modulating its functions according to the tissue environment. It has been suggested to play a role in the pathogenesis of HIV infection (Barouch et al., 2016). sST2, the decoy receptor of IL-33, is reported to be a significant

predictor of all-cause mortality in HIV patients on HAART. IL-33 has gained tremendous importance due to its pleiotropic nature, modulating its functions according to the environment. In addition to its previously known functions in TH2 responses, it has the potential to induce protective immunity against viral infections owing to its ability to direct Th1- type immune responses as well. It enhances the activation and antiviral functionality of ST2L- expressing effector cytotoxic lymphocytes (Bonilla et al., 2012).

Understanding the determinants of persistent HIV-1-associated inflammation in treated patients is an important step in mitigating some of the barriers that prevent HIV-1–infected subjects from returning fully to health. The work presented in this doctoral thesis majorly focuses on certain important factors and mechanisms implicated in mucosal repair and maintenance of intestinal homeostasis in HIV infected patients under treatment. We wondered about the dual role of IL-33 in HIV disease and its impact on tissue Tregs and CTL response. The questions that we raised were, whether a defect in tissue repair involving IL-33/ST2 and tissue Tregs are the cause of the persisting leaky gut and inflammation in HIV infection. Secondly, whether IL-33 can enhance antiviral immune response in HIV infected patients on c-ART owing to its pleiotropic nature.

We divided this introduction into three main parts, starting with the role of the IL-33/ST2 pathway, followed by Tissue Tregs and lastly, focusing on IL-33's role in HIV infection.

## **Part I. IL-33/ST2 axis**

Despite anti-retroviral therapy, HIV-1 infection remains a chronic disease. The morbidity and mortality is high due to the underlying immune activation and systemic inflammation. The GI tract is the main target organ affected with loss of gut homeostasis and impairment of the epithelial barrier, which is a prerequisite for microbial translocation (Brenchley et al., 2006a). Microbial translocation was described as a mechanism for immune activation and an underlying cause of T-cell activation in HIV infection which persists in ART treated patients at significant higher levels as compared to HIV-uninfected controls (Brenchley et al., 2006a). Cross-sectional studies have shown the presence of gut mucosal injury and microbial translocation in long-term ART-treated individuals (Mudd and Brenchley, 2016a).

Tissue Tregs are involved in the maintenance of intestinal homeostasis and wound repair through IL-33/ST2 pathway, by the release of Amphiregulin, a growth factor playing an important role in healing (Arpaia et al., 2015a; Burzyn et al., 2013a). The proinflammatory cytokine (alarmin) IL-33, is released upon tissue damage, cell injury or necrosis, upregulating its receptor ST2L on target cells including tissue Tregs, Th2 cells and ILC2s, in addition to others. IL-33 signalling in tissue Tregs provides a critical signal for Treg accumulation and maintenance in inflamed tissues (Schiering et al., 2014a).

sST2, avidly binds to IL-33 in competition with ST2L and thus functions as a decoy receptor for IL-33. The interaction of sST2 with IL-33 blocks the IL-33/ST2L system (Pusceddu et al., 2019). IL-33 and sST2 have elevated levels in HIV infection (Miyagaki et al., 2011). sST2 is a significant predictor of all-cause of mortality in HIV patients on HAART (Thiébaud et al., 2017). sST2 and IL-33 are also linked to gut damage and microbial translocation in HIV infection

### **1. IL-33 localization and expression**

IL-33 is identified as a member of the IL-1 family. It is a tissue-derived nuclear cytokine, expressed in epithelial, endothelial, and fibroblast-like cells, during homeostasis and inflammation.

Endogenous IL-33 protein and mRNA was visualized for the first time in 2003 in human tissues (Baekkevold et al., 2003). It was initially called ‘nuclear factor from high endothelial venules

(NF-HEV)', due to its abundant expression in HEVs specialized blood vessels that mediate the entry into lymph nodes and other lymphoid organs (Figure 1).

Human IL33 mRNA encodes a protein of 270 residues. In 2005, Schmitz et al, reported that the carboxy-terminal region of the protein corresponds to the IL-1-like cytokine domain and has a three dimensional structure that is similar to other family members with 12 b- strands arranged in a b-trefoil fold (Schmitz et al., 2005)

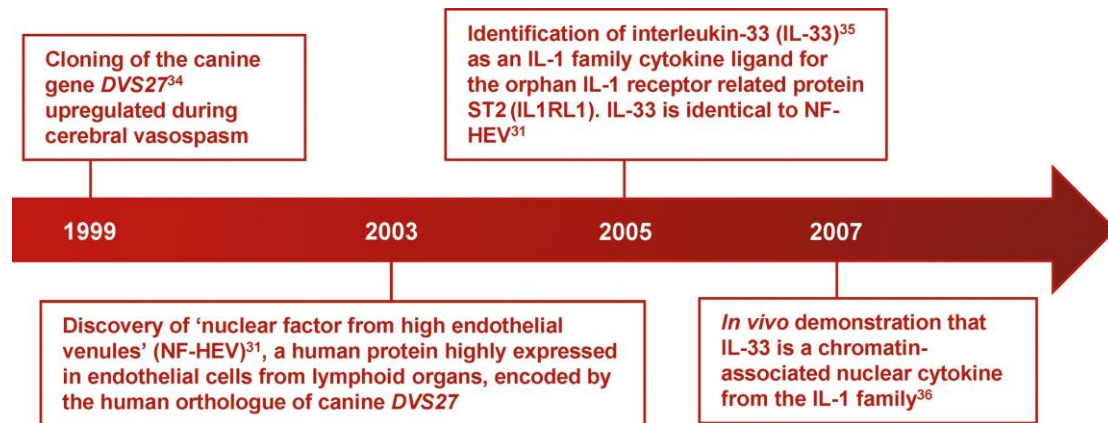


Figure 1. The discovery of IL33. Important dates and events (Cayrol and Girard, 2018)

Full-length (30-kDa) IL-33 is the form predominantly expressed in the cytoplasm and nuclei of IL-33-producing cells, whereas the only detectable form in the serum is the 20–22 kDa cleaved form of IL-33. This cleaved form is reported to possess reduced biologic activity (Pastorelli et al., 2011). IL-33 is produced as a precursor or in full-length form (270 amino acids (aa) in humans and 266 aa in mice) with the typical IL-1 fold in the C terminus. The N terminus contains a nonclassical nuclear localization sequence and a homeodomain-like helix-turn-helix DNA- binding domain as well as a chromatin-binding domain (residues 1–75 in human). IL33 is a chromatin-associated nuclear cytokine *in vivo*. It's amino-terminal 'nuclear' domain (amino acids 1-65) is necessary for nuclear localization and chromatin association. If it is deleted, IL-33 cannot reach the nucleus (Carriere et al., 2007; Martin and Martin, 2016).

IL-33 is encoded by a gene located on chromosome 9 in humans and chromosome 19 in mice (See figure 2 below). Full-length IL-33 is produced in mouse and human from two transcripts, which encode one protein. In a human cell line, a splice product lacking exon 3 was shown to encode a shorter protein that is biologically active in recombinant form, but it is unclear whether this is produced *in vivo*. Human and mouse full-length IL-33 reside in the nucleus.



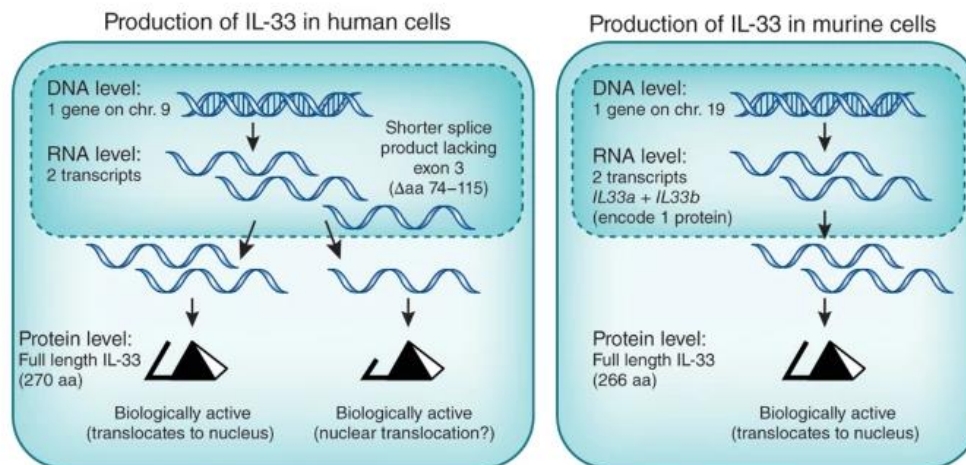


Figure 2. Production of IL-33 in human and murine cells (Martin and Martin, 2016)

IL-33 has two forms, the full-length precursor (IL-33<sub>FL</sub>) and the shorter mature form. Unlike IL-1b and IL-18 (other members of the IL-1 family of cytokines), IL-33 has biological activity as a full-length molecule as well. IL-33<sub>FL</sub> is a bona fide bioactive form of IL-33 that can induce ST2-dependent NF-κB activity and cytokine production in target cells. Inflammatory serine proteases from neutrophils and mast cells can cleave IL-33<sub>FL</sub> precursor to release shorter mature forms (Figure 3). Shorter mature forms have significantly increased biological activity compared to IL-33<sub>FL</sub> precursor. Neutrophil proteases could be critical for IL-33 activation during infection and type-1 inflammatory responses and mast cell proteases during allergic type 2 inflammation (Cayrol and Girard, 2018).

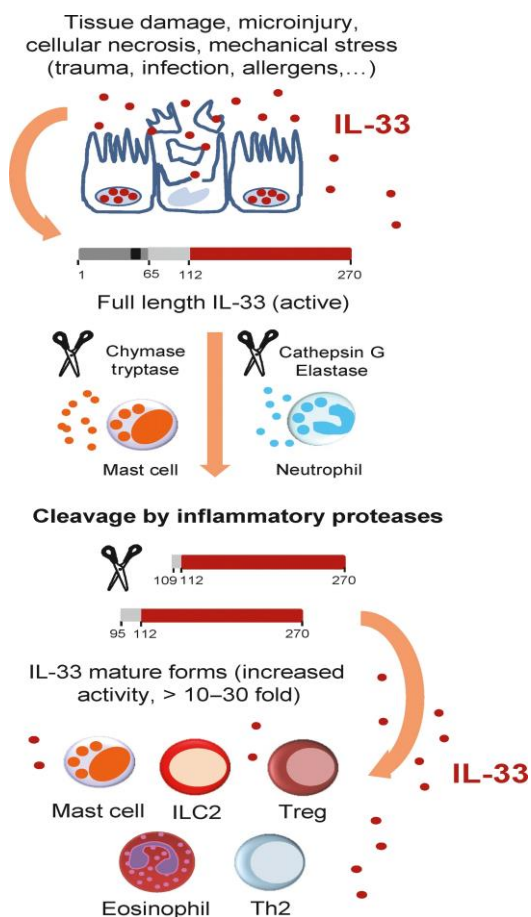


Figure 3. Extracellular release of IL-33 and activation by inflammatory proteases. (Cayrol and Girard, 2018)

IL-33 accumulates in the nucleus of producing cells, binds to histones and chromatin. An essential role of nuclear localization appears to be the regulation of IL-33 cytokine activity through nuclear sequestration (Bessa et al., 2014). IL-33 is constitutively expressed in the nuclei of various cell types in human and mouse tissues, in steady state. Endothelial cells are major source of IL-33 in human body during steady state and during human disease. Epithelial cells are not a major source of IL-33 during homeostasis, but high levels of nuclear IL-33 are observed in epithelial cells at barrier sites exposed to environment (Moussion et al., 2008). Indeed, mucosal epithelial cells constitute major sources of IL-33 in the respiratory and digestive tracts, the skin and the female reproductive system, both in humans and mice (Cayrol and Girard, 2018). Fibroblastic reticular cells in the lymphoid organs and glial cells in nervous tissues are other major sources of IL-33 protein in humans and mice. IL-33 expression can be further increased during inflammatory states. Activated fibroblasts, fibroblast-like cells and myofibroblasts are important sources of IL-33 during inflammation, in addition to epithelial and endothelial cells, especially in diseases associated with fibrosis (Manetti et al., 2010;

Marvie et al., 2010; Masamune et al., 2010), mucosal healing and wound repair (Sponheim et al., 2010).

## 2. Cellular targets of IL-33

ST2 was identified almost three decades ago as an mRNA that is produced by serum-stimulated fibroblasts (Tominaga, 1989), but it remained an orphan receptor until 2005, when IL-33 was identified as the natural ligand of ST2 (Schmitz et al., 2005). The IL1RL1 gene (synonym: ST2 gene) is located on human chromosome 2q12.1. The literature has been misnaming ST2 as “suppressor of tumorigenicity 2”, a gene which is also called ST2, while located on chromosome 11 in humans.

ST2 has four splice isoforms generated from a single transcript dependent on the promoter being used: ST2L, the longest transcript, which is a transmembrane receptor of IL-33; sST2, a secreted soluble factor; ST2V, a variant form of ST2; and ST2LV, another variant form of ST2L, which are differentially regulated through alternative promoter binding. Little is known about ST2V, other than that it is expressed highly in gastrointestinal organs. ST2LV lacks the transmembrane domain found in ST2L; ST2LV is secreted by eye, heart, lung, and liver tissues; and is found during later stages of embryogenesis (Figure 4) (Griesenauer and Paczesny, 2017a; Hodzic et al., 2017; Kakkar and Lee, 2008).

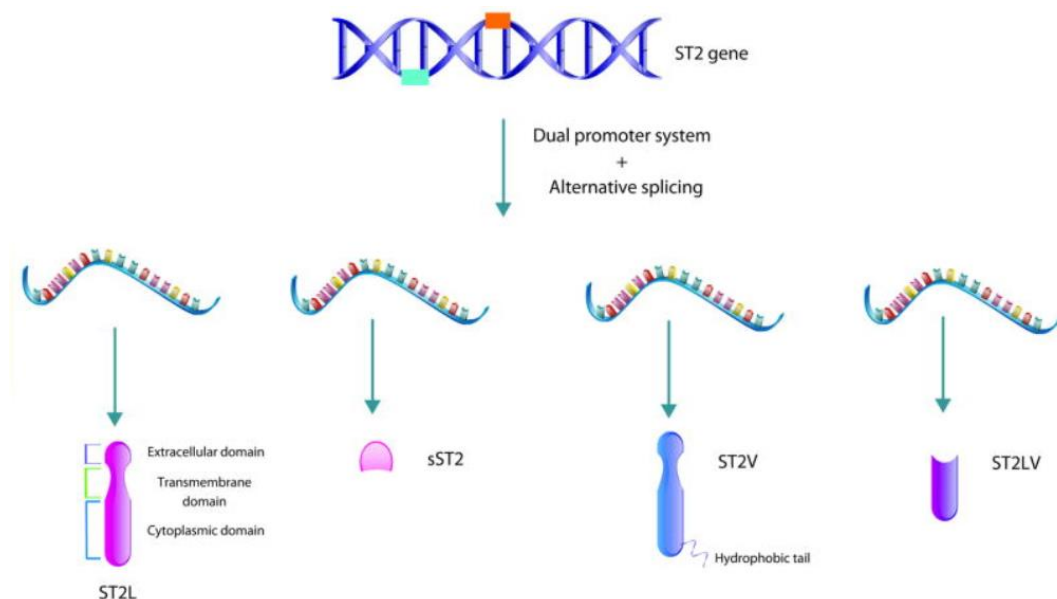


Figure 4. Isoforms of IL-33 receptor ST2 (Hodzic et al., 2017)

The expression of sST2 has mainly been investigated in individuals with diseases rather than in healthy people. In humans, sST2 can be produced spontaneously in the lung, kidney, heart, and small intestine, and after activation with IL-33 in mast cells or with anti-CD3/anti-CD28 in both CD4 and CD8 T cells (Pusceddu et al., 2019). The molecular function of sST2 was predominantly analyzed in animal models, and its role in humans was investigated mainly in clinical settings. Increased circulating concentrations of sST2 in different diseases suggest that sST2 is not specific for a distinct disorder but is rather a marker of inflammatory disease. It represents a general marker of disease and mortality. Accordingly, sST2 is of limited value for diagnostic purposes. However, strong evidence indicates that plasma concentrations of sST2 provide prognostic information in multiple diseases and several settings (Pusceddu et al., 2019). In addition to environmental factors and diseases, serum concentrations of sST2 seem to be regulated by genetic factors for up to 40% of inter-individual variability (Ho et al., 2013; Pusceddu et al., 2019).

The major targets of IL-33 in vivo are tissue-resident immune cells that express ST2 (Liew et al., 2016; Molofsky et al., 2015a) constitutively such as mast cells, group 2 innate lymphoid cells (ILC2s) (Monticelli et al., 2011; Yagi et al., 2014) and tissue regulatory T cells (Tregs) (Schiering et al., 2014a). Other cellular targets include T helper 2 (Th2) cells, eosinophils, basophils, dendritic cells, Th1 cells, CD8<sup>+</sup> T cells, NK cells, iNKT cells, B cells, neutrophils and macrophages (Figure 5) (Griesenauer and Paczesny, 2017a). IL-33 is thus emerging as a crucial immune modulator with pleiotropic activities in type-2, type-1 and regulatory immune responses, and important roles in allergic, fibrotic, infectious, and chronic inflammatory diseases.

IL-33-mediated stimulation of both myeloid and lymphoid cells supports their proliferation and survival, directs their migration and causes their robust production of type 2 immune mediators, particularly IL-5 and IL-13 (Lott et al., 2015; Molofsky et al., 2015a). Th2 cells were the first cells shown to express ST2 and require the transcription factor GATA3 for their generation and type 2 functions. ST2<sup>+</sup> ILC2s are also GATA3 dependent and produce the type 2 cytokines IL-5 and IL-13 in response to IL-33. ILC2 activation by IL-33 not only enables them to support type 2 immune responses but also aids tissue repair through their production of AREG and the generation of reparative M2 macrophages (Liew et al., 2016).

ST2/IL33 signaling in Tregs was first suggested to enhance their protective ability in an experimental colitis model. IL-33 was shown to increase both ST2 and Foxp3 levels and expand Tregs in mice with colitis. ST2<sup>+</sup> Foxp3<sup>+</sup> GATA3<sup>+</sup> Treg cells are found in the primary and secondary lymphoid tissues, adipose tissues, lungs and gut (Schiering et al., 2014a). IL-33 induces ST2<sup>+</sup> Treg cell proliferation and expression of epidermal like molecule amphiregulin (AREG) (Arpaia et al., 2015a), which enhances immune regulatory functions and supports tissue repair (Burzyn et al., 2013a). I will describe these Tregs in more detail in a dedicated chapter.

IL-33 can also support Type 1 immune responses that are dominated by TNF and interferon-gamma (IFN $\gamma$ ). IL-33 stimulates the expression of ST2 by Th1, CD8 T cells and NK cells as a defense mechanism against intracellular pathogens, thereby increasing the capacity of these cells to produce IFN $\gamma$ . This redirects the default type 2 inducing capacity of IL-33 to effector programs in response to life threatening challenges (Liew et al., 2016). I will also describe these CD8<sup>+</sup>T cells in detail in a dedicated chapter.

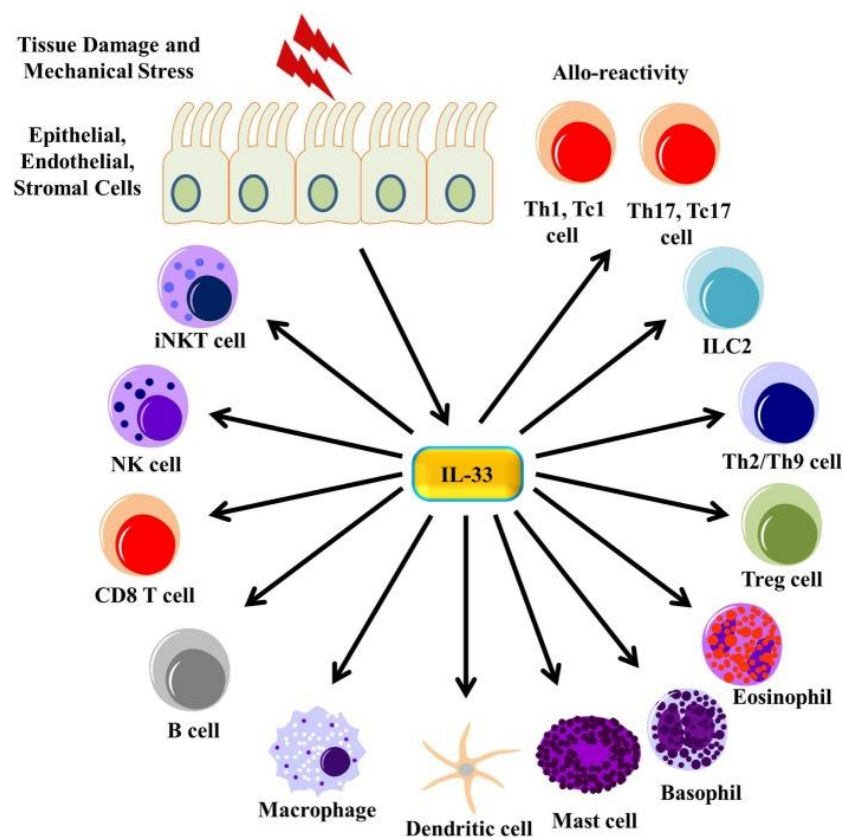


Figure 5. IL-33 signaling on immune cells (Griesenauer and Paczesny, 2017a)

Although, they are not well characterized, non-immune cells may also constitute important cellular targets of IL-33 *in vivo*. These include endothelial cells, epithelial cells, fibroblasts, astrocytes and neurons. However, ST2 expression by these cells is dominated by sST2, particularly following exposure to pro-inflammatory stimuli (Cayrol and Girard, 2018; Liew et al., 2016; Mildner et al., 2010).

### **3. IL-33 induced signaling and immune activation**

IL-33 signaling is typical of other TIR domain-containing family members. IL-33 binds to its transmembrane receptor ST2L, which induces a conformational change that allows ST2L to interact with IL-1 receptor accessory protein (IL-1RAcP), a co-receptor that is shared with other IL-1 family members (IL1a, IL1b, IL-36). The IL-33/ST2/IL1RAcP heterodimeric complex then induces signaling through myeloid differentiation primary response protein 88 (MyD88) adaptor, IL-1R-associated kinase 1 (IRAK1) and IRAK4 kinases, and tumour necrosis factor (TNF) receptor-associated factor 6 (TRAF6), that culminates in the activation of mitogen-activated protein kinases (MAPKs) and nuclear factor- $\kappa$ B (NF $\kappa$ B) transcription factors (Cayrol and Girard, 2018; Liew et al., 2016; Schmitz et al., 2005). These events lead to inflammatory gene transcription, proliferation, survival, cytokine secretion by ST2<sup>+</sup> cells and an immune response (Figure 6) (Liu et al., 2013).

The investigation of the IL-33/ST2L signaling pathway in humans is limited to a few studies as ST2L is a cell surface protein that is activated only upon binding with IL-33. The analyses of the molecular downstream effectors of this signaling pathway were investigated in only *in vitro* studies and not *in vivo*, probably due to the lower availability of tissue samples in humans (Pusceddu et al., 2019).

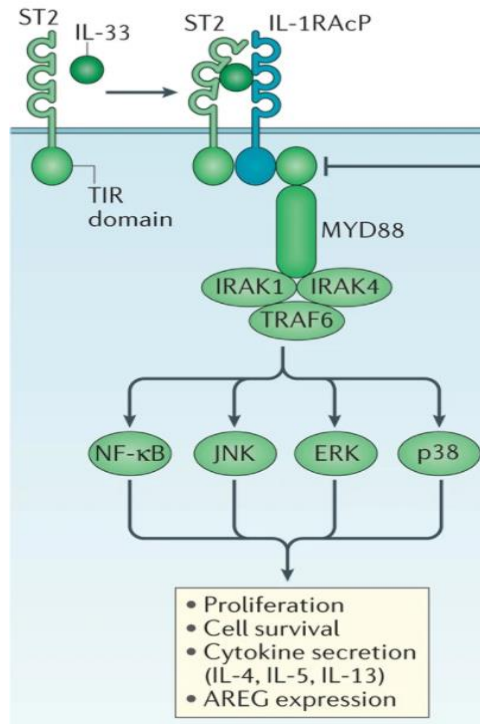


Figure 6. IL-33 Signalling. Cascade of events leading to proliferation, survival, and cytokine secretion (Liew et al., 2016)

#### 4. Regulation of IL-33 activity

IL-33 functions as an alarmin (DAMP, damage associated molecular pattern), being released by tissue damage or injury. The full length biologically active form after being cleaved by inflammatory proteases, generates mature highly active forms, which exhibit 10-30-fold higher activity. Mast cell proteases might generate mature IL-33 in allergic inflammation whereas neutrophil proteases may have a role during infection (Lefrançois et al., 2014, 2012). Because IL-33 is a potent cytokine and alarmin, its nuclear localization or retention is extremely important to avoid lethal multi-organ inflammation and maintaining immune homeostasis. Several mechanisms are in play for limiting IL-33 activity, as depicted in figure 7, and described below:

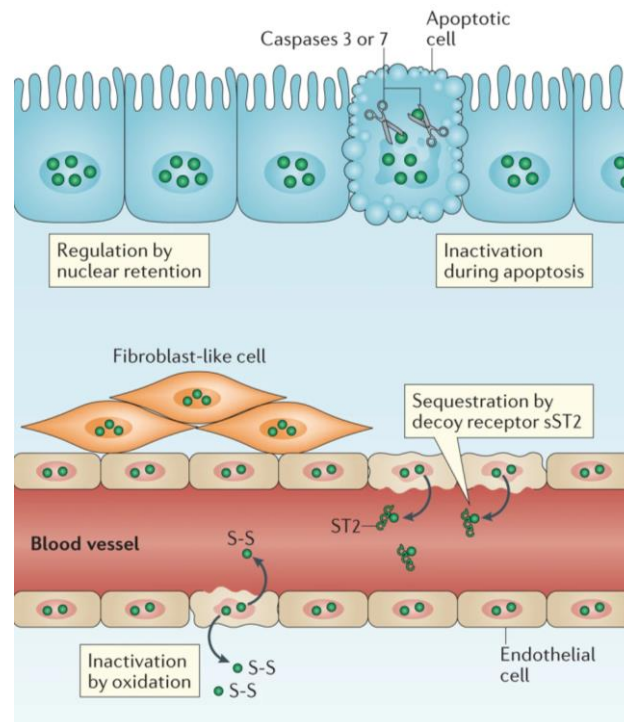


Figure 7. Regulation of IL-33 activity (Liew et al., 2016)

i. Nuclear sequestration.

One of the major mechanisms of regulation is through nuclear retention/sequestration of the protein. The N-terminal part of IL-33 contains a chromatin-binding motif (CBM) and a predicted bipartite nuclear localization sequence (NLS). The N-terminal domain, that contains the IL-33 CBM, has been shown to be necessary and sufficient for nuclear targeting of epitope-tagged (GFP-fused) IL-33 in human cells (Carriere et al., 2007, p. 1). Deletion of the N-terminal nuclear domain containing the CBM has been shown to result in early lethality in a knock-in mouse model. Heterozygous mice bearing the mutation displayed marked multi-organ inflammation with dense infiltrates of eosinophils, inflammatory monocytes, neutrophils, and macrophages. The mice died 3 months after birth, and it was associated with constitutive release of IL-33 in serum and was prevented in ST2-deficient mice, indicating that it was caused by the potent pro-inflammatory effects of extracellular IL-33 (Cayrol and Girard, 2018). Overexpression of mature IL-33 in skin keratinocytes in transgenic mice has been shown to cause an inflammatory skin phenotype, whereas overexpression of full length IL-33 in the same system did not cause spontaneous cutaneous inflammation (Kurow et al., 2017).

ii. Regulation by proteases.



IL-33 is cleaved and inactivated by caspases during apoptosis, a process that does not trigger inflammation *in vivo* (Cayrol and Girard, 2009; Lüthi et al., 2009). In the b4-b5 loop of IL-33 there is an insertion of 10 residues that is not found in other IL-1 family members. It contains a consensus site for cleavage by apoptotic caspases (caspase 3 and 7), which generate two biologically inactive IL-33 products (Figure 8). Caspase 3, caspase 7 and caspase 1, were able to induce the cleavage of IL-33, *in vitro* (Cayrol and Girard, 2009; Lüthi et al., 2009). Studies of caspase 1 and caspase 7 -deficient mice have revealed important role of these caspases in the limitation of type 2 immune responses in the lungs (Madouri et al., 2015, p. 1). Inflammatory proteases are other critical regulators of IL-33 bioactivity. They process IL-33FL in the central domain, upstream of the IL-1 cytokine domain, and generate mature forms (Lefrançais et al., 2014, 2012). When they are present in large amounts, they could also be involved in IL-33 inactivation, through protein degradation. It has been shown in several models that IL-33 accumulates during initial hours after release and is generally no longer detectable after 6 hours (Cayrol and Girard, 2009; Lüthi et al., 2009). IL-33 degradation by inflammatory or other proteases is likely to be one of the important mechanisms that limit IL-33 activity *in vivo*.

iii. Inactivation by oxidation

To limit IL-33 bioactivity following its release into the extracellular microenvironment, there is inactivation by oxidation of cysteine residues and formation of disulphide bridges (S-S) in the IL-1-like cytokine domain. This is even more rapid than protein degradation (~2h) (Cohen et al., 2015). The four critical cysteine residues were identified as Cys208, Cys227, Cys232, and Cys259, and mutation of these four cysteines resulted in prolonged activity of IL-33 in the lung microenvironment (Cohen et al., 2015).

iv. Sequestration by soluble ST2

Another mechanism is through sequestration by soluble ST2 in biological fluids, which functions as decoy receptor, neutralizing IL-33 (Bandara et al., 2015; Hayakawa et al., 2007). High levels of sST2 may significantly inhibit IL-33 activity *in vivo*. The IL-33-ST2 signaling pathway is also inhibited by single immunoglobulin domain IL-1R-related molecule (SIGIRR, or TIR8), which disrupts ST2 and IL-1RAcP dimerization (Bulek et al., 2009), and by the ubiquitin-proteasome system, which degrades ST2 (Zhao et al., 2012).

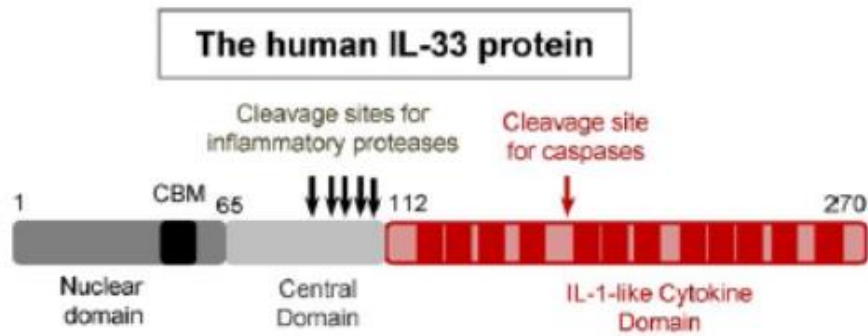


Figure 8. Primary structure of the human IL-33 protein. The different protein domains are indicated and the cleavage sites for proteases involved in IL-33 activation (inflammatory proteases) or inactivation (caspases) are also shown

### 5. Progressive stages of IL-33- ST2 in tissue homeostasis

IL-33 is rarely a lone driver but rather acts to modulate and amplify signals in a local and context-dependent fashion. IL-33 activity was primarily associated in driving TH2- immune responses, particularly in augmenting cytokine levels of IL-4, IL-5 and IL-13, but it has recently emerged as a switch-hitting cytokine adjuvant. It is a multi-talented immune player that promotes both TH2 and TH1 immune responses given the right type of immune cells activated, the microenvironment, and the cytokine milieu.

IL-33 acts both as traditional extracellular cytokine as well as a nuclear transcription factor.

Figure 9 illustrates the progressive stages of IL-33/ST2 axis at homeostasis, acute and chronic injury, along with beneficial and pathological effects of IL-33, as described below:

**In the *homeostatic stage***, constitutive pools of IL-33 become active locally to sustain basal physiology by activating ST2+ resident cells in a cell- and tissue-specific manner. IL-33 is present as a reservoir in the nuclei of a restricted subset of cells, including endothelial and epithelial cells, and perhaps certain fibroblast reticular stromal cells. In response to poorly defined cues, which may be mechanical, hormonal or metabolic, active IL-33 is translocated to the extracellular space to maintain tissue integrity, limit excess inflammation and promote tissue adaptation to remodeling and other stress. ILC2s, some Tregs, and mast cells are the primary tissue-resident cells that constitutively express high levels of ST2, positioning these cells as initial targets of IL-33.

The role of IL-33 during tissue homeostasis is most informed from studies in adipose tissue. IL-33 is abundant in adipose tissue, where expression has been confirmed in endothelial cells and fibroblast-like reticular cells (Kolodin et al., 2015; Molofsky et al., 2015b). White adipose tissue (WAT) is the major storage site for high-energy triglycerides. WAT of lean mice is populated with ST2<sup>+</sup> ILC2s and Tregs. Molofsky and al have shown that IL-33-mediated ILC2 activation typically leads to tissue accumulation of eosinophils and alternatively activated macrophages, and is associated with elevated numbers of tissue Tregs (Molofsky et al., 2015a, 2013). Moreover, IL-33 sustains the architecture and function of ST2<sup>+</sup> resident cells that limit infiltration of adipose tissue by inflammatory lymphocytes and myeloid cells that lead to insulin resistance and metabolic syndrome. Indeed, in mice, loss of IL-33 or ST2 leads to worsening obesity and insulin resistance, a hallmark of type 2 diabetes (Miller et al., 2010; Vasanthakumar et al., 2015). Although further work is needed, these data suggest a model whereby ILC2s, a subset of Tregs and possibly mast cells are positioned in tissues through a developmentally regulated process, where they can respond to local fluctuations in extracellular IL-33.

**In the *amplification stage***, tissue-specific pools of IL-33 increase, accompanied by expansion of ST2<sup>+</sup> immune cells (particularly ILC2s and Th2 cells) in attempting to maintain homeostasis, usually in response to perturbations. Depending on the location of the barrier (skin, lung, gut), the probable cause of perturbation will be different and ranges from bacteria, fungi and viruses to helminths. Although triggers differ, the basic principles of alarming resident sentinel cells by IL-33 may apply.

Helminths induce adaptive Th2 cells and low-affinity IgE antibody production, but these responses typically cause little acute tissue pathology, likely due to the concomitant activation of T regulatory cells. Indeed, expansion of Treg cells repress the pro-inflammatory effects of chronic infestation and promote the healing of involved tissue (Molofsky et al., 2015a).

In contrast, allergic pathology states, such as asthma and atopic dermatitis (Kim and Artis, 2015), are characterized by chronic elevations in tissue IL-33. The expansion of ILC2s becomes dissociated from Treg stimulation, enabling the activation of adaptive immunity and associated accumulation of Th2 cells and the development of high-affinity IgE capable of activating mast cells and basophils. These events are associated with tissue pathology. Inhibition of the IL-33 pathway may be a promising therapeutic approach for limiting these pathologic responses (Fahy, 2015, p. 2).

**In the *Conversion stage*,** infectious, inflammatory or neoplastic triggers elicit tissue damage and epithelial breaches in the context of pathogen-associated molecular patterns. Pre-formed tissue IL-33 stores are released, and IL-33 is further induced in tissue cells. Inflammation and foreign antigen induce dendritic cell activation and IL-12 production, with trafficking of inflammatory leukocytes including neutrophils, macrophages, NK cells, NKT cells, Th1 cells and CD8<sup>+</sup> T cells from blood. There is increased IL-33 responsiveness through induced expression of ST2 on these inflammatory cells, a process termed as *conversion*, resulting in overt manifestations of type 1 immunity, while repressing Treg and type 2-associated responses. This facilitates clearance of microbial and viral pathogens but often at the cost of tissue damage. Indeed, the role of IL-33 in viral infections is complex, ranging from disease exacerbation in respiratory syncytial virus infection (Walzl et al., 2001), to host protection in herpes simplex virus infection (Aoki et al., 2013) in mice. Treatment of mice with IL-33 reduced the viral loads and pancreatitis after coxsackievirus-B infection (Sesti-Costa et al., 2013). The enhanced viral clearance was due to the production of IFN $\gamma$  and TNF from CD8 T cells and NK cells, whereas the inflammatory pancreatitis was attenuated due to the activation of mast cells with resulting enhanced differentiation of M2 macrophages and Treg cells. IL-33 promoted protective CD4<sup>+</sup> and CD8<sup>+</sup> T cells against human papilloma virus (Villarreal et al., 2014; Villarreal and Weiner, 2015). In conclusion, the role of IL-33 in viral infections is highly virus-specific and limits its potential use as an adjuvant to enhance virus immunity.

As for viral infection, IL-33 has diverse effects in cancer models. Direct overexpression of IL-33 in neoplasms promoted NK and CD8<sup>+</sup> T cell infiltration and restriction of cancer growth and metastasis (Gao et al., 2015). However, IL-33 can signal on cancer cells that themselves express ST2L, thereby promoting survival and metastasis (Yu et al., 2015). Indeed, IL-33 is implicated in tumor-associated inflammation and tumor progression through immune regulation. In a recent human study on head and neck squamous cell carcinoma (HNSCC), the IL-33 stromal level was significantly higher in advanced stage of disease and positively correlated with Foxp3<sup>+</sup> cells and with poor prognosis. In contrast, IL-33 in tumor epithelial cells was significantly reduced in the advanced stages compared to early stages. The tumor infiltrating Tregs expressed high levels of ST2, compared with those among PBMCs from healthy controls or HNSCC patients, and there was no significant difference between healthy donor or patient PBMCs. The tumor infiltrating Tregs expressing high levels of ST2, respond to IL-33 signals. IL-33 induced naïve T cells to differentiate into ST2<sup>+</sup> Foxp3<sup>+</sup> T cells and

increased the proportion of total Tregs and levels of cytokines, such as IL-10 and TGFb1, promoting the suppressive function. (Wen et al., 2019).

In colorectal cancer (CRC), the IL-33 signaling promotes the development of CRC by changing the phenotype of Tregs. In mice, it was shown that tumor infiltrating Tregs preferentially upregulate ST2, and this IL-33/ST2 signaling positively correlates with tumor size and number. In humans, the number of activated ST2-expressing Tregs are increased in blood and tumor lesions of CRC patients. (Pastille et al., 2019)

IL-33 not only acts as an alarmin to provide acute initial protection, but its activity could also lead to long term immunosuppression following a systemic inflammatory response via expansion of the Treg population (Nascimento et al., 2017). In an experimental mouse model of septic peritonitis, IL-33 stimulated ILC2-mediated M2 macrophage polarization via type 2 cytokine production, which lead to IL-10 release, activation of Tregs, and ultimately immunosuppression in sepsis survivors. ST2-deficient mice that survived the experimental sepsis model had decreased type 2 cytokine release and Treg expansion, with no subsequent immunosuppression and improved survival following secondary infection. Furthermore, WT mice following sepsis treated with exogenous sST2 had improved survival with this secondary infection compared to WT mice without sST2. These data correlate with human data that shows survivors of severe sepsis and septic shock had elevated serum levels of IL-33, IL-10, and Tregs.

With chronic unresolved inflammation, however, tissue IL-33 pools will increase, and repetitive cycles of IL-33 release, will ultimately contribute to tissue damage. Regulatory mechanisms are suppressed, inflammation is amplified, and fibrosis ensues (systemic sclerosis, liver cirrhosis, allergic diseases). sST2 levels may increase to contain effects of the expanded IL-33 pool, which can be buffered over many years before pathologic effects of fibrosis and other tissue injuries become apparent.

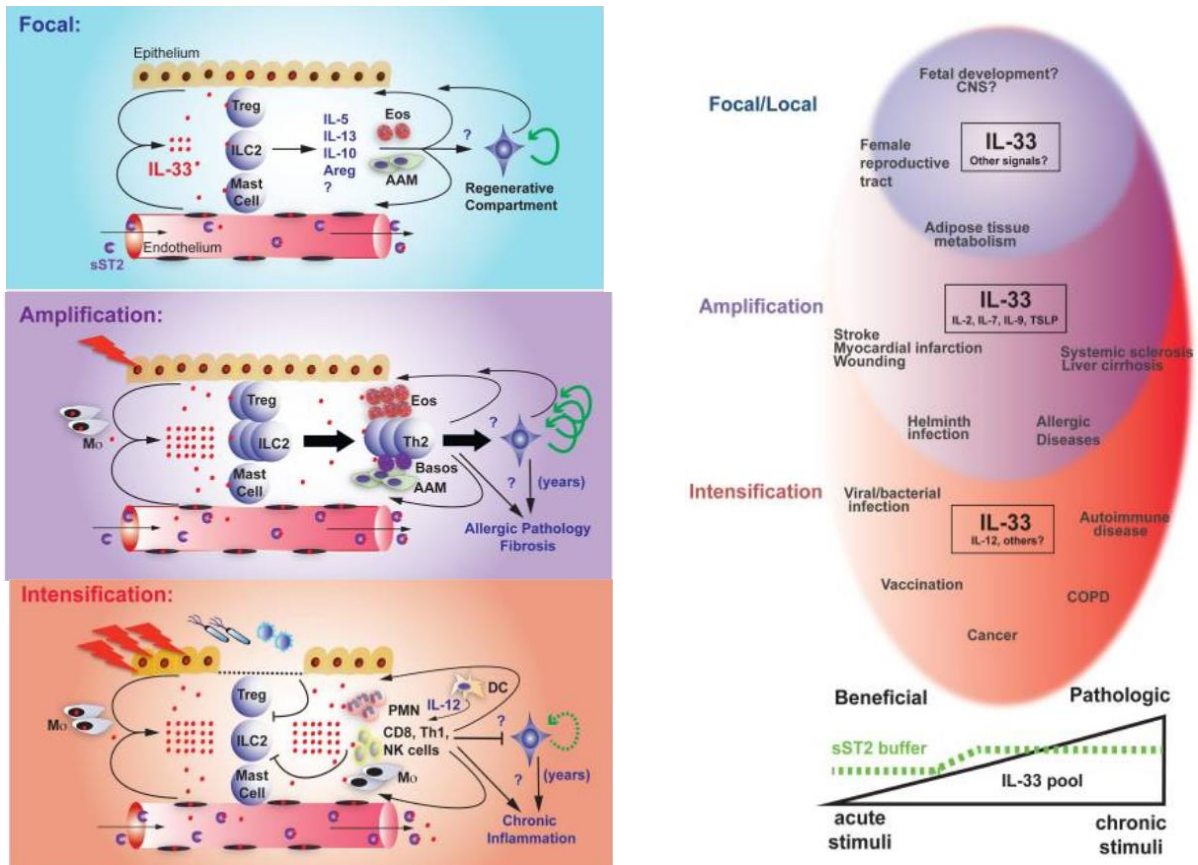


Figure 9. Homeostasis to amplification to conversion stage (Molofsky et al., 2015a)

## 6. IL-33/ST2 in gut

### 6.1 Intestinal mucosal structure

The epithelial barrier of the GI tract has a total surface area of 200m<sup>2</sup>, and it is the most widely exposed organ system to the external environment. The gut epithelium serves as one of the first lines of defense of the intestine against ingested pathogens, antigens and toxins. The gut mucosa is a multilayer system consisting of an external ‘anatomical’ barrier and an inner ‘functional’ immunological barrier. Commensal gut microbiota, a mucous layer, and the intestinal epithelial monolayer constitute the anatomical barrier. The inner layer consists of a complex network of immune cells organized in a specialized system known as ‘gut-associated lymphoid tissue’ or GALT. GALT represents both isolated and aggregated lymphoid follicles and is one of the largest lymphoid organs, containing up to 70 % of the body’s total number of immunocytes. It

is involved in responding to pathogenic microorganisms and in providing immune tolerance to commensal bacteria. The mucosal antigen presenting immune cells have the ability to present antigen to naïve T lymphocytes, with subsequent production of cytokines and activation of mucosal immune responses, when needed. The interaction of these components sustains the maintenance of the delicate equilibrium of intestinal homeostasis. This balance can be altered by many factors, including alterations of the gut microflora, mucus layer, and epithelial damage, leading to increased permeability and translocation of luminal contents to the underlying mucosa. Emerging evidence indicates a paramount role for IL-33 in the maintenance of gut mucosal homeostasis. (Lopetuso et al., 2012)

## **6.2 Role of IL-33 in gut homeostasis**

### *6.2.1 Maintenance of the intestinal epithelial barrier*

IL33/ST2 signaling play an important role in maintaining the physical barrier of the intestinal mucosal epithelium as the first line of defense against pathogens. It has the ability to promote epithelial proliferation and mucus production (Hodzic et al., 2017; Schmitz et al., 2005). In an intact barrier, cellular contacts provide the signals to produce full-length IL-33 constitutively but neither process nor release it. In these barrier cells, full-length IL-33 resides in the nucleus, where it controls gene expression and is possibly involved in maintaining their resting state.

A study by Mahapatro et al. demonstrated that intestinal IL-33 expression is localized to the pericryptal fibroblasts during homeostasis and is increased during infection (Mahapatro et al., 2016). They also elucidated a role for IL-33/ST2 signaling in the differentiation of stem cells in organoid culture. Activation of the IL-33/ST2 pathway in epithelial progenitor cells leads to inhibition of Notch signaling and results in the differentiation of stem cells towards a secretory intestinal cell lineage. These secretory cells include Paneth cells, goblet cells, and enteroendocrine cells. Specifically, goblet cells are known to play an important role in intestinal immunity by producing mucin, the main constituent of mucus, an important barrier mechanism (Liew et al., 2016). Moreover, mice injected with IL-33 in vivo produce more colonic mucin (Monticelli et al., 2015a).

### 6.2.2 *Maintenance of the gut microflora*

It was demonstrated that mice deficient in IL-33 are dysbiotic or have a higher concentration of pro-inflammatory bacteria composition in their microbiome compared to their wild-type (WT) controls. This dysbiosis promoted release of IL-1 $\alpha$ , which drives colitis and tumor formation (Malik et al., n.d.). These mice lacking IL-33 were specifically deficient in IgA but had equivalent levels of other immunoglobulins (Malik et al., n.d.). The lack of IgA resulted in the continued presence of colitogenic bacteria that would normally be identified for removal by the binding of IgA. These findings demonstrated the role of IL-33 in modulating intestinal inflammation through interactions with the gut microbiome (Hodzic et al., 2017).

### 6.2.3 *Activation of local inflammation*

While IL-33 is necessary for maintaining the intestinal barrier, once the epithelial barrier is breached, IL-33 functions as an alarmin/danger signal to alert the immune system of a local threat, by recruiting and activating innate immune cells to mount an effective, inflammatory reaction to promote a type 2 inflammatory response, in order to restore normal gut homeostasis. IL-33 has been shown to enhance mucosal defenses against intestinal parasites and bacteria, as described for *Toxoplasma gondii*, *Pseudomonas aeruginosa* and *Leptospira* infection, indicating a primary role of protection. (Huang et al., 2007; Jones et al., 2010; Wagenaar et al., 2009).

When tissue damage is accompanied by pathogen or commensals infiltration, IL-33 functions are skewed towards pathogen removal through Type 1 immune responses (Bonilla et al., 2012) (See Figure 10). PAMP-activated myeloid derived antigen presenting cells (APCs) secrete IL-12 and IL-23. IL-12 upregulates ST2 expression by T cells and NK cells and facilitates IL-33 mediated augmentation of IFN $\gamma$  and TNF production. The Type 1 cytokines can promote the secretion of IL-33 antagonist, sST2. IL-23 counteracts the IL-33 driven signaling in ST2<sup>+</sup> Treg cells. Once the pathogens are eliminated, IL-33 then supports the tissue repair (Liew et al., 2016) Tissue repair is mediated through the release of AREG by ILC2s and Tregs, which binds to its receptor EGFR to restore mucosal integrity



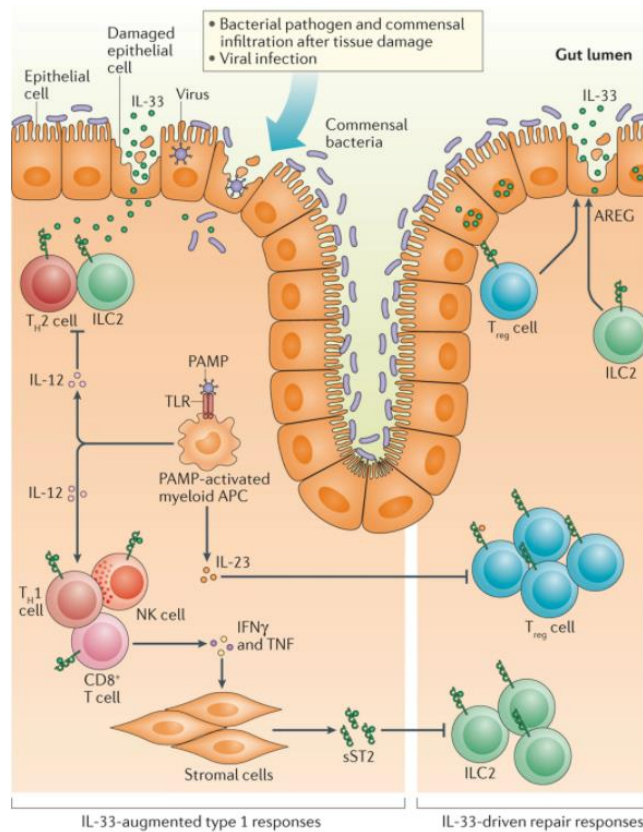


Figure 10. IL-33 activity in the Gut (Liew et al., 2016)

#### 6.2.4 Regulation of balance between TH17 and Tregs

IL-33 appears to influence both Tregs and Th17 cells to reduce inflammation and protect the gut mucosa from self-inflicted damage.

Th17 cells promote inflammation to help combat invading pathogens (pathogenic bacteria and some fungi, microbes probably not well covered by Th1 or Th2 immunity) (Weaver et al., 2007). These cells produce IL-17A (also referred to as IL-17), IL-17F, and IL-22, cytokines involved in neutrophilia, tissue remodeling and repair, and production of antimicrobial proteins. Th17 cells differentiate in response to the STAT3-activating cytokines IL-6, IL-21, and IL-23 along with TGF- $\beta$  and IL-1 $\beta$ . The differentiation of CD4+ T cells that produce IL-17 and IL-22 is influenced by the composition of the intestinal microbiota and by the presence of innate immune cells that amplify the Th17 cell response (Littman and Rudensky, 2010).

A study elucidated a role for IL-33 in regulating Th17 cells in the gut by downregulating pro-inflammatory cytokine expression and limiting expansion of Th17 cells (Pascual-Reguant et al., 2017). These authors demonstrated that IL-33 is released by epithelial cells of the small intestine, using a model of intestinal inflammation, via injection of anti-CD3 antibody into

mice. Intestinal epithelial cells were the predominant source of IL-33 in the small intestine during inflammation. This finding is important because it highlights the context-dependent expression of IL-33. In the steady state, the intestinal epithelium is not a principle source of IL-33, but in the setting of inflammation, intestinal epithelial cells are indeed significant sources of IL-33. Moreover, while Th17 cells do not express the ST2 receptor at baseline, these cells from mice injected with anti-CD3 express ST2. Once they express ST2, the IL-33/ST2 axis induces a downregulation of pro-inflammatory markers and an upregulation of the anti-inflammatory cytokine IL-10. Furthermore, they also demonstrated that mice deficient in the ST2 receptor have a more robust expansion of the TH17 cell population in the setting of inflammation compared to WT control. These data indicate that via the suppression of TH17 cells, IL-33 is an important modulator of inflammation in the small intestine and can help attenuate inflammation in the setting of TH17-mediated cell injury.

On the other hand, in another study it was shown that IL-33 signaling axis promotes an anti-inflammatory Treg phenotype in the setting of colonic inflammation in mice as a means of protecting the intestinal epithelium from ongoing damage (Schiering et al., 2014a). ST2 expression on Tregs is constitutive, and it is highly expressed on Tregs of the colon. IL-33 promotes expansion of this Treg population and acts as a “homing beacon” for ST2-expressing Tregs in the setting of inflammation. Intriguingly, IL-23, a pro-inflammatory cytokine with a clearly defined pathogenic role in IBD, inhibits the IL-33/ST2 signaling axis. This data suggests that IL-33 plays an important role in preventing secondary tissue damage in inflammatory reactions, particularly in the large intestine.

### **6.3 Role of IL-33 in IBD**

Inflammatory bowel disease (IBD) is characterized by chronic, relapsing inflammation with global epithelial barrier dysfunction and persistent leakiness. Crohn’s disease (CD) and ulcerative colitis are the two major forms of inflammatory bowel disease. Although the etiology of both CD and ulcerative colitis remains obscure, the imbalanced cytokine production and T-cell dysfunction are considered the key factors in inflammatory bowel disease pathogenesis (Baumgart and Carding, 2007).

In chronic inflammatory disease, such as active ulcerative colitis (UC), IL-33 expression is markedly increased in intestinal epithelial cells and infiltrating lamina propria mononuclear

cells, belonging to the monocyte/macrophage and B-cell lineages. IL-33 mucosal tissue levels also showed a positive correlation with the severity of gut inflammation. Increased IL-33 serum concentrations have also been detected in IBD patients in comparison with age-matched healthy controls (Beltrán et al., 2010; Pastorelli et al., 2010). The increased serum levels of IL-33 in IBD patients reflect an active inflammatory state and represent a potential biomarker for disease activity. IL-33 mRNA has also been associated with UC disease activity.

The intestinal tissue expression pattern of ST2 is different in healthy mucosa compared to that found in chronically inflamed IBD patients, wherein ST2 is abundantly expressed in macroscopically noninflamed colon epithelium. During chronic inflammatory processes characterizing either UC or CD, its expression is lost/decreased and redistributed. Recruitment of ST2 positive cells into the lamina propria is a common feature shared by different intestinal inflammatory conditions, whereas epithelial changes in ST2 expression appear to be specific for IBD (Lopetuso et al., 2012; Pastorelli et al., 2011).

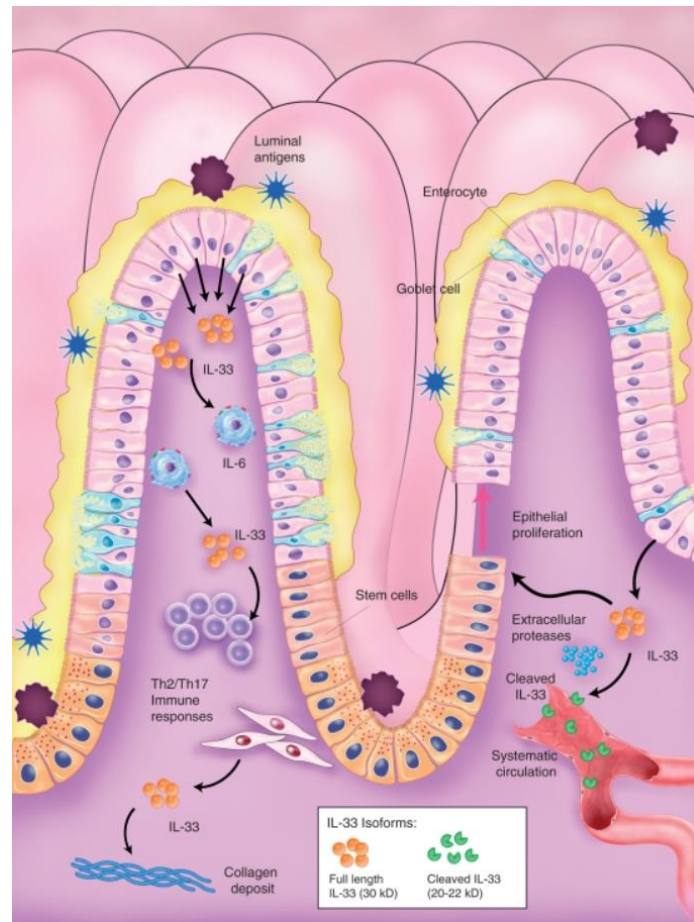


Figure 11. Working hypothesis of IL-33's role in inflammatory bowel disease (Pastorelli et al., 2011) The bioactive IL-33 is produced primarily by intestinal epithelial cells, as well as lamina propria mononuclear cells. Potent levels of IL-33 are released from stressed gut epithelial cells as a danger signal either by intrinsic defects (e.g. structural impairment affecting cell integrity, barrier dysfunction, etc.) or extrinsic exposure to harmful stimuli, as in the case of DSS (Dextran Sodium Sulphate) challenge. This initiates an inflammatory cascade that leads to intestinal inflammation. IL-33 has the ability to promote pro-inflammatory cytokine production, such as IL-6, as well as Th2 and Th17 adaptive immune responses. Intestinal myofibroblasts have also recently been reported as a source of IL-33 in inflammatory bowel disease (IBD). Bioactive IL-33 can potentially be cleaved by extracellular proteases within the gut mucosa, dampening the pro-inflammatory effects of IL-33. The cleaved products enter the systemic circulation, where they can serve as a bioactivity disease marker for IBD. IL-33 has also been shown to promote epithelial proliferation and can alternatively be involved in epithelial repair and wound healing. Loss and/or redistribution of ST2 expression on the epithelium of IBD patients may indicate disruption of IL-33/ST2 signaling, leading to ineffective restoration of epithelial barrier integrity, facilitating translocation of luminal, bacterial products and the perpetuation of chronic intestinal inflammation.

IL-33 knockout (KO) mice were subjected to dextran sodium sulfate (DSS). Colonic inflammation induced by DSS occurs in a T-cell-independent manner and results from disruption of the epithelial barrier and consequent exposure of innate immune cells to gut luminal contents, including the bacterial microflora. IL-33 KO mice displayed less prominent inflammation, with a significant decrease in granulocyte infiltration during the acute phase of

the colitis compared with wildtype littermates (Oboki et al., 2010). Subsequently, during the recovery phase of the experiment, weight recovery was markedly delayed in IL-33 KO mice, whereas no significant difference in colonic inflammation was observed between IL-33 KO and wild-type littermates. It was suggested that IL-33 plays an important role in driving acute, innate immune responses, but is dispensable in the maintenance of a chronic inflammatory state. Alternatively, with the delayed weight recovery observed in IL-33 KO mice, the possibility of the potential role of IL-33 in restoration of gut homeostasis and mucosal healing could also exist (Pastorelli et al., 2011).

As opposed to the aforementioned results, another study showed that IL-33 administration during repeated, chronic cycling of DSS caused a reduction of colitis, suppressed interferon gamma (IFN $\gamma$ ), and decreased bacterial translocation (Groß et al., 2012). This supported a protective role of IL-33 that was suggested to occur by switching from Th1-to Th2-driven immune responses. These results were supported by a study using the trinitrobenzene sulfonic acid (TNBS)-induced model of colitis, which represents another chemically induced model of colonic inflammation, reported to elicit Th1 immune responses (Duan et al., 2012). IL-33 expression was found to be elevated. With exogenous administration of IL-33, the TNBS-induced colitis ameliorated and induced the production of Th2-type cytokines. In addition, the protective effect of IL-33 was found to be diminished after depletion of Treg cells. It was suggested that the IL-33-induced expansion of Foxp3<sup>+</sup> Treg cells facilitates the decrease in the severity of colitis. Moreover, administration of exogenous IL-33 to mice with DSS-mediated intestinal epithelial injury resulted in the activation of ILC2 cells and amelioration of the clinical manifestations of intestinal inflammation (Monticelli et al., 2015a). In addition, they had improved restoration of normal colonic crypt architecture and increased expression of genes involved in tight junction formation as compared to PBS-treated mice subjected to DSS colitis.

In conclusion, IL-33 possess dichotomous functions within the gut mucosa, where IL-33 can possess both classic pro inflammatory properties as well as protective anti-inflammatory functions, depending primarily on the presence of receptor bearing cells during a particular setting. The figure 12 depicts the potential dichotomous function of IL-33 in intestinal inflammation, where pro-inflammatory stimuli, such as tumor necrosis factor (TNF), IL-1b and pathogens-associated molecular patterns (PAMPs), increase intracellular IL-33 expression in intestinal epithelial cells. IL-33 is subsequently released by necrotic epithelial cells as a potential alarmin. Depending on the presence and abundance of ST2-bearing target cells, IL-33

may have different global effects within the gut mucosa. Encounter with activated immune cells, IL-33 may enhance immune responses that can further exacerbate the severity of inflammation. Concomitantly, IL-33 can also act on intestinal epithelial cells and myofibroblasts to induce epithelial proliferation and repair as well as promote wound healing. (Lopetuso et al., 2012; Pastorelli et al., 2011). The recruitment of IL-33-positive fibroblasts to ulcers is considered a protective process driven by the loss of the epithelial barrier and by the presence of the gut microflora that acquires a critical role in modulating mucosal healing. IL-33 initiates a tissue-repair or wound-healing process to ensure quick sealing of the breach in the barrier.

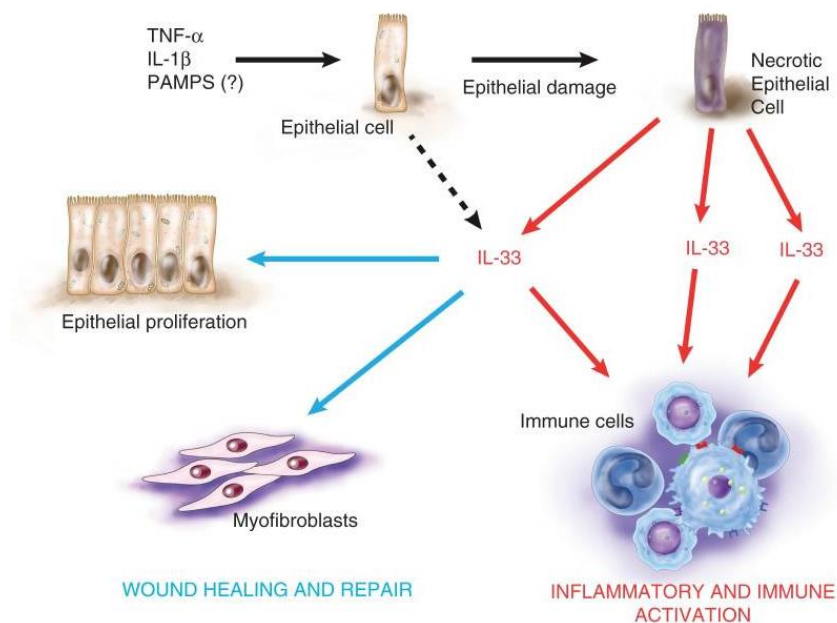


Figure 12. Potential dichotomous functions of IL-33 in intestinal inflammation (Pastorelli et al., 2011)

## 7. IL-33 in tissue repair and fibrosis

IL-33 has an important role in resolution of inflammation and repair of tissue damage. This is achieved either by its direct effect on subepithelial myofibroblasts and IEC (as mentioned in the previous section) or indirectly, by targeting ST2 expressing cells (Tregs and ILC2s) (Burzyn et al., 2013a; Monticelli et al., 2011).

IL-33 has been found to participate in the early inflammatory process of wound healing as other members of the IL-1 family. Increased IL-33 mRNA and protein expression have been demonstrated after scratching *in vivo*, suggesting its role in wound healing (Lee et al., 2016;

Yin et al., 2013). Moreover, administration of rIL-33 accelerated wound healing with enhanced re-epithelialization. IL-33 upregulation also promoted the collagen deposition and the expression of extracellular matrix (ECM)-associated genes such as fibronectin and collagen IIIa, which implies a direct effect of IL-33 on matrix synthesis. Furthermore, IL-33 facilitated the development of alternatively activated macrophages in incisional wound tissue, which closely related to resolution of inflammation and promotion of wound repair (Yin et al., 2013). IL-33 signalling for repair induced by Treg cells and ILC2s is mediated in part by amphiregulin (AREG), the epidermal growth factor (EGF) like growth factor (Burzyn et al., 2013a; Monticelli et al., 2011). The role of amphiregulin is described below in a dedicated section.

When the IL-33/ST2 axis is dysregulated, this important ligand-binding pair can also play a critical role in the progression of chronic inflammation and fibrosis. Fibrosis is commonly believed to represent the end stage of the repair process during chronic and/or relapsing tissue damage, wherein inflammatory cell infiltration and resident fibroblast activation persists, while the reparative ability of mesenchymal stem cells is diminished (Lopetuso et al., 2012). Activation of quiescent fibroblasts to ECM-producing myofibroblasts is a normal part of wound-healing mechanisms and is driven mainly by transforming growth factor- $\beta$  (TGF- $\beta$ ). Myofibroblasts are the major producers of extracellular matrix components and an expansion of myofibroblasts is observed during fibrosis, inducing permanent tissue scarring and eventually organ failure. The progressive replacement of parenchymal tissues with ECM is observed in fibrotic diseases, such as systemic sclerosis, idiopathic pulmonary fibrosis, and liver cirrhosis, leading to impaired organ function. Fibrosis is estimated to contribute to nearly 45% of deaths in the developed world (Rankin et al., 2010).

Repeated subcutaneous administration of IL-33 to WT mice has been shown to lead to the accumulation of inflammatory cells and development of subsequent cutaneous fibrosis. IL-33-induced fibrosis developed through an IL-13-dependent mechanism, involving ST2 expressing ILC2 and eosinophils (Rankin et al., 2010). Human patients with systemic sclerosis have elevated IL-33 levels that correlated with the extent of skin and lung fibrosis (Yanaba et al., 2011).

Gut mucosal damage leading to release of IL-33 upregulates the Th2 cytokine response along with induction of proliferation of SEMFs. Chronic and repetitive epithelial damage leads consequently to fibrosis (Lopetuso et al., 2012) (See Figure 13). Within the gut mucosa, colonic

subepithelial myofibroblasts SEMFs are primary sources of IL-33, particularly in ulcerated lesions from patients with UC. In conclusion, sustained repair efforts driven by IL-33 may lead to type 2 cytokine supported fibrotic diseases.

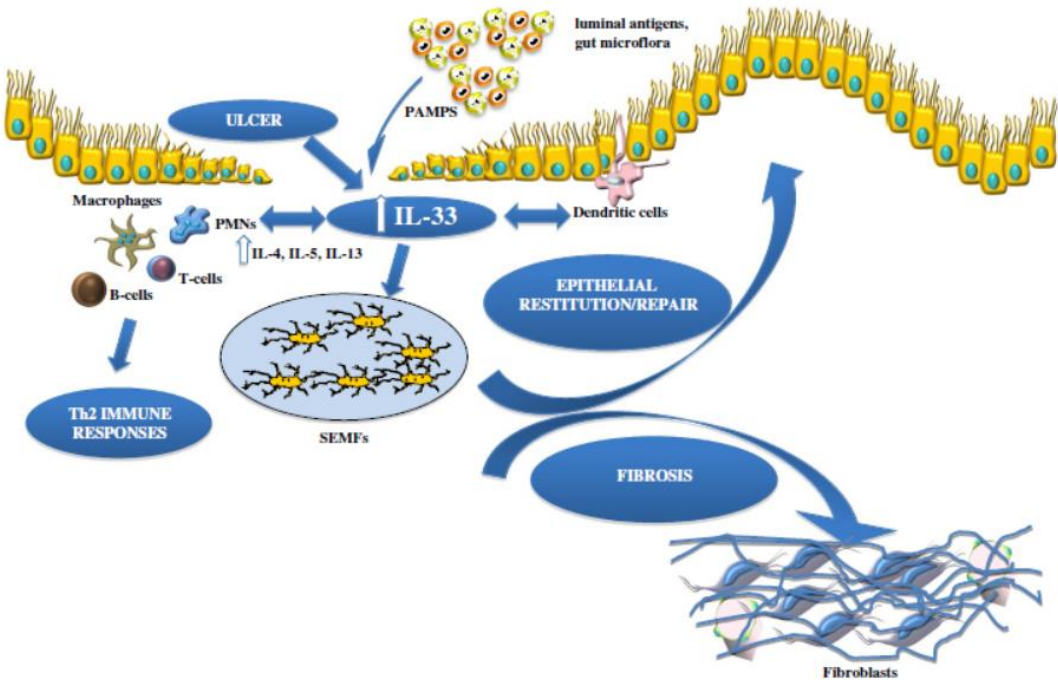


Figure 13. Working hypothesis of role of IL-33 in gut mucosal repair and fibrosis (Lopetuso et al., 2012)



## **Part II. Tissue Treg**

Regulatory T cells are a unique subset of helper T cells, which maintain a delicate balance between overactive responses and immunosuppression. Contrary to initial interpretations of Tregs being a universal immunosuppressive population, in recent years, they have also been identified as key players in tissue inflammation resolution and repair. They are defined by stable expression of the lineage-specific transcription factor Foxp3 and high amounts of the interleukin (IL2) receptor  $\alpha$ -chain (CD25). They represent 5-10 % of peripheral CD4<sup>+</sup> T cells. In addition to serving as a Treg cell marker, CD25 expression is essential for the differentiation and proliferative fitness of Treg cells. Mice lacking the IL2R chains suffer from massive systemic autoimmunity that can be reversed by the adoptive transfer of CD4<sup>+</sup>CD25<sup>+</sup> T cells from wild-type mice (Furtado et al., 2002, p. 2). The central role of Treg cells in peripheral tolerance is highlighted by the development of fatal autoimmunity in scurfy mice, which lack the gene encoding Foxp3, and in mice with Foxp3 deficiency specifically in T cells. Moreover, people with IPEX syndrome (immune dysregulation, polyendocrinopathy, enteropathy, X-linked), who have mutations in FOXP3, are susceptible to many autoimmune diseases. Notably, Treg cell-deficient mice and patients with IPEX syndrome often develop gastrointestinal and skin manifestations, which highlights the critical role of Treg cells in the maintenance of homeostasis at barrier sites.

### **1. Classification and differentiation of Treg cells**

#### **1.1 Development pathways**

Like the development of all T cells that express the  $\alpha\beta$  T cell antigen receptor (TCR), the development of Treg cells begins in the thymus, where two distinct pathways begin to diverge. Thymus-derived Treg cells (tTreg cells) differentiate into Foxp3<sup>+</sup> Treg cells in the thymus following recognition of self-antigen by the TCR. In contrast, peripherally derived Treg cells (pTreg cells) exit the thymus as naive CD4<sup>+</sup> T cells and differentiate into Foxp3<sup>+</sup> Treg cells in the periphery following recognition of their cognate antigen, which is regarded as being non-self. Specifically, strong TCR signaling, suboptimal costimulation, and high amounts of TGF $\beta$  and retinoic acid favor the induction of Foxp3 in peripheral naïve CD4<sup>+</sup> T cells (Chen et al., 2003; Josefowicz et al., 2012a; Plitas and Rudensky, 2016). Helios and neuropilin-1 (Nrp1)

have been widely used as markers to distinguish tTreg (Helios<sup>+</sup> Nrp1<sup>+</sup>) and pTreg (Helios<sup>-</sup> Nrp1<sup>-</sup>) cells (Weiss et al., 2012; Yadav et al., 2012). The roles of tTreg and pTreg cells are distinct, as mice deficient for a Foxp3 cis regulatory element—conserved non-coding sequence (CNS) 1, in which generation of pTreg cells, but not tTreg cells, is specifically impaired, with development of spontaneous inflammation in lung and gastrointestinal tissues (Josefowicz et al., 2012b). Gut is the main site of extrathymic differentiation of Treg cells, where short-chain fatty acids, in particular butyrate produced by commensal microorganisms, facilitates extrathymic generation of Treg cells. The differences between these Treg cell subsets suggest that tTreg cells are likely responsible for tolerance to self-antigens, whereas pTreg cells restrain immune responses to “non-self” antigens including allergens, commensal microbiota, and dietary antigens at barrier sites and paternal alloantigens (Plitas and Rudensky, 2016).

## **1.2 Activation status**

Treg could also be classified on Treg’s activation status into two separate populations: central Treg (cTregs) cells (also known as resting Treg cells) and effector Treg (eTregs) cells (also known as activated Treg cells or effector memory Treg cells). cTregs are comparatively quiescent Tregs in the lymphoid tissues. They express the lymphoid homing molecules CD62L and CCR7 and are dependent on IL2 secreted by Tconv in T cell zones of the lymphoid tissues. On the other hand, eTregs are primarily non-lymphoid Tregs which downregulate lymphoid homing molecules and upregulate CD44, inducible costimulator (ICOS), Glucocorticoid-induced tumor-necrosis-factor-receptor-related (GITR) as well as expression of various chemokine receptors (e.g., CCR4, CCR6, and CCR10) and adhesion molecules (e.g., KLRG1, CD103) and other activation induced markers. Most of these cells express the transcription factor BLIMP1 and produce IL-10, akin to the population of ICOS<sup>+</sup>IL10<sup>+</sup> Tregs in humans (Koizumi and Ishikawa, 2019; Sharma and Rudra, 2018).

As illustrated in the figure 14, thymic-derived central Tregs recirculate through secondary lymphoid organs. Upon TCR and CD28 signaling, central Tregs differentiate into effector Tregs. In response to inflammatory signals, the effector Tregs adopt additional transcription factors, which provide the means to suppress specific subsets of effector helper T cells. The effector Tregs gain expression of specific chemokine receptors that promote their migration to the targeted tissues. In non-lymphoid tissues, the effector Tregs and the tissue-resident Tregs expand upon tissue injury, suppressing inflammation and immunity, and promoting

homeostasis and repair. For example, visceral adipose tissue (VAT)-Treg cells accumulate in and suppress the inflammation of adipose tissue, thereby regulating insulin resistance (Feuerer et al., 2009; Kolodin et al., 2015). In the colon, Treg subsets expressing RAR-related orphan receptor (ROR)t and GATA-binding protein 3 (GATA3) are involved in inhibiting inflammation and tissue repair, respectively (Schiering et al., 2014a, p. 33; Sefik et al., 2015a). Thus, each tissue-specific Treg subset exhibits distinct functions and phenotypes, likely due to tissue-specific environmental cues. In a Th1 inflammatory context, Tregs express transcription factor Tbet, responsible for Th1 differentiation, and express CXCR3 to accumulate at such sites. Transcription factor STAT3 is expressed in Tregs in a Th17 context, which helps in upregulation of CCR6 for migrating to the intestine and for production of IL10. While Bcl6 expression in Tregs was shown to be important for regulating Tfh cells and expression of CXCR5 (Rothstein and Camirand, 2015; Sharma and Rudra, 2018).

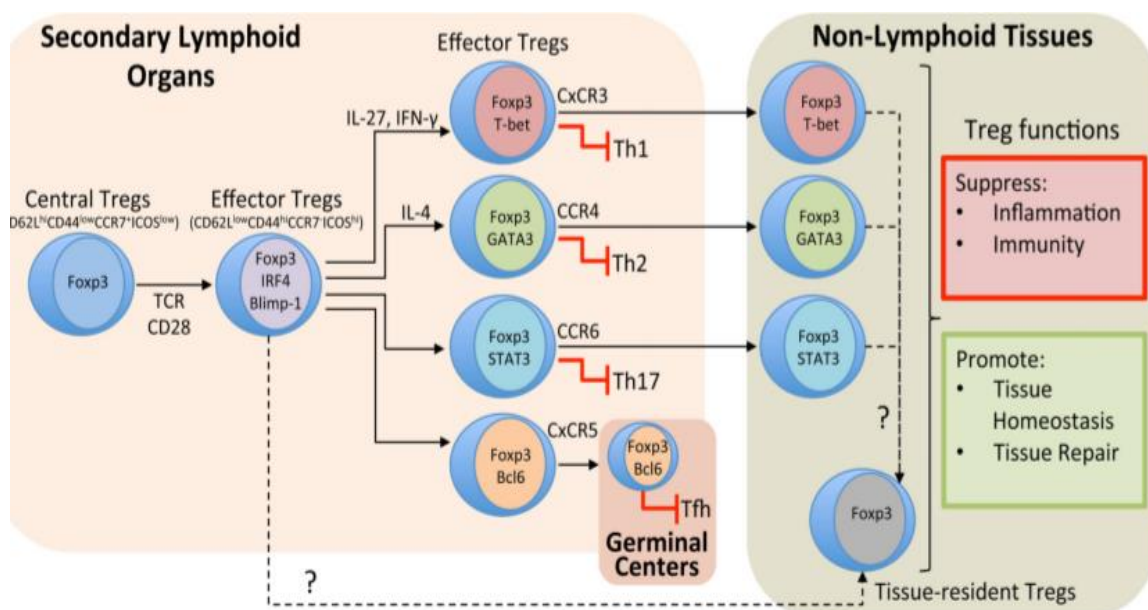


Figure 14. Diversity of Treg subsets and specialization of function (Rothstein and Camirand, 2015)

Recent studies have revealed substantial heterogeneity in gene expression profiles within each Treg subset, suggesting a considerable functional diversity of Treg cells. In fact, single-cell RNA-sequencing (RNA-seq) analysis of Treg cells has demonstrated the complexity of cellular states in both cTreg and eTreg cells. It has shown that eTreg cell diversity is affected by TCR signal strength. In addition, comparative single cell RNA-seq analysis of Treg cells in lymphoid and non-lymphoid tissues (skin and colon) suggested that Treg heterogeneity is associated with the progressive trajectory of Treg states from the lymph nodes to non-lymphoid tissues and the

adaptation of Treg cells to each peripheral tissue. Furthermore, that differentiation and functional states of distinct Treg subsets are regulated by various transcription factors as shown in figure 15 (Koizumi and Ishikawa, 2019).

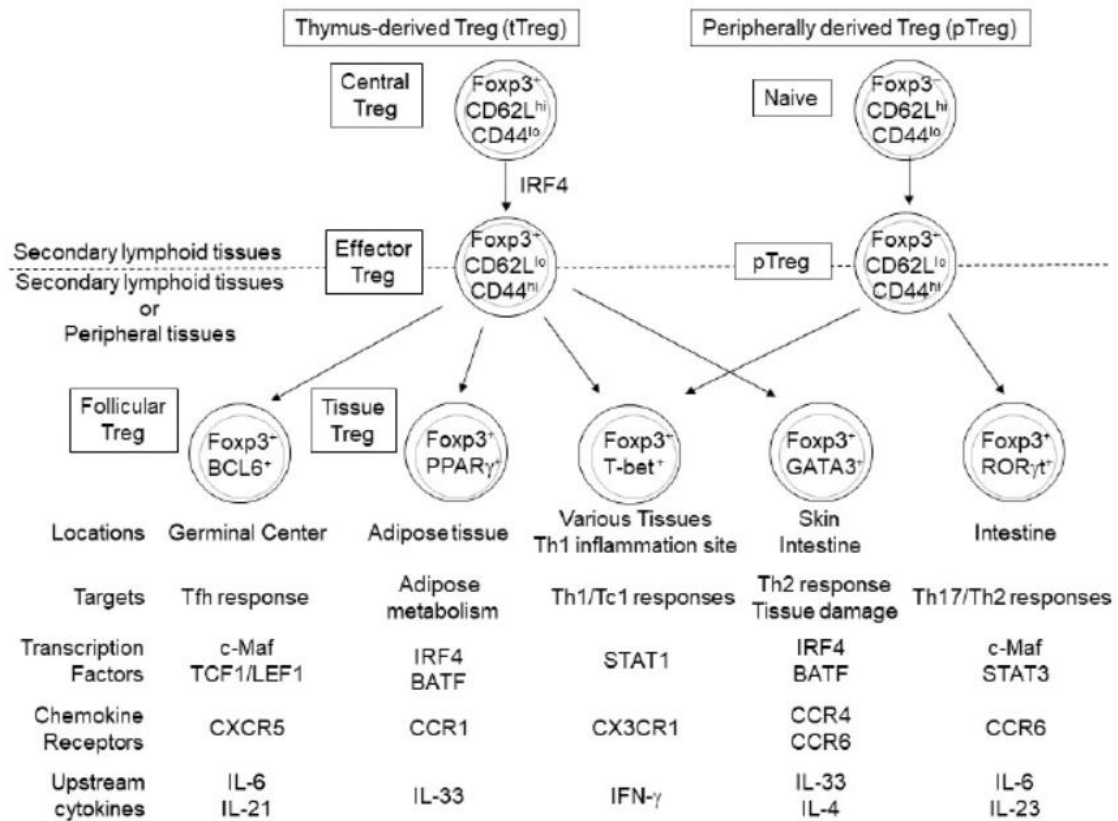


Figure 15. Diversity of Treg subsets and specialization of function

### 1.3 Suppression mechanisms

The suppressor function of Treg cells is essential for the restraint of fatal autoimmune and inflammatory responses. This was demonstrated by the generation of knock-in mice expressing diphtheria toxin receptor (DTR) under control of the Foxp3 locus. Elimination of Treg cells in Foxp3DTR mice in adult healthy mice upon repeated exposure to diphtheria toxin (DT) resulted in a disease similar to that found in Foxp3null mice and death within 10 to 14 days with massive expansion and activation of immune cells including granulocytes, dendritic cells (DCs), NK cells, CD4+ and CD8+ T cells, and B cells (Kim et al., 2007).

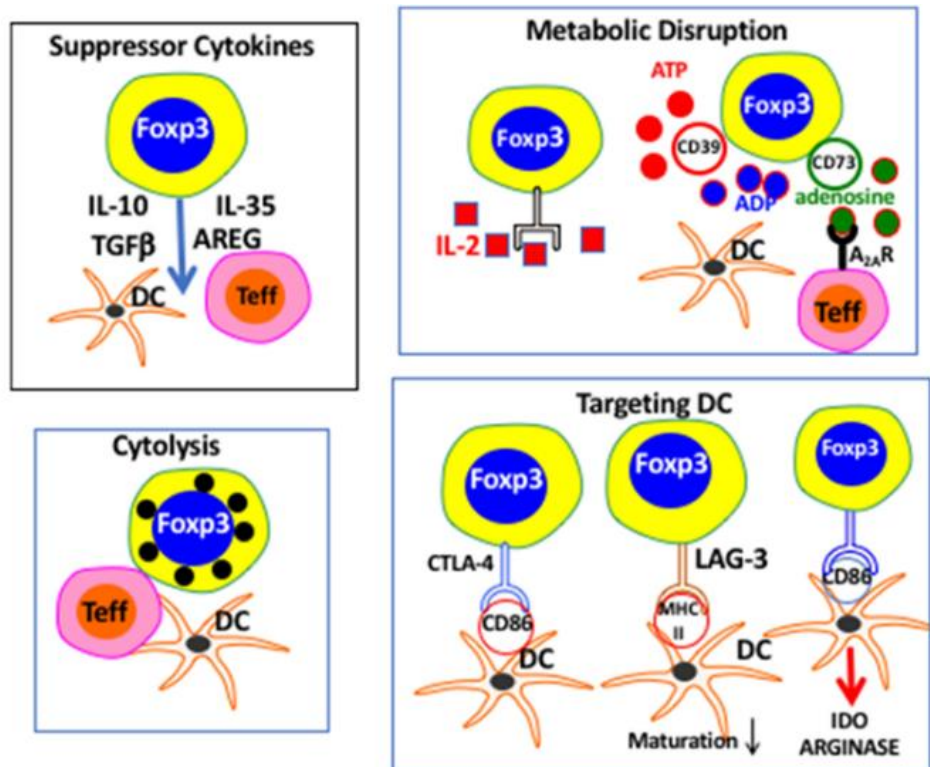


Figure 16. Proposed pathways of Treg-mediated suppression. (Shevach, 2018)

Figure 16 summarizes the proposed pathways by which Tregs may manifest their suppressor effector functions. These include: 1) the release of soluble suppressor cytokines, like IL-10, TGFb and AREG, 2) cytolysis, direct killing, 3) disruption of metabolic pathways, and 4) pathways that target DCs. There is not one universal pathway by which Tregs mediate suppression. It depends on what is suitable for a particular situation or inflammatory niche.

i) Release of suppressor cytokines

Production of immunomodulatory cytokines such as IL-10, TGFb, IL-35 and amphiregulin (AREG) by the Treg cells, are capable of suppressing pro-inflammatory responses of T cells, B cells, NK cells dendritic cells and macrophages (Plitas and Rudensky, 2016). Selective ablation of IL-10 in Foxp3<sup>+</sup> Treg cells revealed that IL-10 production by Treg cells is essential for keeping the immune response in check at environmental interfaces such as colon and lungs. Another immunosuppressive cytokine secreted by Treg cells, IL-35, has also been suggested to maintain immunological tolerance in the gut. TGF-β produced by Treg cells can suppress Th1 responses (Josefowicz et al., 2012a).

AREG also has a critical role for the efficient CD4<sup>+</sup> T regulatory cell function. In the absence of AREG, the Treg cells cannot suppress local inflammation. The activated CD4 Treg cells

migrate to the site of inflammation, where they are exposed to AREG, which enhances their suppressive capacity, possibly through the secretion of immunosuppressive exosomes. In chronic inflammation, mast cells derived AREG enhances Treg function (Dietmar M.W. Zaiss et al., 2013), whereas, in acute inflammation, like hypersensitivity reaction in the skin, basophils are described as the main source of AREG (Meulenbroeks et al., 2015). In addition, tissue resident Treg cells can also produce AREG that may act in autocrine fashion enhancing their function further (See Figure 17) (Burzyn et al., 2013a; Zaiss et al., 2015).

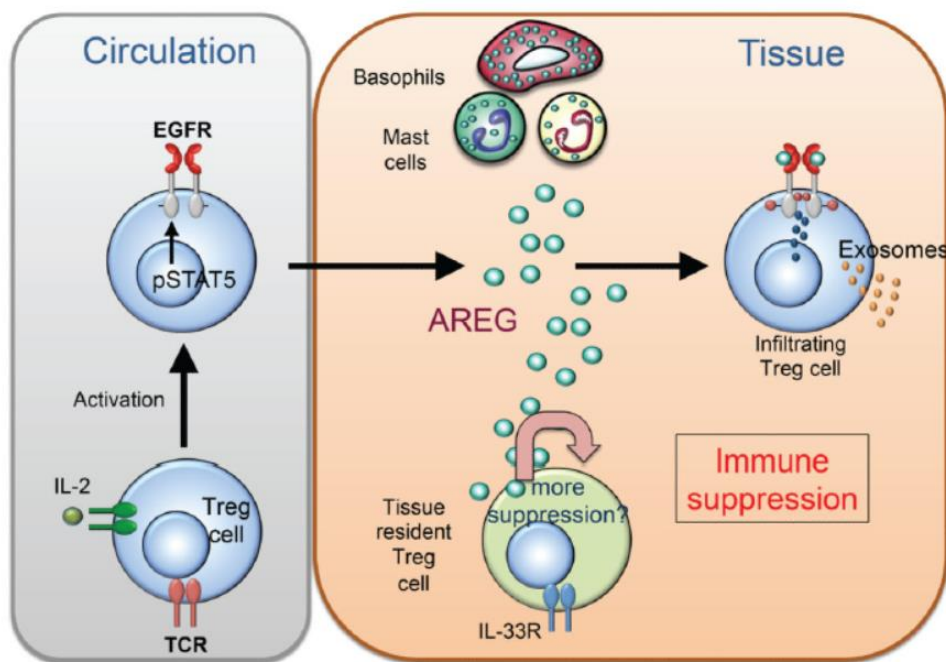


Figure 17. Amphiregulin enhances regulatory T cell function (Zaiss et al., 2015)

## ii) Cytolysis

Cytolysis of target cells as a means for Treg cell-mediated suppression was first suggested by the finding, in human Treg cells, granzyme A can be induced by a combination of CD3 and CD46 stimulation, resulting in the induction of apoptosis in activated target cells. Later several groups identified granzyme B as being highly upregulated in mouse Treg cells. Upon activation, Treg cells can kill either responder T cells or APCs in a granzyme B-dependent manner in vitro. These findings have been further confirmed by the in vivo studies in which granzyme B has been critical in maintaining Treg cell-dependent long-lived skin graft tolerance as well as in Treg cell-mediated suppression of tumor clearance (Josefowicz et al., 2012a).

### iii) Metabolic disruption

Several cell-surface molecules were proposed to play a role as mediators of Treg cell-mediated suppression; CD25, a subunit of IL-2 receptor (IL-2R) and the original Treg cell marker, is upregulated on effector T cells and is constitutively expressed at a high level on Treg cells. While indispensable in Treg cell homeostasis (Furtado et al., 2002, p. 2), high-level IL-2R expression on Treg cells could deprive effector T cells of IL-2 and inhibit their proliferation (Josefowicz et al., 2012a).

Tregs may also play their inhibitory role using ectoenzymes including the ectonucleoside triphosphate diphosphorylase-1 (CD39) and the ecto-5-nucleotidase (CD73) for their suppressive activity. CD39 and CD73 are highly expressed on the surface of Foxp3+ Tregs and have been increasingly used as markers of Tregs (Deaglio et al., 2007; Mandapathil et al., 2009; Schuler et al., 2011). Human Tregs highly express CD39 on their surface but negligible CD73 (Mandapathil et al., 2010). These CD39 and CD73 enzymes hydrolyse extracellular pools of inflammatory ATP into adenosine diphosphate (ADP) and/or adenosine monophosphate (AMP) to adenosine. Adenosine promotes a depressive action on the immune cell activity and exerts a potent anti-inflammatory effect. Adenosine signaling not only directly inhibits the proliferation of effector T cells, but also negatively impacts the function of dendritic cells (Antonioli et al., 2013, p. 73; Josefowicz et al., 2012a). The inhibitory control by Treg-derived adenosine is mediated by the engagement of A2A receptors on effector T cells. In another study, it was observed that the CD73-derived adenosine produced by Tregs downregulates NF- $\kappa$ B activation in effector T cells through A2A receptors, thereby reducing the release of a broad spectrum of proinflammatory cytokines and chemokines. In addition, a self-reinforcing loop in the immunosuppressive activity of Tregs was also highlighted, driven by adenosine generation. In particular, the activation of A2A receptors, expressed on Tregs, promoted the expansion of these cells, which in turn acquired an increased immunoregulatory activity (Ohta et al., 2012)(Antonioli et al., 2013, p. 73).

### iv) Targeting DCs for immunosuppression

Treg cell-specific surface molecule, cytotoxic T-Lymphocyte Antigen 4 (CTLA-4), is implicated in Treg cell-mediated suppression function. High expression of CTLA-4 enables downregulation of costimulatory molecules CD80 and CD86 expression on DCs. The reduced suppression capacity of CTLA-4-deficient Treg cells is due to their inability to downregulate

CD80 and CD86 via trans-endocytosis. Mice with a selective loss of CTLA-4 in Treg cells were analyzed. In these studies, selective CTLA-4 deficiency resulted in greatly increased numbers and activation of Treg cells under inflammatory conditions, yet the suppressive activity of CTLA-4-deficient Treg cells was impaired (Josefowicz et al., 2012a; Sakaguchi et al., 2010, p. 3; Wing et al., 2008).

In addition, Treg can also increase the expression of the enzyme indoleamine 2, 3-dioxygenase (IDO) in DCs via CTLA-4 induced signaling, resulting in starvation of Tconv and arrest of cell cycle, as IDO can catalyze degradation of the essential amino acid tryptophan, and also induces iTreg generation (Lei et al., 2015).

There are also other molecules, which Treg cells might use to control APC function. For instance, LAG-3, a CD4 homolog that exhibits a high binding affinity with MHC class II, is suggested to be required for maximal suppressive activity of both natural and induced Treg cells (Huang et al., 2004, p. 3). Moreover, TIGIT is expressed, like CTLA-4, at a high level on Treg cells and on activated conventional T cells. Upon Treg cell interactions with dendritic cells, TIGIT appears to induce production of immunosuppressive cytokines IL-10 and TGF- $\beta$  by dendritic cells (Josefowicz et al., 2012a). Furthermore, TNF receptor family members such as GITR are also important players in controlling Treg cell function. It seems likely that engagement of GITR on the surface of effector T cells enhances their responses, effectively releasing these cells from Treg cell-mediated suppression. Consistent with these results, GITR deficiency does not result in noticeable aberrations in immune homeostasis or tolerance (Josefowicz et al., 2012a).

Further increasing the complexity and diversity of Tregs, several Treg subpopulations are discovered in non-lymphoid tissues (tissue resident Tregs). These tissue Tregs are not only instrumental in suppression of inflammatory responses but also benefit organ homeostasis. They can be further identified by the virtue of tissue specific phenotypic and functional characteristics. We will elaborate these Treg populations in the context of adipose tissues, skin, muscle, and liver, with the primary focus on the intestine.

## **2. Tissue Tregs**

Several current works have linked IL-33/ST2 to controlling Tregs homeostasis in the non-lymphoid tissues (Kuswanto et al., 2016; Schiering et al., 2014). IL-33/ST2 drives positive



feedback loops in Treg activation. IL-33 either directly or indirectly enhances expression and function of the transcription factors Foxp3, GATA3 and STAT5, which in turn promote the expression of ST2 in Tregs.

## **2.1 Intestinal tissue Treg**

Like other non-lymphoid tissues, Tregs are enriched in the intestinal LP with the colon harboring 25-35% and the small intestine harboring 10-15% Treg among the total CD4 T cells (Sharma and Rudra, 2018).

Recent studies have identified multiple subsets of Tregs in the intestine by transcriptomic and functional analyses. The three specialized subsets are based on the expression of ROR $\gamma$ t and GATA3 expression. The figure 18 below illustrates the three subsets according to the expression of the transcription factors and the surface markers.

- i. More than 65% of the colonic Treg cells and around 35% of small intestinal Treg cells express ROR $\gamma$ t and are negative for Helios and neuropilin 1 (NRP1). They are considered to be peripherally derived Tregs (pTregs) that develop from naïve T cells in response to microbiota-derived antigens and metabolites, including short chain fatty acids (SCFAs). TGF $\beta$  along with DC generated vitamin A metabolite all-trans retinoic acid also differentiates naïve T cells into Tregs in presence of microbial antigens. These Tregs deploy various mechanisms towards establishing dominant tolerance with IL-10 production and direct suppression of Th17 cells as most prominent ones.
- ii. A second subtype, Foxp3<sup>+</sup> ROR $\gamma$ t<sup>-</sup> NRP1<sup>-</sup> constitutes 10% of the colon Tregs, while 40% of the Tregs in the small intestine. They are also considered to be pTreg cells that are induced by dietary antigens and have a role in tolerance to dietary antigens.
- iii. The third subtype is GATA3<sup>+</sup> Treg cells that do not express ROR $\gamma$ t and constitute about 30% of the colon Tregs. They are thought to be thymus-derived, expressing Helios and NRP1. The Gata3<sup>+</sup> Helios<sup>+</sup> subpopulation is less sensitive to the composition of the intestinal microbiota. Accumulation of these cells is promoted by IL-33 upon tissue damage through selective expression of ST2. Gata3<sup>+</sup> Treg cells are associated with protection against aberrant chronic inflammation, and GATA3 is important for Foxp3 expression and Treg cell accumulation in the intestine. Along those lines, intestinal ST2<sup>+</sup> Treg cells have high expression of the gut homing

receptors CCR9 and  $\alpha 4\beta 7$  (Whibley et al., 2019). These Foxp3<sup>+</sup> GATA3<sup>+</sup> Treg cells express ST2 and are important in the suppression of inflammation and facilitation of tissue repair by secretion of amphiregulin (Sharma and Rudra, 2018) (Tanoue et al., 2016). ST2<sup>+</sup> Tregs are superior to ST2<sup>-</sup> Tregs in suppressing CD4 T cell proliferation in vitro independent of IL-33, partially mediated by enhanced production and activation of the anti-inflammatory cytokines IL10 and TGF $\beta$  (Siede et al., 2016).

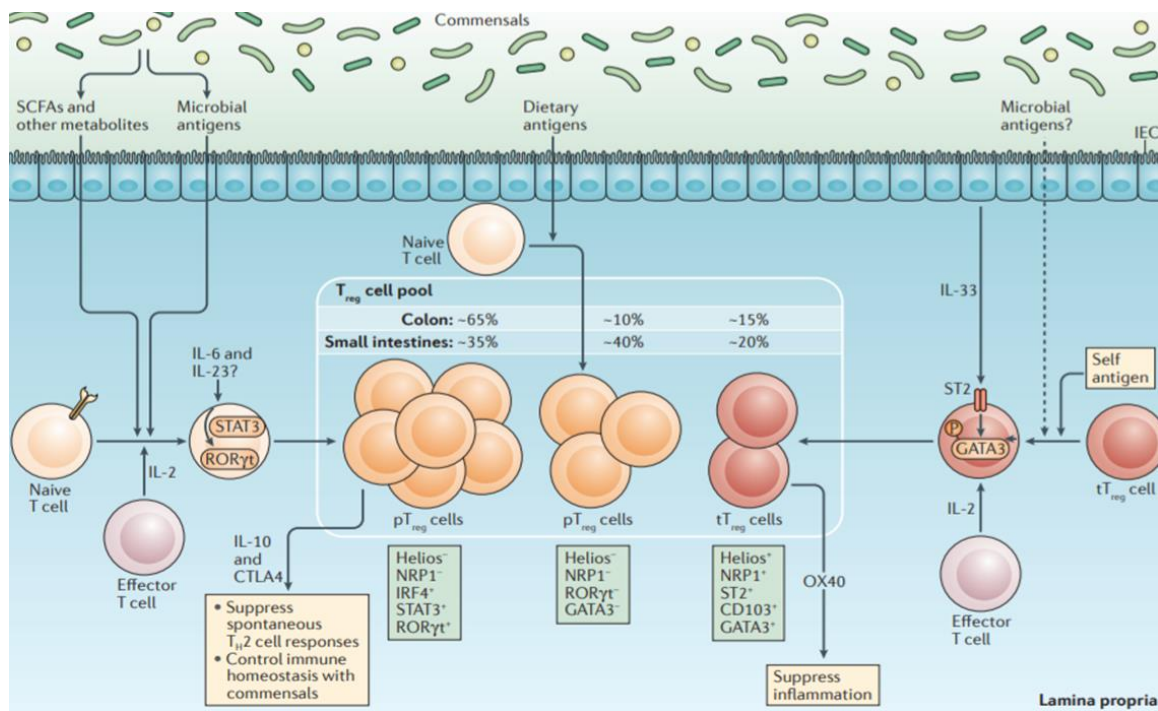


Figure 18. Three subpopulations of intestinal Tregs. Peripherally derived RORgt<sup>+</sup>NRP1<sup>-</sup> Helios<sup>-</sup> Treg subset, peripherally derived RORgt<sup>-</sup> helios-NRP1<sup>-</sup> Treg subset, thymic derived RORgt<sup>-</sup>Helios<sup>+</sup>NRP1<sup>+</sup>GATA3<sup>+</sup> Treg subset (Tanoue et al., 2016)

These subpopulations of intestinal Tregs in humans are still under exploration. Gata3<sup>+</sup> Tregs have been observed in human blood, but not the intestine (Wohlfert et al., 2011, p. 3), while RORgt<sup>+</sup> Tregs can be found in human colonic lamina propria from both healthy controls and patients with Crohn disease (Sefik et al., 2015a).

In humans, another subpopulation of intestinal CD161<sup>+</sup> Tregs has been recently described. This sub-population is a distinct, highly suppressive bona fide retinoic-acid dependent Treg enriched in lamina propria. They can enhance wound healing through soluble mediators including *IL17A* and *IL22*. The transcriptional control involves a transcription factor network in which

BACH2, ROR $\gamma$ t, RUNX1, FOXL2 and BATF, all play a role. Neutralization of IL-17 alone, IL-17 together with IL-22, IL-4 or IL-10 impeded the wound healing capacity of supernatants of activated CD161<sup>+</sup> Tregs, implicating these cytokines as mediators of wound healing. CD161<sup>+</sup> Treg cells are either induced to develop or specialize in the periphery and have relatively small circulating numbers. Human CD161<sup>+</sup> Treg cells have a retinoic acid-regulated gene signature and that retinoic acid directly regulates KLRB1, the gene encoding CD161. Bowel is rich in retinoic acid, an area for Treg generation and since CD161<sup>+</sup> Treg cells expressed CCR9, a gut-homing chemokine receptor, the lamina propria was enriched in CD161<sup>+</sup> Treg cells, particularly in IBD. The CD161 ligand, LLT1, is expressed on activated DCs and in actively inflamed areas. (G. A. Povoleri et al., 2018).

## **2.2 Adipose tissue Treg**

Feuerer et al identified unique fat residing Tregs in mouse and human adipose tissues (ATs) (Feuerer et al., 2009). Unlike the peripheral lymphoid compartment where normally 10-15% of CD4<sup>+</sup> T cells are Foxp3<sup>+</sup> Tregs, almost half of the fat CD4<sup>+</sup> T cells are Foxp3<sup>+</sup> Tregs. They are found to accumulate primarily in visceral fat over a period of time since birth, peaking at 25 weeks of age. In one study, it was found that this characteristic accumulation of Tregs was accompanied by a sudden drop in mice aged further, suggesting a negative correlation between the frequency of visceral WAT resident Tregs and age-related metabolic inflammation (Cipolletta et al., 2015). In contrast, in another study, the Tregs were even greater in number in aged mice (Bapat et al., 2015). This discrepancy could be due to a number of factors, including microbial and dietary composition as well as husbandry practices. These visceral WAT Tregs were still found to be functional.

These WAT Tregs appear to be largely of thymic origin. Adoptive transfer of congenically marked Tregs confirmed that visceral WAT Tregs are not significantly derived from circulating Tregs. In addition, very high expression of both Helios and neuropilin1 (Nrp1) as well as a transcriptomic analysis of visceral Tregs suggest their thymic origin, while little or no conversion of naïve T cells into visceral WAT Tregs (Kolodin et al., 2015). Most of these WAT Tregs fall in the category of eTregs. Visceral WAT Tregs specifically express ST2. Both IL-33 deficiency as well as ST2 deficiency was shown to result in specific reduction in number and percentage of visceral WAT Treg compartment. Development and proliferation of visceral WAT Tregs appears to be dependent on two signals, namely, the TCR crosslinking, inducing PPAR- $\gamma$  (transcription factor important in the differentiation and function of adipocytes) and

ST2 expression. IL-33 is expressed by human adipocytes and stromal cells, implying that adipose tissue itself creates a niche for the resident Treg population (Vasanthakumar et al., 2015). Other than those mentioned, these Tregs also express high levels of GATA3, KLRG1, CD69 and adipose signature chemokine, receptor CCR2 (Sharma and Rudra, 2018).

VAT Tregs prevent obesity-associated inflammation and preserve insulin sensitivity and glucose tolerance. Insulin resistance and metabolic syndrome has been suggested to be caused by adipose originated proinflammatory cytokines, like TNF $\alpha$ , IL6 and type 1 IFNs, leading to increased recruitment of proinflammatory immune cells. This proinflammatory polarization of immune signaling is accompanied by depletion of resident Treg cells. Human obese subjects were found to be deficient in circulating Tregs, whose levels were inversely correlated with body weight and BMI. Treg specific deletion of PPAR $\gamma$  resulted in more than 80% depletion of visceral fat Tregs. This decrease in Treg population was accompanied with marked inflammatory cell infiltration in visceral WAT. Contrary to obese animals, depletion of visceral WAT Tregs in aged animals, improved metabolic parameters and rescued aging induced insulin resistance (Bapat et al., 2015; Sharma and Rudra, 2018).

### **2.3 Muscle tissue Treg**

Muscle Tregs accumulated in the injured skeletal muscle of mice. They promoted a shift from pro- to anti-inflammatory myeloid population (Burzyn et al., 2013a; Castiglioni et al., 2015). However, paradoxical pathogenic role of Tregs was reported in chronic muscle injury infected with *Toxoplasma gondii*. Tissue damage was associated with prolonged and persistent pro-inflammatory macrophage accumulation, whereas ablation of Tregs rescued the macrophage homeostasis and skeletal muscle fiber regeneration (Jin et al., 2017).

ST2 is constitutively expressed on muscle Tregs. Mice with specifically ablated ST2 in Tregs showed impaired Treg accumulation in injured muscle. Muscle Tregs were expanded by IL-33, which exhibited a typical muscle Treg profile after injury, but IL-33 did not enhance the recruitment of circulating Tregs to the muscle. It suggests that IL-33 effects in muscle injury seems to primarily be local. Muscle Treg cells expressed the growth factor Amphiregulin, which acted directly on muscle satellite cells in vitro and improved muscle repair in vivo (See Figure 19) (Burzyn et al., 2013; Sharma and Rudra, 2018; Zhang et al., 2017).

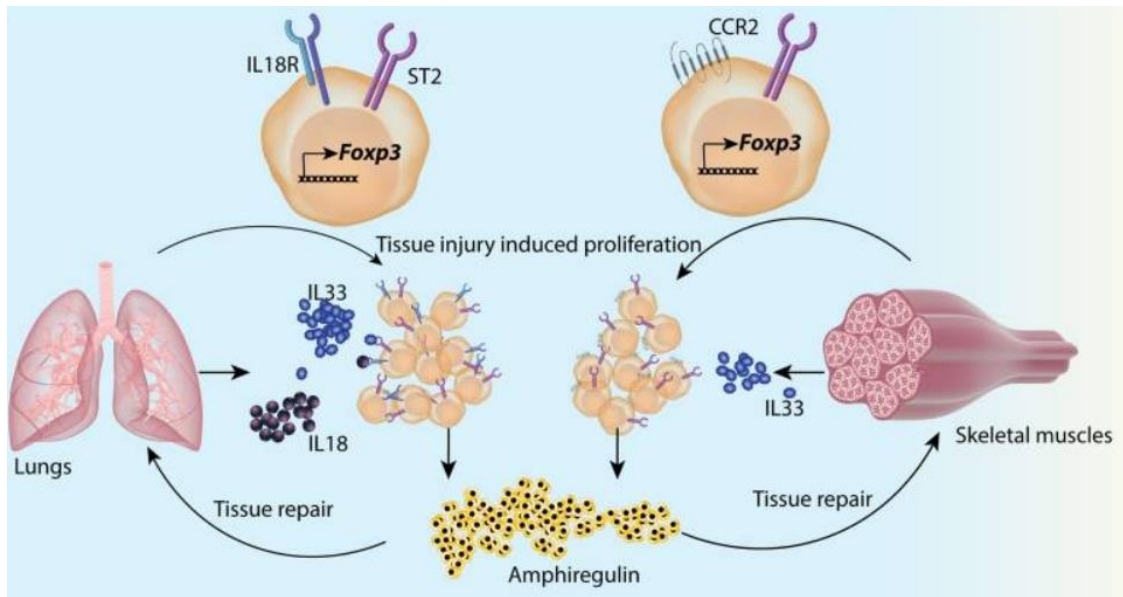


Figure 19. IL-33 mediated repair by Tissue Tregs in lungs and skeletal muscles (Sharma and Rudra, 2018)

The transcriptome of muscle Tregs was most similar to that of VAT Tregs, exhibiting 91% of the canonical Treg signature. However, the gene expression profile of muscle Treg was distinguishable from those of lymphoid organ Tregs and Tregs at several inflamed sites. They also displayed a distinct TCR which again suggested that such tissue Tregs may be responding to local signals. Genes encoding IL-10, CCR1, ST2 and AREG and platelet-derived growth factor were unregulated (Zhang et al., 2017).

Foxp3+CD4+ T cells have been shown to reside in human skeletal muscle, with an increased representation in muscle lesions of patients with Duchenne's muscular dystrophy.

## 2.4 Skin tissue Treg

The skin has a particularly high proportion of Treg in the steady state. Normally, 30–50% and 20–30% of total CD4+ T-cells are Tregs in mouse and human skin, respectively (Sharma and Rudra, 2018). Treg in the skin are CD44+ and CD103high, and express the chemokine receptors CCR4, CCR5, CCR6 and CCR7 (Sather et al., 2007). The skin Treg cell pool is dominated by tTreg cells that express GATA3, as well as Nrp1 and Helios, which can constitute up to 80% of all skin Treg cells. A Helios+ tTreg cell population that expresses the transcription factor ROR $\alpha$  but not ROR $\gamma$ t has also been found in the skin, although whether these Treg cells co-express GATA3 has not been reported.

Thymic Treg cells seem to be central in the prevention of aberrant inflammatory responses in the skin. Mice with deletion of GATA3 or ROR $\alpha$  in Treg cells develop heightened type 2 skin inflammation spontaneously or after challenge, respectively (Whibley et al., 2019a). In addition to Treg cells functions in the skin of maintaining homeostasis by controlling aberrant immune responses, tolerance to microbiota, defense against pathogens, they also induce tissue repair following injury.

Treg cells have been shown to facilitate the healing process following skin wounding by attenuating IFN- $\gamma$  production and pro-inflammatory macrophage accumulation. The expression of the epidermal growth factor receptor (EGFR) by Treg cells is required for their accumulation into wounds (Nosbaum et al., 2016). Indeed, specific deletion of EGFR in Tregs resulted in delayed skin wound closure. Treg cells have also been shown to stimulate the regeneration of hair follicles by promoting the proliferation and differentiation of stem cells in the skin (Ali et al., 2017).

## **2.5 Liver tissue Treg**

Tregs have also been shown to accumulate in the HCV-infected liver. The intrahepatic Tregs express high levels of HLA-DR, CTLA-4, CD45RO, and CCR7, as opposed to their peripheral blood counterparts. Liver Tregs are highly activated and differentiated. They were more numerous in HCV-infected livers showing only limited fibrosis, suggesting a role in limiting fibrosis in HCV-infected livers (Claassen et al., 2010). Whereas, in another study in patients with chronic hepatitis C virus infection, Tregs were enriched in the areas of fibrosis and their number correlated with the stage of fibrosis. These Tregs were upregulated in IL-8 expression and acted on hepatic stellate cells, functioning as a pro-fibrogenic factor. Moreover, these Tregs upregulated the expression of TIMP1, MMP2, TGF-b1, alpha-SMA, collagen and CCL2 in primary human hepatic stellate cells (HSC) (Langhans et al., 2013)

Similar to muscle Tregs, the liver Tregs constitutively express high levels of ST2, which was further increased in the liver Tregs responding to infection-induced injury. The ST2<sup>+</sup> Tregs have a high proliferative capacity and exhibited elevated expression of CD103, KLRG1 CTLA-4, GITR and PD-1 in mice infected with murine CMV. Unlike in the muscles, the IL-33 is mainly produced by the endothelial cells in the liver of uninfected mice and is produced by macrophages in infected livers. IL-33 signaling was shown to be crucial for Treg cell accumulation after MCMV infection, while ST2-deficient mice showed a more pronounced

liver pathology and higher mortality compared to infected control mice (Popovic et al., 2017, p. 2).

### 3. Amphiregulin producing Treg

Tregs contributing to tissue repair focus on two main aspects. First, immune mediated regulation, where in the context of tissue injury, Tregs restrict the infiltration of inflammatory cells through the release of immunosuppressive cytokines, as had been earlier mentioned in the context of mechanisms of action of T regulatory cells. Tregs produce high levels of IL-10, which act directly on fibroblasts. TGFb1 produced by Tregs promotes their differentiation and enhances the formation of tough fibrous tissues at the healing sites.

Second, Tregs also contribute in non-immunological manner, by directly acting on parenchymal cells. For example, by the release of Amphiregulin, a growth factor that plays an important role in healing and regeneration in the intestinal mucosa, lung epithelium and skeletal muscle (Zhang et al., 2017). Figure 20, is showing the possible characterization of tissue repair Tregs and the two pathways contributing to the repair process.

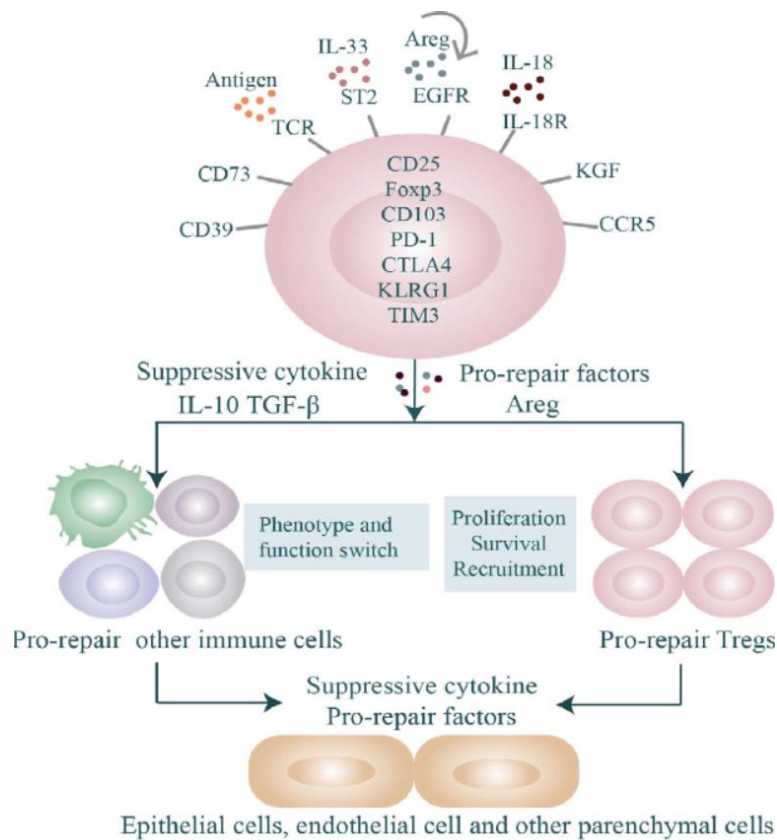


Figure 20. Characteristics of 'repair' Tregs in local tissues and their functions in the repair process (Zhang et al., 2017)

Amphiregulin (AREG), a member of the epidermal growth factor (EGF) family, is constitutively expressed by a number of epithelial and mesenchymal cell types during development and homeostasis. It has been shown to play a central role that can contribute to host resistance but is primarily a key factor that induces tolerance by promoting the restoration of tissue integrity following damage associated with acute or chronic inflammation (Burzyn et al., 2013; Monticelli et al., 2011). Although, originally it was described as an epithelial cell-derived factor, AREG can be expressed by multiple populations of activated immune cells, including basophils, eosinophils, mast cells, ILC2, dendritic cells, activated CD4 T cells, CD8 T cells, and a distinct subset of CD4 T regulatory cells (Table 1) (Zaiss et al., 2015). Furthermore, multiple immune mediators including TGFb1 (Zhou et al., 2012), can induce AREG expression, suggesting that its production can be regulated by the activation of multiple cell types during an immune response.

Arm of Immune system	Cell type	Host	Inflammatory setting	Reference
Innate	Basophils	Human	Allergy	Qi et al. <i>JACI</i> 2010
		Mouse	Contact hypersensitivity	Meulenbroeks et al. <i>J Inv Derm</i> 2014
	Eosinophils	Human	<i>Ex vivo</i> GMCSF stimulation	Matsumoto et al. <i>Int Arch Allergy Immunol</i> 2009
	Mast Cells	Human	<i>Ex vivo</i> IgE cross-linking	Wang et al. <i>JACI</i> 2005
		Human	<i>Ex vivo</i> FcεRI aggregation	Okumura et al. <i>JACI</i> 2005
		Mouse	Dermatitis, T cell transfer colitis, cancer	Zaiss et al. <i>Immunity</i> 2013
	Neutrophils	Human	Cystic fibrosis	Adib-Conquy et al. <i>Mol Med</i> 2008
	Group 2 innate lymphoid cells (ILC2)	Human	Atopic dermatitis	Salimi et al. <i>J Exp Med</i> 2013
		Mouse	Influenza	Monticelli et al. <i>Nature Immunol</i> 2011
	Dendritic cells	Human & mouse ( <i>in vitro</i> )	ATP stimulation	Bles et al. <i>Blood</i> 2010
Adaptive	CD4 <sup>+</sup> T cells	Human	<i>Ex vivo</i> TCR stimulations	Qi et al. <i>PLoS ONE</i> 2012
		Mouse	<i>Trichuris muris</i>	Zaiss et al. <i>Science</i> 2006
	Regulatory CD4 <sup>+</sup> T cells (subset)	Mouse	Muscle injury	Burzyn et al. <i>Cell</i> 2013
	Tumor infiltrating CD8 <sup>+</sup> T cells	Mouse	Chemical carcinogenesis	Kwong et al. <i>J. Invest. Derm.</i> 2010

Table 1. Innate and adaptive immune cell sources of Amphiregulin (Zaiss et al., 2015)

Amphiregulin expression in Tregs was first described by Burzyn et al, who found that Tregs recruited to acutely injured skeletal muscles of mice produced high amounts of this epidermal growth factor. Administration of recombinant AREG improved muscle repair while deletion of



Treg cells impaired the healing process and prolonged the proinflammatory infiltrate (Burzyn et al., 2013)

During influenza virus infection, there is an accumulation of lung tissue Tregs in the damaged lungs. AREG-producing Tregs expressing IL-18R were found to be the major contributor of the repair function. They were mostly CD44<sup>hi</sup>CD62<sup>lo</sup> and exhibited elevated expression of CD103, PD1, GITR, CTLA4 and KLRG1. Transcriptional profiling revealed that IL-10<sup>+</sup>IL18R<sup>-</sup> effector Tregs and IL10-IL18R<sup>+</sup> repair Tregs exhibited similar activation phenotype, with >90% of shared differentially expressed genes. The most pronounced genes were related to Treg suppressive function. Analysis of transcripts selectively up regulated in ‘repair’ Tregs showed that these cells were proficient in extracellular matrix remodeling and tissue repair (Arpaia et al., 2015).

The function of AREG on Tregs has also been described in colitis and tumour models. Mice lacking amphiregulin had insufficient numbers of Tregs to the site of inflammation, which showed immune regulatory dysfunction. The presence of amphiregulin enhanced the suppressive capacity of Tregs but had no influence on the overall survival or proliferation of Tregs. A positive feedback loop was observed whereby amphiregulin produced by Tregs promote tissue repair, and autocrine or paracrine amphiregulin signals activate Treg function by the EGFR pathway (Zaiss et al., 2015).

Figure 21 summarizes similar associations between Tregs and the repair process, which are also evident in heart muscle, skin, liver, kidney, lungs, bone, and CNS. In the context of myocardial infarction (MI), Tregs limit tissue injury and promote regeneration. Tregs improved scar tissue formation and survival. General ablation of Tregs resulted in aggravated cardiac inflammation. (Zhang et al., 2017)

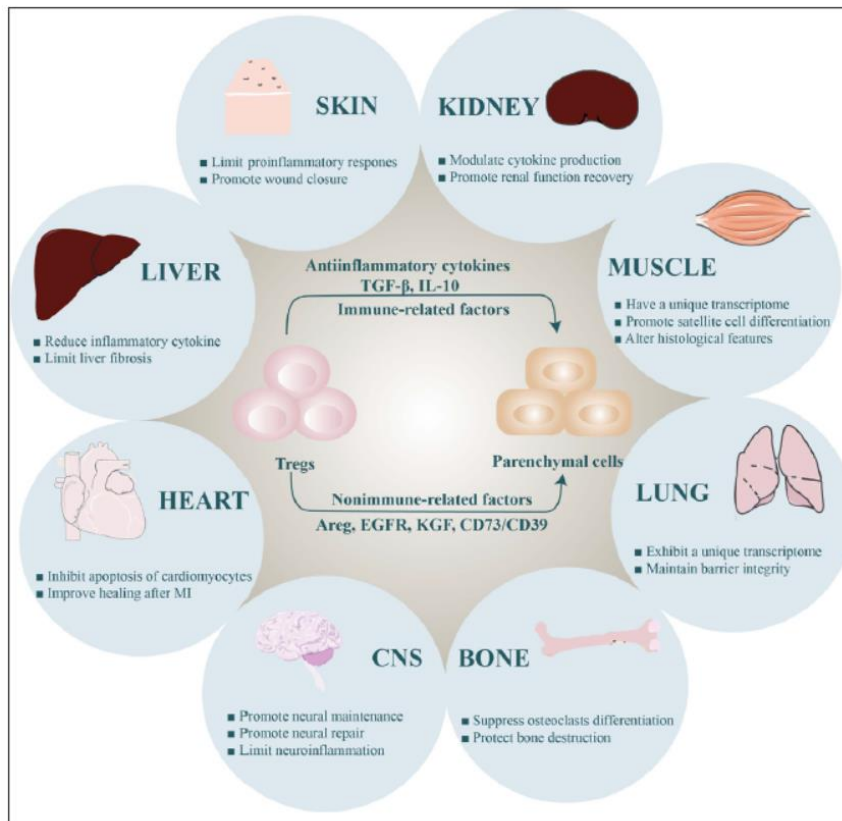


Figure 21. Tregs contribution to tissue homeostasis by promoting wound healing and repair at multiple tissue sites (Zhang et al., 2017)

The receptor of Amphiregulin, the EGF receptor (EGFR), contributes to wound healing. In contrast to high-affinity EGFR ligands, such as EGF, TGF $\alpha$  or heparin-binding EGF-like growth factor (HB-EGF), which activate the proliferation-stimulating mitogen-activated protein kinase (MAPK) signalling pathway, the low-affinity EGFR ligand Amphiregulin preferentially induces the phosphorylation of EGFR-Y992 and thus the activation of the PLC $\gamma$  signalling pathway. As a consequence, Amphiregulin is a poor mitogen for many epithelial and mesenchymal cell types and it appears highly unlikely that Amphiregulin substantially contributes to wound healing by inducing the proliferation of epithelial cells at the site of wounding (Zaiss, 2020). Zaiss and al recently discovered that Amphiregulin induced the local activation of TGF $\beta$ . In mouse models of CCl $_4$ -induced acute liver damage and of *Nippostrongylus* infection-induced lung damage, they demonstrated that during wound healing Amphiregulin induced the TGF $\beta$ -mediated differentiation of tissue stem cells and in this way critically contributed to the restoration of tissue homeostasis. TGF $\beta$  is secreted in a latent form, and only by releasing it from this latent complex becomes the bio-active form of TGF $\beta$  exposed. One way of releasing bio-active TGF $\beta$  is the activation of integrin- $\alpha$ V-containing complexes.

They found that Amphiregulin induced the activation of integrin- $\alpha$ V-containing complexes and thus the local activation of TGF $\beta$ . This local activation of TGF $\beta$  induced the differentiation of blood vessel-associated mesenchymal precursor cells, so called pericytes, into collagen-producing myo-fibroblasts; a process that critically contributed to the restoration of injured blood vessels and thus wound healing.

Epithelial associated solid tumor microenvironments can have high local concentrations of AREG and tumor infiltrating EGFR<sup>+</sup> CD4<sup>+</sup> Treg cells. Treatment with EGFR antagonists or Treg cell depleting agents promotes tumor regression. EGFR antagonists may also function in part to inhibit local CD4 Treg cells function, thereby alleviating immunosuppression and enhancing anti-tumour immune responses, including tumour specific CD8 cytotoxic T cells (Dietmar M.W. Zaiss et al., 2013). Conservation of this pathway is suggested in a murine model of Hepatitis B virus infection, where AREG expression is associated with the presence of hyper-suppressive EGFR<sup>+</sup> CD4 Treg cells that could limit antiviral CD8 T cell function (Dai et al., 2015). While, EGFR antagonists could impact therapeutic treatment of multiple disease states, including chronic viral infection and cancer (Zaiss et al., 2015).

## **Part III. HIV and IL-33**

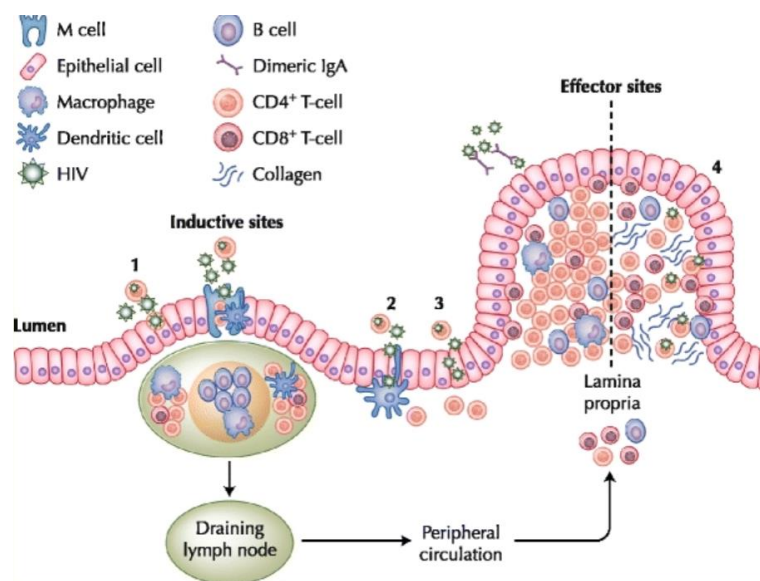
### **1. Short introduction on HIV infection**

HIV, the human immunodeficiency virus, continues to be a major global public health issue, having claimed almost 33 million lives so far. There were an estimated 38.0 million people living with HIV at the end of 2019 (“HIV/AIDS,” n.d.).

The main route of transmission of HIV infection is through unprotected sexual contact, HIV can be transmitted via the exchange of a variety of body fluids from infected people, such as blood, breast milk, semen and vaginal secretions. HIV can also be transmitted from a mother to her child during pregnancy and delivery. The sexual transmission occurs via HIV-infected body fluids coming in contact with genital, oral or rectal mucosa. Mother-to-child transmission is an additional route of transmission (“HIV/AIDS,” n.d.).

The GI (gastrointestinal tract) is the most important target organ in the pathogenesis of SIV and HIV infection. The intestinal mucosa serves as a ‘feeding ground’ for HIV amplification in early infection and the gut associated lymphoid tissue (GALT) has the largest and most important site of persistence for virus in chronic infection (Xu et al., 2013).

Figure 22 illustrates the mechanisms by which HIV may cross the gut barrier leading to infection and its consequences. HIV may cross the epithelium and initiate infection (1) by transcytosis across intact epithelial cells or M cells; (2) by adhering to dendrites of mucosal dendritic cells; or (3) by direct passage through epithelial breaches. Intestinal CD4<sup>+</sup> T cells (in both inductive and effector sites) are rapidly infected and depleted during acute HIV infection. (4). Tight junctions in the intestinal epithelium are compromised. The lamina propria loses most of its CD4<sup>+</sup> T cells but there is an influx of CD8<sup>+</sup> T cells. Collagen deposition occurs and may hinder reconstitution of CD4<sup>+</sup> T cells (Shacklett and Anton, 2010)



**Figure 22.** Intestinal inductive and effector sites in HIV infection (Shacklett and Anton, 2010)

The rate and severity of disease transmission is dependent on multiple factors, including the level of virulence and the quantity of the viruses in the infected individual, as well as factors related to the host, such as age, genetic differences, immune responses and environmental factors. The progression of the disease can take up to several years without treatment in HIV-1-infected individuals (Langford et al., 2007).

Although, effective antiretroviral therapy (ART) has prolonged life expectancy and decreased the morbidity and mortality of people living with HIV (PLWH), but there is not a complete eradication nor a complete restoration of depleted CD4+ T-cell count (Jacob D Estes et al., 2008). Consequently, there is a persistence of chronic immune activation and inflammation. In addition, persistent inflammation exacerbates tissue damage in PLWH, particularly in the gastrointestinal tract, allowing microbial components to enter into circulatory system, further increasing inflammation (Brenchley et al., 2006a; Zevin et al., 2016; Zicari et al., 2019). This immunological status leads to a vicious cycle (Figure 23) and increases the risk for PLWH to develop non AIDS-related co-morbidities, mainly cardiovascular disease, renal impairment and CNS disorders.(Klatt et al., 2013a; Zicari et al., 2019).

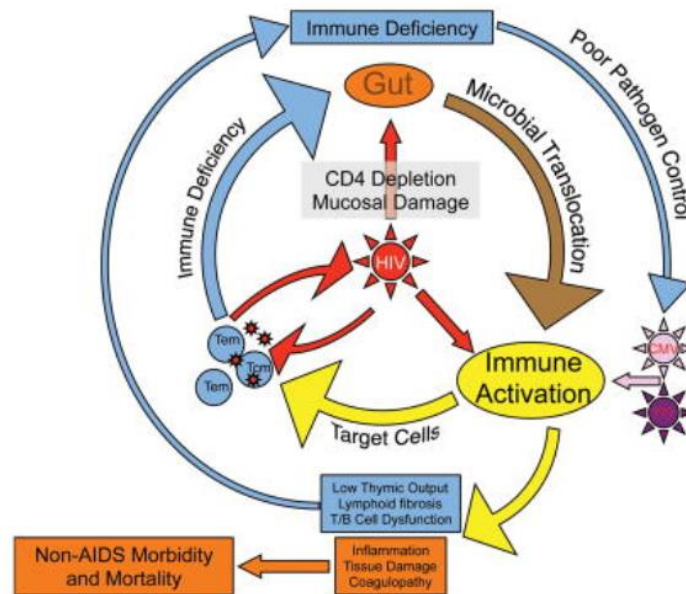


Figure 23. HIV initiates and sustains a vicious cycle in chronic state (Klatt et al., 2013a)

## 2. Immune activation in HIV infection

The cumulative immunological abnormalities of both the innate and adaptive arms of the immune system are referred to as immune activation. The degree to which the immune system is activated in HIV is of extreme importance, as it represents the strongest correlate of disease progression, morbidity and mortality than either plasma viral load or peripheral blood CD4 T cell counts (Deeks and Walker, 2004; Giorgi et al., 1999; Sodora and Silvestri, 2008). Intestinal mucosal damage and microbial translocation from the intestinal mucosa to the circulation, together with HIV-1 replication, may be the cause of the resulting chronic immune activation characteristic of HIV/SIV infection (Brenchley et al., 2006a; Klatt et al., 2013b; Marchetti et al., 2013). Systemic activation in SIV/HIV patients is characterized by, a high cell turnover and proliferation of T cell targets supportive of continued viral replication, high levels of immune activation and exhaustion of B and T cell clones, increased frequencies of apoptotic T cells, increased production of pro-inflammatory and pro-apoptotic cytokines (termed the cytokine storm) (Xu et al., 2013).

Antiretroviral therapy has transformed HIV infection into a chronic disease that persists for many decades. Despite effective therapy, there is persistent inflammation and immune dysfunction, leading to the ‘non-AIDS morbidity’.

The root causes/mechanisms of inflammation and systemic immune activation can be both direct and indirect (summarized in Figure 24). Direct contributing factors include the ongoing HIV replication from reservoirs as well as immunomodulatory functions of viral proteins and innate and adaptive immune responses against the virus and its antigens. In chronic immunocompromised state, high levels of other co-pathogens (CMV and EBV) normally controlled by the immune system, also contribute to the immune activation via recognition of virally infected cells by antigen-specific T and B cells and viral products binding to pattern recognition receptors.

There is CD4 T cell depletion with altered balance of critical CD4<sup>+</sup> T-cell subsets (altered TH17/Treg balance) leading to irreversible damage to immunoregulatory system (see below for details). With activated immune system there is secretion of proinflammatory cytokines, such as IFN- $\alpha$ , TNF- $\alpha$ , IL-1, IL-6 and IL-18. These cytokines in turn can lead to additional immune activation and apoptosis of immune cells. Additionally, increased secretion of systemic proinflammatory cytokines can also disrupt the capacity of immune system to control healthy levels of lymphocytes that normally produce homeostatic effector cytokines such as IL-17 and IL-22, which are critical to maintain the integrity of the epithelial barrier (Brenchley et al., 2006a; Hunt et al., 2014; Klatt et al., 2013b). An inability to maintain immunological and epithelial integrity of the gastrointestinal (GI) tract results in translocation of luminal antigens, including altered gut microflora and microbial products, into peripheral circulation (Deeks et al., 2013; Paiardini and Müller-Trutwin, 2013).

Studies have also found alterations in the composition of the commensal microbiota, contributing to the increased immune activation observed in HIV infected individuals (Ellis et al., 2011).

In 2008, Arthos and colleagues reported that the major HIV-1 envelope glycoprotein, gp120, can interact directly with the activated form of  $\alpha 4\beta 7$  (Arthos et al., 2008). Of note, the presence of  $\alpha 4\beta 7$  is not a requirement for HIV-1 entry into the host cells.  $\alpha 4\beta 7$ -integrin is expressed by various lymphocytes and is responsible for T cell homing into GALT. In fact, the interaction between  $\alpha 4\beta 7$ -integrin and mucosal adhesion, cell-adhesion molecule 1 (MADCAM1) which is expressed on high endothelial venules, facilitates the migration of activated T cells from Peyer's patches and/or MLNs to effector sites. Studies based on in vivo mice model has shown that

naïve T cells express low level of  $\alpha 4\beta 7$ -integrin whereas in memory T cells the expression of  $\alpha 4\beta 7$ -integrin is higher.

The ability of HIV to bind to  $\alpha 4\beta 7$  integrin via gp120 recognition enables to colonize GALT and participate to intestinal reservoir constitution. In a cohort study, a higher frequency of circulating  $\alpha 4\beta 7^{\text{hi}}$  CD4<sup>+</sup> T cells prior to infection was associated with a higher risk of HIV-1 acquisition and disease progression. During the first step of HIV infection, the virus targets the integrin  $\alpha 4\beta 7$ . At day 10 post-infection,  $\alpha 4\beta 7^+$  CD4<sup>+</sup> T cells contain higher levels of SIV RNA than other CD4<sup>+</sup> T cells. A high frequency of  $\alpha 4\beta 7^+$  CD4<sup>+</sup> T cells in the rectal mucosa greatly increased susceptibility to SIV infection. Of note, Th17 cells express high level of  $\alpha 4\beta 7$ . In vitro exposure of retinoic acid-treated  $\alpha 4\beta 7^{\text{hi}}$  CD4<sup>+</sup> T cells to trimeric gp120 was reported to increase LFA-1 activation, which is known to promote the formation of virologic synapses and HIV-1 infection, an effect that was selectively inhibited by an anti- $\alpha 4$  antibody that blocks gp120 binding. To validate the importance of  $\alpha 4\beta 7$ -gp120 interaction in vivo, infusion of an antibody to  $\alpha 4\beta 7$  in rhesus macaques prior to and during the acute phase of SIVmac239 infection significantly reduced the viral load in plasma and gastrointestinal tissues and delayed the peak of viremia (Girard et al., 2017; Liu and Lusso, 2020). Girard et al. found that when treating HIV<sup>+</sup> patients with suppressive antiretroviral therapy (ART), immunological non-responders exhibited a significantly higher rate of gut-homing  $\alpha 4\beta 7$  CD4<sup>+</sup> T cells compared to immunological responders (Girard et al., 2016). Lastly, 6 HIV-infected individuals who also had mild inflammatory bowel disease received vedolizumab concomitant with suppressive ART. anti- $\alpha 4\beta 7$  therapy led to a significant and unexpected attenuation of lymphoid aggregates in the gut compartment, most notably in the terminal ileum, along with a reduction in naïve T cells and a decrease in CD4<sup>+</sup> T-cell activation. Since lymphoid aggregates are key sites for maintaining the latent HIV-1 reservoir, these immunologic effects of anti- $\alpha 4\beta 7$  therapy suggest a possible long-term beneficial effect in reducing the size of the viral reservoir.

HIV-mediated deposition of collagen in lymphoid tissues is also another well-established cause of persistent immune dysfunction and inflammation (J. Estes et al., 2008; Zeng et al., 2011).

Each mechanism may feed to the others, thus creating an uncontrolled positive feedback. This inflammatory environment causes end-organ damage. As HIV-mediated breakdown in the integrity of the gut mucosa and chronic microbial translocation in the systemic circulation is widely assumed to be a major cause of inflammation (Brenchley et al., 2006a), a series of clinical trials reversing this process have been performed, with variable success.



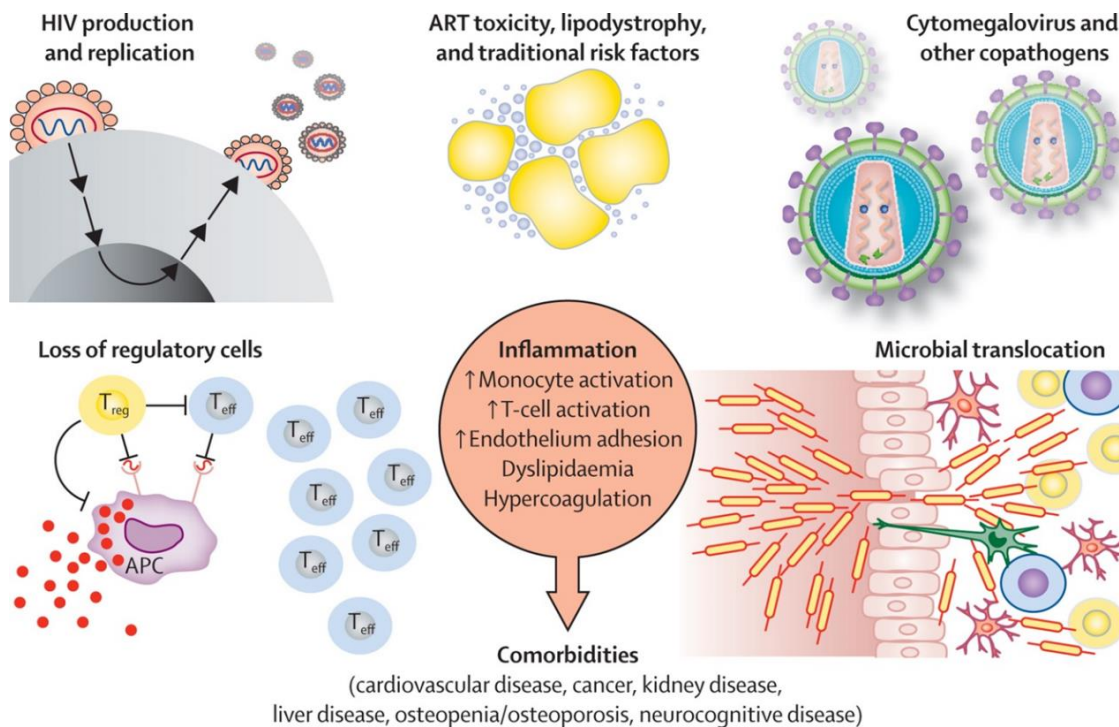


Figure 24. Causes and consequences of immune activation and chronic inflammation during antiretroviral treatment (Deeks et al., 2013)

### 3. Microbial translocation due to a leaky gut in HIV infection

#### 3.1 Microbial translocation

Microbial translocation is defined as the translocation of commensal microbial products from the intestinal lumen into the systemic circulation in the absence of overt bacteremia.

Microbial translocation occurs even in healthy individuals but increases with damage to the intestinal barrier. At steady state, microbial translocation is blocked with the structural integrity of the epithelial barrier and the functional gut resident immune cells.

It has a key role in driving the persistent immune activation in individuals with chronic HIV infection (Brenchley et al., 2008; Sandler and Douek, 2012; Tincati et al., 2016). Microbial translocation, is measured by blood concentrations of lipopolysaccharide (LPS), detection of universally-conserved microbial 16S ribosomal RNA gene sequences (rDNA) in plasma, and increased levels of peripheral blood CD8<sup>+</sup> T-cell subsets with an activated phenotype (Brenchley et al., 2006a; Sandler and Douek, 2012). These bacterial and fungal products may include peptidoglycan, lipoteichoic acid, lipopolysaccharide (LPS), flagellin, ribosomal DNA (rDNA) and unmethylated CpG-containing DNA. These products elicit potent pro-

inflammatory responses by activating a number of receptors: including nucleotide-binding oligomerization domain 1 (NOD1) and NOD2, as well as Toll-like receptor 2 (TLR2), TLR4, TLR5, TLR6 and TLR9, which are expressed by many cell types. In innate immune cells (such as monocytes, macrophages and dendritic cells), binding of these receptors to microbial products activates a signalling cascade, leading to the production of the pro-inflammatory cytokines Interleukin-1 $\beta$  (IL-1 $\beta$ ), IL-6, tumour necrosis factor (TNF) and type I interferons (IFN $\alpha$  and IFN $\beta$ ). These responses may be beneficial to the host in the setting of acute infections, but they can contribute to disease pathology in chronic, as in HIV infection (Sandler and Douek, 2012).

Pro-inflammatory cytokines such as IL-1 $\beta$ , IL-6 and TNF can induce inflammation and tissue damage in the vasculature, as well as the proliferation of local monocytes or macrophages and smooth muscle cells, resulting in cardiovascular disease (Hansson and Hermansson, 2011). They also increase the proliferation of and impair apoptosis of pre-malignant cells and malignant cells, and stimulate bone resorption, which leads to osteoporosis (Sandler and Douek, 2012).

Microbial translocation has a role in predicting clinical events and outcomes in HIV. Activated monocytes/ macrophages secrete soluble CD14 (sCD14), which binds LPS. Plasma levels of sCD14 and LPS were first described as independent predictors of mortality and disease progression in chronically infected individuals. Based on the conflict in the existing published data, the link between sCD14 and LPS has been shown to be complex in HIV-infected individuals (Marchetti et al., 2013; Sandler and Douek, 2012; Tincati et al., 2016). One study demonstrated that measures of LPS-induced innate immune activation (sCD14), gut epithelial barrier dysfunction (I-FABP, zonulin-1), coagulation (D-dimer) and inflammation (hs-CRP, sTNFRI) were able to predict mortality in individuals on c-ART (Hunt et al., 2014). In contrast, however, administration of cART in elite controllers did not affect markers of microbial translocation and inflammation (Tenorio et al., 2014). Collectively, studies have shown that microbial translocation markers may predict clinical outcome in the setting of untreated HIV infection but may not be as valuable in individuals under c-ART treatment.

Microbial translocation arises from gut structural as well as immunological disruption. Structural disruption is caused by loss of epithelial tight junctions, apoptosis of enterocytes and lymphoid tissue fibrosis. Whereas immunological disruption includes loss of TH17 subset

preferentially, dysfunctional phagocytosis and B cell dysfunction. This is illustrated in figure 25 and detailed as follows.

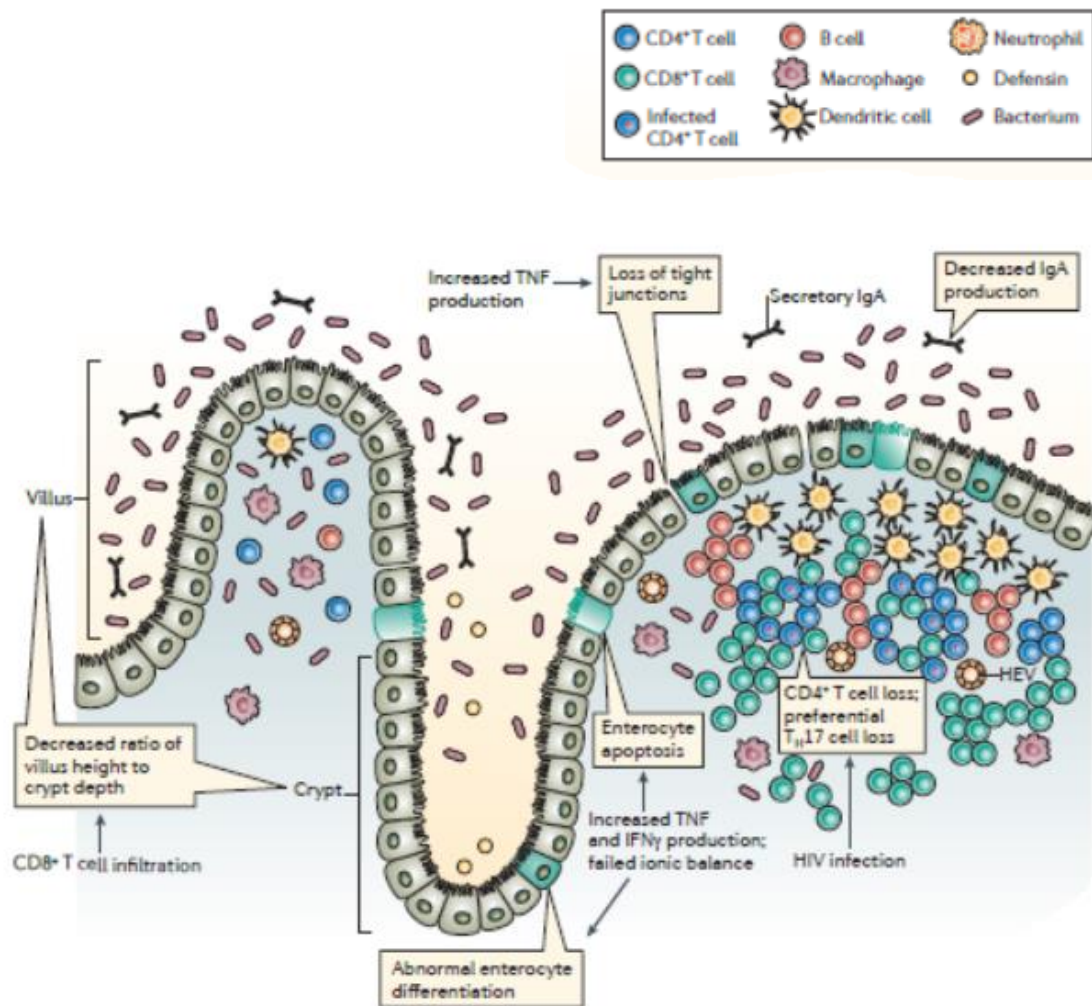


Figure 25. Altered intestinal epithelial integrity with structural, histological and immunological changes during HIV infection (Sandler and Douek, 2012)

### 3.2 Gut mucosal damage

One defense mechanism which protects against microbial translocation is the structural barrier of the GI tract. The tight epithelial barrier is a single layer of cells, which serves to protect the body from translocating bacteria and to absorb luminal nutrients. Impairment of gut barrier is a prerequisite for microbial translocation. During HIV or SIV infection, the integrity of the tight epithelial barrier of the GI tract is compromised, which has been demonstrated by focal breaches in the barrier, decreased expression of epithelial repair tight junction genes, and

increased GI tract permeability (Mohan et al., 2012; Nazli et al., 2010). The bacterial and fungal disseminated products along with HIV itself, activates the TLRs, which consequently activate the innate immune cells, such as monocytes, macrophages, and dendritic cells (as discussed in section 2). This initiates a signaling cascade, activating the adaptive immune system, eliciting the production of pro-inflammatory cytokines including interleukin-1b (IL-1 $\beta$ ), IL-6, tumor necrosis factor (TNF), and type 1 interferons (IFN- $\alpha$  and IFN- $\beta$ ). The persistence of these pro-inflammatory cytokines can induce tissue damage, and increase the proliferation of local immune cells (Klatt et al., 2013b; Xu et al., 2013).

The epithelial barrier integrity is interrupted by the loss of enterocytes and disruption of the tight junctions. Loss of enterocytes may be caused by the virus itself (Brenchley et al., 2006a; Nazli et al., 2010; Sandler and Douek, 2012). HIV replication can drive epithelial damage, likely due to the production of proinflammatory cytokines which can directly induce apoptosis of epithelial cells (Nazli et al., 2010).

Bystander effects may also contribute to enterocyte loss. It may also be caused by damage inflicted by pro-inflammatory cytokines such as TNF. Studies have found evidence of damage to the intestinal epithelium in HIV infected individuals. Citrulline levels are decreased, indicating loss of small intestine enterocytes, which produce most citrulline (Crenn et al., 2009). Moreover, HIV-infected individuals, regardless of whether they have received ART and whether they show virologic suppression, have increased plasma levels of intestinal fatty acid binding protein (I-FABP) (Sandler et al., 2011), which is made exclusively by enterocytes and is released into the bloodstream on enterocyte loss.

Loss of enterocytes results in loss of epithelial tight junctions, as is evidenced by the discontinuous staining of the tight junction protein claudin 3 in rhesus macaques with chronic SIV infection (Estes et al., 2010). Tight junction loss occurs within 14 days post-infection (Estes et al., 2010) and leads to increased epithelial barrier permeability. Furthermore, the expression of genes that induce epithelial barrier maintenance is decreased in acute HIV infection (Sankaran et al., 2008).

Histologically, the gut epithelium is characterized by villous atrophy, crypt hyperplasia and villous blunting. Intestinal permeability has been measured by absorption of d-xylose and fat as well as by increased villous atrophy indicated by increased urinary lactulose/mannitol ratios,

has been shown to contribute to structural damages in the gut mucosa, leading to malabsorption of nutrients and diarrhea (Klatt et al., 2013b).

Even in the absence of lentiviral infection, damage to the tight epithelial barrier correlates with local levels of LPS, demonstrating the importance of maintaining barrier integrity to avoid microbial translocation (Klatt et al., 2013b).

### **3.3 Decreased clearance of bacterial products**

In a healthy individual, if microbes traverse the epithelial barrier and enter the lamina propria, several immune mechanisms exist to kill or transport the bacteria back to the lumen. However, the massive dysfunction of mucosal immune system during HIV and SIV infection fails to prohibit systemic microbial translocation once microbial products enter the lamina propria.

Studies have shown reduced phagocytic activity of mucosal macrophages in untreated HIV-infected patients. This low phagocytic function of mucosal macrophages in HIV infection may play a role in the inefficient defense against pathogens and antigens that cross the epithelial layer. Consistent with this, *in situ* and *ex vivo* data obtained from chronically SIV-infected rhesus macaques and from a humanized mice model of HIV infection, respectively, indicate insufficient elimination of translocated luminal products by tissue macrophages (Allers et al., 2014). Specialized GI tract macrophages are essential in the clearance of microbial products from the lamina propria, however, these dysfunctional macrophages during HIV infection, no longer phagocytose bacterial products. Instead, they are hyperactive and respond with inflammatory cytokines. The mechanisms whereby HIV-1 impairs phagocytosis are not well known. There are a number of phagocytic receptors, and engulfment of specific opportunistic pathogens is often receptor-specific. Most studies have shown that HIV-1 infection results in either elevated or unchanged surface expression of Fcγ receptors (FcγRs) or complement receptors (CRs), indicating that the inhibition of phagocytosis by HIV-1 occurs during or after receptor binding. It was demonstrated that HIV-1 infection of human monocyte-derived macrophages markedly affects FcγR-mediated phagocytosis. This occurs perhaps because the infection inhibits the tyrosine phosphorylation of cellular proteins that is necessary for the initial receptor activation (Kedzierska et al., 2003).

The local production of IgA is believed to be one of the main defense mechanisms within the GI tract, yet there have been few studies of the effect of HIV-1 infection on IgA<sup>+</sup> B cells in GALT. Another immunological irregularity observed during HIV and SIV infection is B cell dysfunction, including significantly lower levels of IgA, which are a key component of the mucosal immune system. Particularly, they are essential in protection against microbial translocation as IgA can directly bind bacteria, prohibiting their binding to the epithelium, thus neutralizing their bioactivity. In addition, IgA can transport bacteria from the lamina propria back into the lumen (Klatt et al., 2013b). HIV-specific IgA are generally absent or present at very low levels in plasma and mucosal secretions of chronically HIV-infected patients. HIV-1 infection preferentially leads to potent IgG responses in body fluids. Decreased levels of IgA in intestinal fluids associates with reduced proportions of IgA plasma cells within the lamina propria (LP) of the duodenum and colon of chronically HIV-infected patients. In AIDS patients, depletion of IgA plasma cells in the small intestine also correlates with decreased secretion of IgA in saliva. In supernatants of short-term cultured duodenal biopsies from SIV-infected macaques, there is a decrease in total IgA and a lack of SIV-specific IgA for up to 6 months post-infection. Moreover, IgA secretion is highly dependent on the integrity of epithelial cells, which is frequently impaired during acute and chronic HIV/SIV infections. Overall, the lack of IgA in intestinal fluids of chronically HIV-infected patients or SIV-infected macaques can be explained by a default in shedding, impaired homing of IgA plasmablasts to LP or their impaired terminal differentiation into plasma cells (Chaoul et al., 2012). In addition, recent data of Xu et al. suggested that an impaired isotype switching towards IgA occurs in inductive sites (Xu et al., 2009), preventing the generation of IgA plasmablasts.

### **3.4. CD4<sup>+</sup> T cell depletion: preferentially Th17 cells**

The mucosal T cells, even at physiological state, are baseline activated, and are mainly (>98%) of the memory (CD45RO<sup>+</sup>) phenotype. The mucosal CD4<sup>+</sup> T cells are predominantly CCR5<sup>+</sup>/CXCR4<sup>+</sup>, constitutively expressing the co-receptors for HIV infection. Fewer than 20% of these CD4<sup>+</sup> T cells exist in the blood. Thus, the majority of the HIV target cells lie in the intestinal tract, making it a primary focus of immunological investigations. Within days of infection, there is massive infection and depletion of activated, memory CD4 T cells in the

intestine of SIV infected macaques and HIV infected humans, regardless of the route of infection (mucosal or intravenous) (Shacklett and Anton, 2010; Xu et al., 2013).

Certain subsets of CD4<sup>+</sup> T cells are selectively infected and eliminated in HIV infection, whereas others are relatively spared resulting in marked changes in immune homeostasis and the inflammatory response to HIV and other pathogens.

A major CD4<sup>+</sup> T cell subset that is targeted and eliminated early are Th17 cells. Th17 cells are important mediators of immune defenses in the gut and are induced by the microbiota in the intestinal lamina propria. Th17 cells are significantly depleted during HIV and SIV infection, which is associated with GI tract structural damage, microbial translocation and immune activation (Gordon et al., 2010; Klatt et al., 2013b). Th17 cells produce IL-17 and IL-22, which promote recruitment of neutrophils to sites of bacterial infection, induce enterocyte proliferation, maintain mucosal epithelial integrity, induce production of antibacterial defensins, and thus play an important role in antibacterial immunity. Selective depletion of Th17 may play a key role in loss of mucosal integrity. Of the CD4<sup>+</sup> T cells, Th17 cells, which express both CCR5 and the gut homing marker CCR6, are preferentially depleted, probably because they are more susceptible to HIV infection than CCR5<sup>+</sup>CCR6<sup>-</sup> cells (Brenchley et al., 2008; El Hed et al., 2010). Furthermore, TH17 cells produce IL-22, which enhances epithelial regeneration (Liu et al., 2009), so their loss may also cause impaired mucosal healing and consequently increased intestinal permeability and microbial translocation. Gut resident Th17 cells are preserved in natural hosts of SIV, which may contribute to the lack of microbial translocation and chronic immune activation in SIV infected, non-progressing hosts (Brenchley et al., 2008).

Moreover, the ratio of Th17 to Treg cells is decreased in both blood and rectosigmoid biopsy samples of HIV-infected individuals and correlates with increased plasma markers of microbial translocation and higher level of activated CD8<sup>+</sup> T cells (Favre et al., 2010a; Sandler and Douek, 2012). The change in Th17/Treg ratios is associated with the induction of the immunomodulatory enzyme indoleamine 2,3-dioxygenase 1 (IDO1), which is an intracellular haem-containing enzyme that catalyses the oxidative catabolism of tryptophan to kynurenine (Figure 26). Increased concentrations of 3-hydroxyanthranilic acid (a consequence of IDO1 upregulation) result in decreased natural killer (NK) cell production of IL-17 and IL-22. These two cytokines, which control extracellular bacteria and promote mucosal repair, are already

decreased owing to Th17 cell depletion (Reeves et al., 2011). IDO1 can be upregulated by type I IFNs and TLR agonists such as LPS and bacterial and viral DNA. In HIV-infected individuals, IDO1 induction is associated with increased levels of LPS and sCD14, increased CD8+ T cell activation, a greater CD4+ T cell decline and a reduction in CD4+ T cell counts (Favre et al., 2010a). Thus, increased microbial translocation in HIV infection and systemic immune activation may further perpetuate bacterial overgrowth by inducing the production of the tryptophan catabolite 3-hydroxyanthranilic acid. This, in turn, increases Treg cell frequency to counteract the heightened immune activation as a negative feedback mechanism, drives further Th17 cell depletion, and decreases IL-17 and IL-22 production by NK cells.

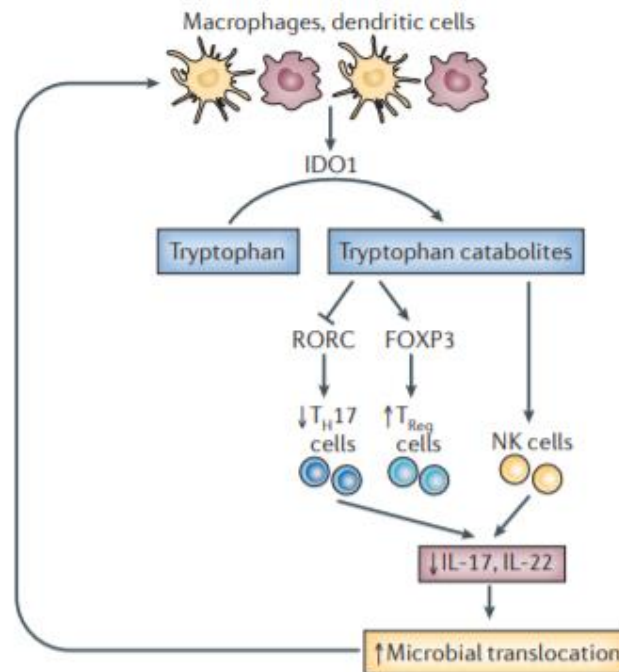


Figure 26. Th17/Treg balance and microbial translocation (Sandler and Douek, 2012).

### 3.5. Impaired CD4 T cell recovery

The reduction in circulating CD4+ T cells may impair GALT restoration by decreasing the number of T cells that could traffic to the intestine, leading to bacterial overgrowth and increased microbial translocation. Thus, there is a tight interplay among low nadir CD4+ T cell counts, microbial translocation, immune activation, and immunologic non-response.



One potential mechanism by which microbial translocation may impair CD4<sup>+</sup> T cell recovery in patients on ART is by causing lymphatic tissue fibrosis, which can be quantified by visualizing collagen deposition (Schacker et al., 2002). Tregs secrete Transforming Growth Factor- $\beta$  (TGF- $\beta$ ), triggering collagen production and deposition by fibroblasts; resulting in structural damage and fibrosis of the lymph node reducing the regeneration and survival of CD4<sup>+</sup> T cells thus worsening immunodeficiency (Estes et al., 2007).

In patients with chronic HIV infection on ART, a greater density of collagen I deposition in mesenteric and axillary lymph nodes and GALT is associated with a smaller CD4<sup>+</sup> T cell population in the lymphatic tissue and with reduced CD4<sup>+</sup> T cell recovery (J. Estes et al., 2008; Schacker et al., 2002). This collagen deposition is driven by TGF $\beta$ 1, which is produced in response to local immune activation induced by LPS. Indeed, in chronic SIV infection, LPS can be detected in the mesenteric and axillary lymph nodes and in the colon (Estes et al., 2010). The degree of fibrosis in the GALT correlates directly with CD4<sup>+</sup> T cell activation and inversely with CD4<sup>+</sup> T cell count (J. Estes et al., 2008; Jacob D Estes et al., 2008). Collagen deposition in the T cell zone is associated with destruction of the fibroblastic reticular cell network, resulting in loss of access of T cells to IL-7, which naive CD4<sup>+</sup> T cells require for survival, and obstruction of antigen presentation by dendritic cells to T cells. Thus, microbial translocation drives the immune activation that causes lymphatic tissue fibrosis and thereby impairs CD4<sup>+</sup> T cell recovery (Sandler and Douek, 2012; Zeng et al., 2011).

#### **4. Persistence of GI abnormalities despite suppressed viremia with ART**

Current cART treatments can suppress viremia to undetectable levels for the life of an individual and their use vastly improves the life expectancy of HIV-infected individuals. Further, suppressed viremia diminishes the majority of immune activation contributed to direct HIV replication. However, despite this virus suppression, cART-treated individuals still have increased morbidity and mortality compared to HIV-uninfected individuals, which, is associated with ongoing immune activation.

During cART, individuals can be broadly classified as immune responders (IR), exhibiting CD4 reconstitution and slightly decreased immune activation, and immune non responders (INR), who maintain high immune activation and low levels of CD4 T cells. Several studies have shown that lack of immune restoration in these INRs is associated with increased levels of microbial translocation (Marchetti et al., 2008; Merlini et al., 2011; Piconi et al., 2010).

Although cART induces peripheral CD4 reconstitution, this reconstitution is limited in the GI tract. Collagen deposition in the GI tract is not resolved during cART treatment, which may be the cause of this limited reconstitution (Jacob D Estes et al., 2008). Moreover, during cART, damage to the tight epithelial barrier of GI tract still occurs, including decreased epithelial repair genes, and increased enterocyte apoptosis. Thus, the mucosal immune dysfunctions persist despite suppressed viremia, and are likely the underlying cause of microbial translocation (Klatt et al., 2013b)

Studies have demonstrated profound alterations of the intestinal epithelium, with marked impairment of gut junctional complex proteins (JC; cadherin, claudins, zonula occludens 1) which can be found in the peripheral blood. cART is able to correct gut abnormalities only partially. Persistent defects in JC protein expression, microbial composition and immune function have in fact been described in aviremic individuals. Importantly, these defects appear to be differentially expressed according to the degree of CD4+ T-cell recovery on cART (Figure 27) (Tincati et al., 2016).

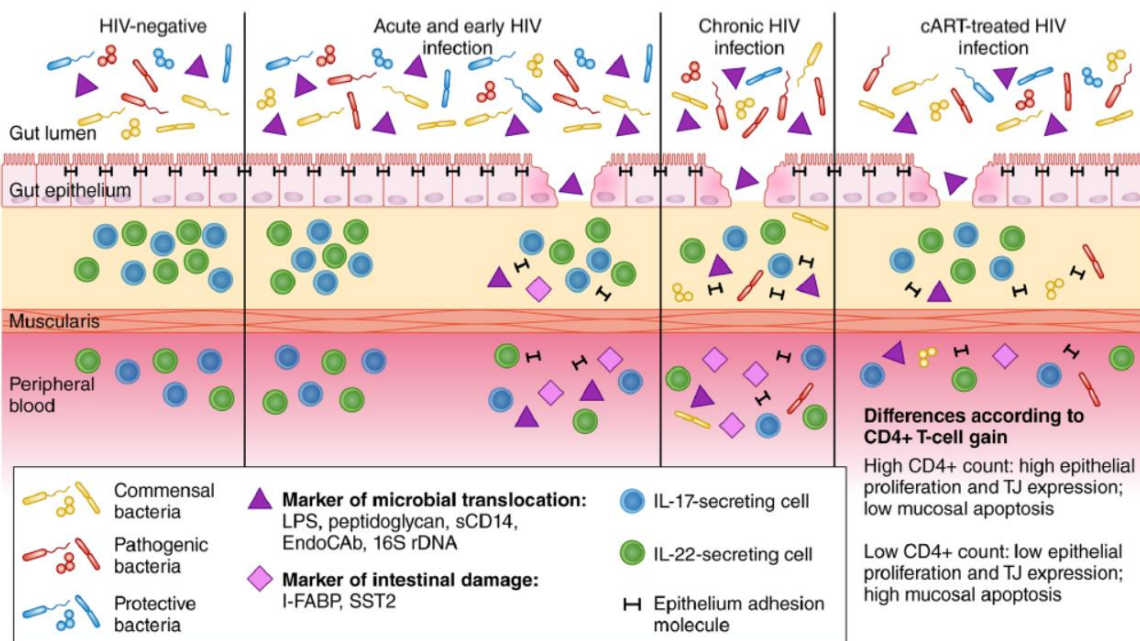


Figure 27. GI abnormalities in untreated and treated HIV disease (Immunological Non Responder (INR) and Immunological Responders (IR) (Tincati et al., 2016)

Thus, ART can facilitate partial intestinal healing and decrease microbial translocation, but not to the level observed in HIV-uninfected individuals.

## 5. Dynamics of Tregs in HIV

The data on the levels of Tregs during HIV-infection are conflicting, mainly due to the lack of Treg-specific markers (different markers used in different studies), the multiplicity of distinct Treg subsets and to whether frequencies or absolute numbers were assessed. The majority of findings indicate that the relative frequency of CD25<sup>+</sup> Tregs is increased in blood, while their absolute counts are reduced as a consequence of lower total CD4<sup>+</sup> T-cell counts during progressive HIV-1 infection. The number of Tregs decrease over time but at a lower rate than the whole CD4 T-cell population, meaning that Treg frequency increases. Treg expansion is observed both in the periphery and, to a greater extent, in mucosal and lymphoid tissues in HIV-infected patients with progressive disease. In contrast, HIV controllers (that spontaneously control viral replication) and Long-term-non-progressors (LTNPs) (that remain asymptomatic for many years maintaining high CD4 cell counts without ART) exhibit similar or even lower Treg levels. (Chevalier and Weiss, 2013; Paiardini and Müller-Trutwin, 2013)

In the situation of viral control under ART, treatment was able to significantly decrease or even normalize Treg frequency at levels similar to that of healthy donors. In most cross-sectional studies, peripheral Treg frequency was reported to be lower in ART-treated patients compared with untreated, chronically infected patients. Treg frequency was found to be negatively correlated with residual immune activation. Interestingly, the negative relationship between Treg frequency and CD8 T-cell activation was lost after treatment interruption (Chevalier and Weiss, 2013).

Since tissue samples are not easy to recover, only scarce studies have been performed to assess Tregs in tissues of HIV-infected patients. Foxp3<sup>+</sup> Tregs are maintained or accumulate in the lymphoid tissues and mucosa of HIV-infected patients according to some but not all studies (Chase et al., 2007; Epple et al., 2006a). FoxP3 expression levels were found to be higher in rectal and lymph node (LN) Tregs compared with peripheral Tregs in patients and in healthy controls. In chronic untreated HIV infection, most studies have reported highly elevated Treg levels in patients compared with healthy donors in different tissues including duodenal and rectal mucosa. Long-term non progressors (LTNPs) and HIV controllers were reported to have low levels of Tregs in tissues, similar to healthy controls. As in the periphery, tissue Treg levels were found to be lower in treated compared with untreated patients. In a longitudinal study, ART resulted in normalization of mucosal Treg frequency (Chevalier and Weiss, 2013) (Epple et al., 2006a). Tregs were shown to increase membrane expression of the homing receptors CD62L and integrin  $\alpha 4\beta 7$  following HIV-1 binding in vitro, which could explain the

accumulation of Tregs in peripheral lymph nodes and lymphoid mucosal tissues in vivo (Loiseau et al., 2016, pp. 6-).

How Tregs impact HIV-associated chronic immune activation is still intensively debated (Paiardini and Müller-Trutwin, 2013). Tregs seem to have a dual role in HIV infection (Figure 28). On one hand, Tregs seem to be detrimental, since they are able to suppress HIV-specific immune responses contributing to viral persistence (Aandahl et al., 2004, p. 4; Kinter et al., 2004; Weiss et al., 2004). In this scenario, interventions aimed at decreasing Tregs numbers and functions may limit chronic immune activation. On the other hand, Tregs could be 'beneficial' since they might reduce the generalized pro-inflammatory environment typical of HIV infection (Eggena et al., 2005; Karlsson et al., 2011; Tsunemi et al., 2005; Weiss et al., 2010). In this case, interventions aimed at increasing their numbers and functions would limit chronic immune activation and thus ameliorate the course of HIV-disease. This was supported by a study showing that depletion of Treg numbers strongly correlate with both CD4+ and CD8+ T-cell activation in HIV-infected individuals (Kinter et al., 2004, p. 25). Tregs, however, might be efficient in limiting residual immune activation in the context of controlled viral load but unable to suppress strong immune activation in viremic patients (Weiss et al., 2010).

During the chronic phase of HIV infection, Tregs produce TGF- $\beta$ , which mediated collagen deposition, limiting CD4 reconstitution. TGF $\beta$ 1+ Treg response appeared to induce distinct deleterious outcomes by i) dampening the antiviral immune response and ii) causing harmful effects on CD4+ T cell homeostasis by inducing collagen deposition in lymphatic tissues. Expression of TGF $\beta$  by Tregs was directly linked to collagen deposition in an in vitro model, in which TGF $\beta$ 1+ Treg cells, but not conventional activated T cells, stimulated collagen type I protein expression in primary fibroblasts. In a non-human primate model of SIV infection, TGF $\beta$  has been linked to collagen deposition in the T-Zone of lymphatic tissue. The deposition of collagen was shown to be both rapid and progressive, so that by 28 days after infection (comparable to the beginning of the presymptomatic phase of human infection) the mean fold increase from the preinfection level was 20.5 (J. Estes et al., 2008). Collagen deposition continued unabated throughout all stages of disease in the absence of intervention until AIDS defining illness and death ensued, and the extent of collagen deposition was significantly related to decreased numbers of CD4 T cells. Immune activation and inflammation appeared to be the driving stimulus to collagen deposition. These changes do not appear to reverse with

antiretroviral therapy (J. Estes et al., 2008). The deposition of collagen disrupts migration and access of CD4 T cells to cytokines such as IL-7. IL-7 is critical for their survival and proliferation, contributing to CD4 T cell loss in proportion to the degree that different subsets depend on IL-7, with greatest impact on naïve CD4+ T cells, but also depletion of central memory and effector memory CD4+ T cell populations.

CD39 expressing Tregs are also associated with HIV disease and are correlated with progressive disease, HIV viral load and immune activation (Schulze zur Wiesch et al., 2011, p. 39). Interestingly, Tregs of elite controllers exhibited not only the highest expression of CCR5, CTLA-4, and ICOS but also the lowest level of CD39 (Schulze zur Wiesch et al., 2011, p. 39). Increased ectonucleotidase activity and enhanced CD39 expression has been observed on lymphocytes of individuals infected with HIV. Our group has shown a higher number of CD39+ Treg in HIV-positive patients. The effector T cells of these patients exhibited an increased sensitivity in vitro to the suppressive effect of adenosine, which was related to the elevated expression of immunosuppressive A2A receptors (Nikolova et al., 2011).

Taken together, data suggest that Tregs, although capable of controlling low residual T-cell activation somewhat in ART-treated patients, they are not sufficient, in terms of numbers and/or activity, to dampen the generalized immune activation associated with high levels of HIV replication. There is now clear evidence that Treg expansion was observed both in the periphery and, to a greater extent, in mucosal and lymphoid tissues in HIV-infected patients with progressive disease. In contrast, HIV controllers and LTNPs exhibit lower Treg levels. In this regard, Treg may be considered nonprotective in HIV infection.

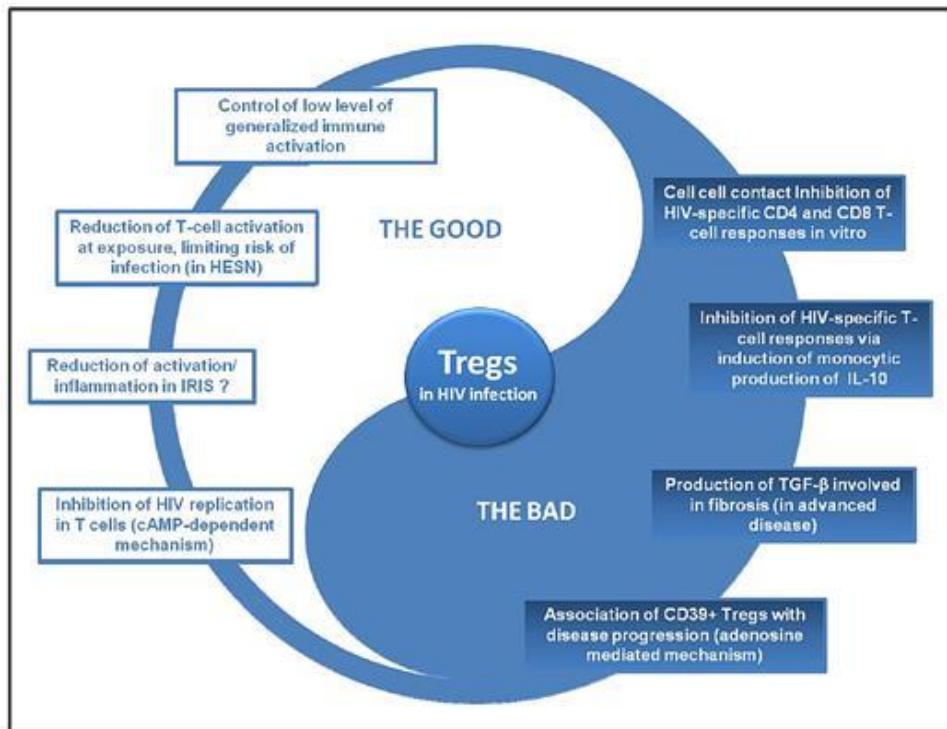


Figure 28. Dual role of Tregs in HIV infection (Chevalier and Weiss, 2013).  
HESN (HIV-exposed seronegative individuals)

## 6. CD8 T cells characterization and response in HIV

CD8<sup>+</sup> T-cells are part of the adaptive immune system, playing a critical role for protection against foreign organisms and malignancies. Powerful and effective HIV-1- specific CD4<sup>+</sup> and CD8<sup>+</sup> T cell immune responses are generally regarded as the principal antiviral immune activity against HIV. However, T cell responses are less functional and persist in an exhausted state in chronic HIV infection.

### 6.1. Activation of CD8 T cells

After exposure to antigen, CD8<sup>+</sup> CTL are activated and can induce death of infected targets. The activation of naïve CD8<sup>+</sup> T-cells requires three signals, provided by antigen-presenting cells (APC). Initially, there is the recognition of the antigen by the T-cell receptor (TCR); in the case of CD8<sup>+</sup> T-cells, the peptides are 8–10 amino acids in length and are presented by major histocompatibility complex (MHC) class I molecules. Second, costimulatory signals are

required, such as the binding of CD80 or CD86 from the APC to the CD28 molecule expressed by the CD8<sup>+</sup> T-cell, and finally an alarm signal produced in response to pathogens, including IL-12 and type I interferon (IFN), among others (Perdomo-Celis et al., 2019).

Naive CD8<sup>+</sup> T cells then differentiate into memory and effector T cells. There is clonal expansion, generating a large pool of effector cells. In humans, this is most likely mediated by a linear differentiation process that eventually results in the formation of fully differentiated effector T cells (Hamann et al., 1999; Tomiyama et al., 2002). Different stages of CD8<sup>+</sup> T-cell differentiation have been characterized based on the expression of TNF-receptor family members like CD27 (Lens et al., 1998; Romero et al., 2007). Expression of CD27 is irreversibly lost upon differentiation to CD8<sup>+</sup> effector T cells. These cells exhibit high effector functions, such as the expression of cytokines, cytotoxic molecules and a high capacity for degranulation. Additionally, at this point, effector CD8<sup>+</sup> T-cells acquire the ability to migrate to peripheral tissues. After clonal expansion, effector cells suffer massive apoptosis, in a period known as contraction phase. Finally, the remaining antigen-specific CD8<sup>+</sup> T-cells constitute the pool of memory cells, which decrease their effector profile, remaining in a quiescent state expecting a new antigenic challenge. This period, which can last the whole life of the individual, is known as the memory phase. Several CD8<sup>+</sup> T-cells differentiation models propose that memory cells can differentiate into effector cells once they are exposed to activation signals, acquiring a high capacity for cytokine production and cytotoxic potential (“Memory CD8 T-Cell Differentiation during Viral Infection,” n.d.) (Jansen et al., 2004; Perdomo-Celis et al., 2019)

## **6.2. CD8<sup>+</sup> T cells effector mechanisms**

The CD8 T cells mediate their response and induce infected target death by several effector mechanisms, including Fas-FasL pathway, whereby the caspase cascade is activated. Effector mechanisms could be divided into lytic (cytotoxicity) and non-lytic (cytokine production) (Figure 29). These effector functions are primarily regulated by the balance between the T-bet, Eomes, and Runx families of transcription factors. The cytotoxic capacity of CD8<sup>+</sup> T-cells depends on the content of their lytic granules and their degranulation capacity (Betts and Koup, 2004). Lytic granules are secretory lysosomes containing effector molecules, such as granzymes and perforin. The core of the granule is surrounded by a lipid bilayer containing lysosome-associated membrane glycoproteins (LAMP), including LAMP-1 (CD107a), LAMP-2 (CD107b), and LAMP-3 (CD63). During the degranulation process, the granule membrane is

fused with the plasma membrane of the activated CD8<sup>+</sup> T-cell, and the content of the granule is released into the immunological synapse between the CD8<sup>+</sup> T-cell and the target cell (Betts and Koup, 2004). LAMP molecules are not found on the surface of resting CD8<sup>+</sup> T-cells. Therefore, the evaluation of the surface expression of CD107a/b allows to identify the degranulation of CD8<sup>+</sup> T-cells. Moreover, apoptosis-inducing Fas ligand (CD95L) is stored in specialized secretory lysosomes of CD8<sup>+</sup> T-cells and the degranulation process controls its expression at the cell surface (Khaitan and Unutmaz, 2011; Perdomo-Celis et al., 2019).

The main mechanism by which CD8<sup>+</sup> CTL are able to induce death of their targets is the granule exocytosis pathway that is mediated by the pore-forming protein perforin and granzyme B. Granzyme B, the principle granzyme in CTL-mediated apoptosis, belongs to the family of the neutral serine proteases and induces apoptosis by triggering activation of caspases within target cells (Khaitan and Unutmaz, 2011). Perforin and granzymes are the most abundant proteins within the lytic granules of CD8<sup>+</sup> T-cells and cooperate for inducing death of target cells. Pore formation by perforin facilitates the entry of granzymes into target cells (Lopez et al., 2013). Cytokines secreted by CD8<sup>+</sup> T-cells include IFN- $\gamma$ , TNF- $\alpha$ , and IL-2, important for promoting antiviral, inflammatory responses, and T-cell survival and proliferation, respectively. Additionally, CD8<sup>+</sup> T-cells secrete the  $\beta$ -chemokines macrophage inflammatory protein (MIP)-1 $\alpha$  (CCL3), MIP-1 $\beta$  (CCL4), and regulated upon activation, normal T-cell expressed and secreted (RANTES; CCL5) (Perdomo-Celis et al., 2019).

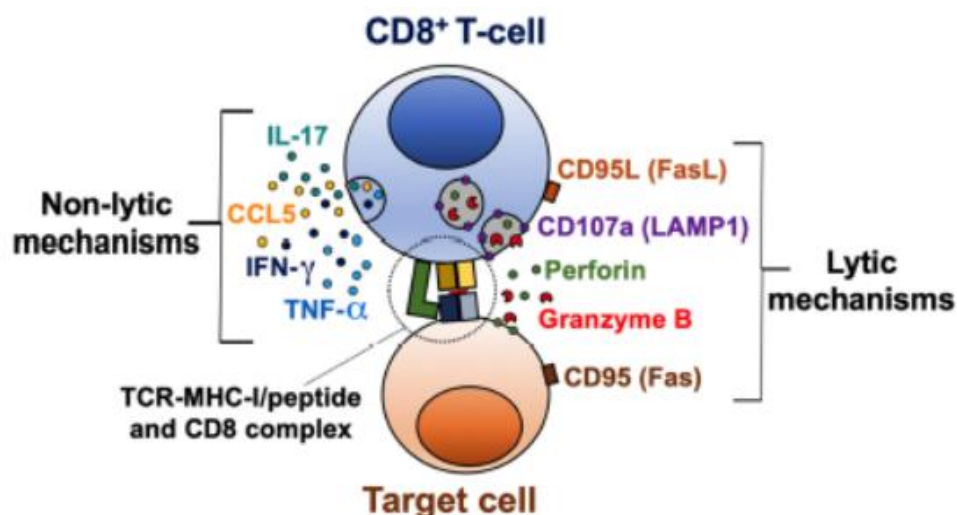


Figure 29. Lytic and non-lytic effector mechanisms of CD8<sup>+</sup> T-cells (Perdomo-Celis et al., 2019)



### 6.3. CD8+ T cell subpopulations

Several CD8+ T-cell subsets have been described, based on the expression of the differentiation markers CD45RA, CD45RO, CCR7, CD62L, CD27, and CD28, among others. Initial characterization of these subsets was based on expression of CD45RA and CD27 (Hamann et al., 1997). While later studies distinguished Tem and Tcm subsets based upon expression of CCR7 and staining for CD45RA and either CD27 or CCR7.

CD27 and CD28 have been used as cell surface markers to discriminate naïve and memory CD8+ T cells from effector CD8+ T cells. These molecules are well known as T cell costimulatory molecules. CD28 interacts with CD80(B7-1) and CD86(B7-2), which are expressed on APCs, and has a dual role as an adhesion and a signaling molecule. Co-engagement of CD28 with the TCR is required for T cell activation. CD28 is downregulated after TCR stimulation. CD27 is less well characterized than CD28. Its expression is up regulated upon TCR stimulation. The CD27 ligand CD70 is transiently up-regulated by Ag receptor stimulation on both T and B cells (Lens et al., 1998). CD27 ligation enhances TCR-induced expansion of both CD4+ and CD8+ T cells. CD45RA, which is one of two CD45 isoforms, has also been used as a marker of naïve CD8+ T cells as well as effector cells. It is an important marker of cellular differentiation and senescence. CCR7 is a chemokine receptor that functions as a homing receptor in the migration of naïve and memory CD8+ T cells to secondary lymphoid tissues. Analysis of chemokine receptor expression may be useful for discriminating among naïve, memory, and effector CD8+ T cells (Larbi and Fulop, 2014).

This identifies naïve (CD45RA+/CD27+/CCR7+), T effector memory (Tem) (CD45RA-/CD27-/CCR7-), T central memory (Tcm) (CD45RA-/CD27+/CCR7+), and T effector memory RA+ (Temra) (CD45RA+/CD27-/CCR7-) CD8 T cells. The most extended classification divides them into naïve, central memory, effector memory, and terminal effector cells (Temra); each of them has different effector capacities, particularly evaluated by the expression of granzymes, perforin, IFN- $\gamma$ , the degranulation ability, and in vitro cytotoxicity. The lowest functional capacity is observed for naïve CD8+ T-cells, which have low expression of effector molecules, reduced degranulation capacity and low in vitro cytotoxicity. Central memory cells have a low/intermediate cytotoxic potential. However, these cells can degranulate and express de novo effector molecules after polyclonal or antigen-specific stimulation. Finally, effector memory and terminal effector cells are characterized by a high cytotoxic capacity (See table 2) (Hamann et al., 1999; Martin and Badovinac, 2018; Perdomo-Celis et al., 2019; Romero et al., 2007).

Cell subset	Phenotype	Degranulation (CD107a/b expression)	Cytotoxic molecules expression (granzymes and/or perforin)	<i>In vitro</i> cytotoxicity	<i>De novo</i> synthesis of granzymes and/or perforin*	IFN- $\gamma$ production*	Main location
Naive	CD45RA <sup>+</sup> CD45RO <sup>-</sup> CCR7 <sup>+</sup> CD62L <sup>+</sup> CD28 <sup>+</sup> CD27 <sup>+</sup> CD57 <sup>-</sup>	-	-	-	-	-	Secondary lymphoid tissues
Central memory	CD45RA <sup>-</sup> CD45RO <sup>+</sup> CCR7 <sup>+</sup> CD62L <sup>+</sup> CD28 <sup>+</sup> CD27 <sup>+</sup> CD57 <sup>-</sup>	++	++	++ <sup>*</sup>	++	+++	Secondary lymphoid tissues
Effector memory	CD45RA <sup>-</sup> CD45RO <sup>+</sup> CCR7 <sup>-</sup> CD62L <sup>-</sup> CD28 <sup>+/-</sup> CD27 <sup>+/-</sup> CD57 <sup>-/+</sup>	+++	+++	+++	+	+++	Blood and inflamed tissues
Terminal effector	CD45RA <sup>+</sup> CD45RO <sup>-</sup> CCR7 <sup>-</sup> CD62L <sup>-</sup> CD28 <sup>-</sup> CD27 <sup>-</sup> CD57 <sup>+</sup>	+++	+++	+++	+	+++	Blood and inflamed tissues

\*After 5 h stimulation.

Table 2. Effector capacity of CD8<sup>+</sup> T-cells according to their differentiation stage (Perdomo-Celis et al., 2019)

Memory CD8 T cells of distinct phenotypes accumulate with age, and accumulation of Temra cells in humans is influenced by chronic infections, such as CMV. (Martin and Badovinac, 2018). Romero et al, studied more extensively the memory subsets. They subdivided the population based initially on CD45RA and CCR7 expression: naive (RA+CCR7+), CM (RA-CCR7+), EM (RA-CCR7-), and effector (EMRA; RA+CCR7-). Naive and CM cells uniformly co-expressed CD27 and CD28. In contrast, the RA+CCR7- EMRA T cells were split into three subsets: pE1 (27+28+), pE2 (27+28-), and effector (27-28-) T cell subpopulation. EM T cells also exhibited high heterogeneity with a differential expression of CD27 and CD28 cell surface molecules. Thus, they identified, within the RA-CCR7- EM T compartment, four phenotypically separate subsets referred to as EM1 (27+28+), EM2 (27+28-), EM3 (27-28-), and EM4 (27-28+) (figure 30) (Romero et al., 2007). EM1 (CD27+CD28+) and EM4 (CD27-CD28+) T cells express low levels of effector mediators such as granzyme B and perforin and high levels of CD127/IL-7R $\alpha$ . EM1 cells also had a relatively short replicative history and display strong ex vivo telomerase activity. Therefore, these cells were closely related to central-memory (CD45RA-CCR7+) cells. In contrast, EM2 (CD27+CD28-) and EM3 (CD27-CD28-) cells expressed mediators characteristic of effector cells, whereby EM3 cells displayed stronger ex vivo cytolytic activity and had experienced larger numbers of cell divisions, thus resembling differentiated effector (CD45RA+CCR7-) cells. These data indicate that progressive up-regulation of cytolytic activity and stepwise loss of CCR7, CD28, and CD27 both characterize CD8<sup>+</sup> T cell differentiation (Romero et al., 2007)

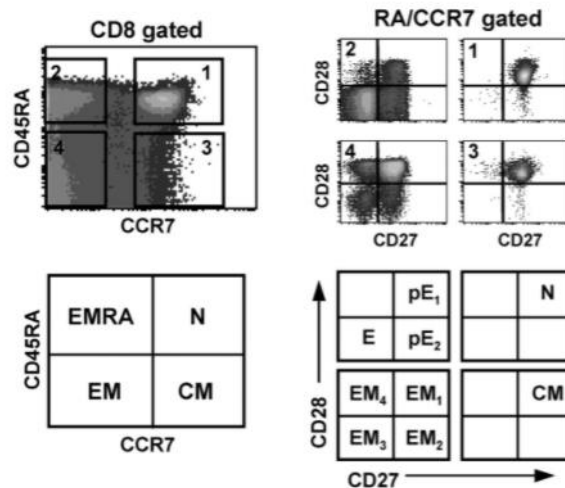


Figure 30. Differential expression of CD45RA, CCR7, CD27, and CD28 cell surface molecules on total CD8+ T cells from healthy blood donors (Romero et al., 2007)

#### 6.4. CD8 T cells dysfunction during HIV infection despite cART

HIV-specific CD8+ T-cells are able to control the virus during acute HIV/SIV infection, but their cytotoxic potential dramatically decreases along with disease progression and are no longer capable of exerting an appropriate antiviral response (Demers et al., 2016). CD8+ T-cells suffer important alterations in their frequency, differentiation, and activation profile. Persistent antigenic stimulation leads to immune exhaustion, a phenomenon in which effector T cells become dysfunctional and lose effector functions and proliferative capacity (Fenwick et al., 2019; Khaitan and Unutmaz, 2011; Larbi and Fulop, 2014).

Compared with seronegative individuals, the total pool of circulating CD8+ T-cells is persistently increased in untreated HIV-infected patients, with higher frequency of memory subsets and reduction of naïve cells. In addition, patients exhibit higher expression of the activation markers HLA-DR, CD38, and Ki-67, and immune exhaustion markers such as programmed death (PD)-1 and T-cell immunoglobulin and mucin-domain containing-3 (TIM-3), lymphocyte activation gene protein (LAG-3), and cytotoxic T lymphocyte antigen-4 (CTLA-4) (Fenwick et al., 2019; Larbi and Fulop, 2014). The expression of these molecules during chronic HIV infection is accompanied by the impairment of CD8+ T-cell lytic and non-lytic mechanisms, as well as the proliferative ability and survival (Perdomo-Celis et al., 2019). The progressive loss of CD8 T-cell function starts with an initial loss of proliferative capacity, cytotoxic potential, and a restricted IL-2 production. In more pronounced stages of exhaustion associated with chronic exposure of T cells to viral antigen, T cells eventually lose the ability

to produce IFN- $\gamma$  (Figure 31) (Fenwick et al., 2019; “Memory CD8 T-Cell Differentiation during Viral Infection,” n.d.).

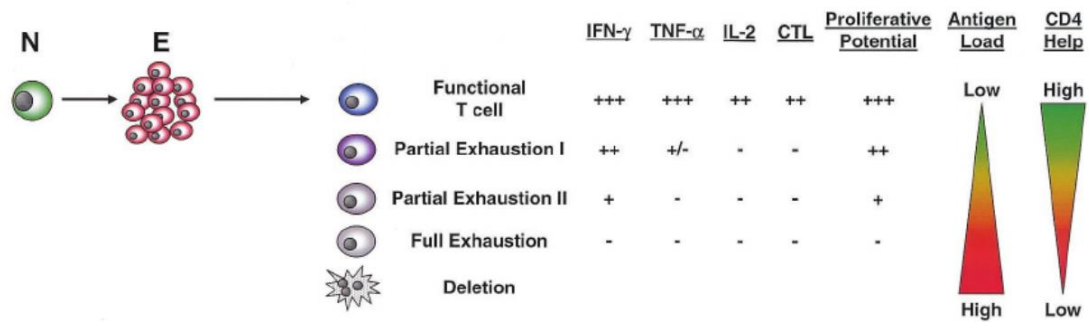


Figure 31. Model for hierarchical exhaustion of CD8 T-cell functions during chronic viral infections (“Memory CD8 T-Cell Differentiation during Viral Infection,” n.d.)

Among the various T cell surface markers described above, CD57, KLRG1, and PD1 have each been associated with replicative senescence, describing T cell populations with reduced proliferative capacity and impaired function. CD57 expression identifies senescent human T cells displaying a terminally differentiated phenotype and increases during HIV infection, probably as a result of chronic immune activation (Larbi and Fulop, 2014; Simonetta et al., 2014). It has been suggested that Effector Memory cells are the subset most predisposed to express PD1, whereas TEMRA display higher levels of CD57 and KLRG1. At present, CD57 and KLRG1 are considered to be inhibitors of proliferation, whereas PD1 is primarily associated with reversible immune exhaustion. In particular, the TEMRA populations (which are defined as the latest stage of T cell differentiation) express CD57 but not PD1, whereas the effector memory-like cells (CD28-CD45RA-) displays the highest levels of PD1 often in the absence of CD57 (Larbi and Fulop, 2014).

Treatment with c-ART induces suppression of viral load and increase of CD4+ T-cell counts. In addition, it induces improvement of some of the CD8+ T-cell alterations found in HIV-infected patients, whereas treatment discontinuation causes the increase of CD8+ T-cell activation and dysfunction. However, compared with seronegative individuals, c-ART does not fully reconstitute the CD8+ T-cell counts (Tanko et al., 2018), the proportions of memory subsets, the levels of activation and exhaustion markers, and their functional capacity (Figure 32B) (Perdomo-Celis et al., 2019; Tanko et al., 2018)

Three major determinants of CD8 T cell dysfunction during chronic infections including HIV are: i) persistent antigen exposure, negative co-stimulation, and chronic inflammation. Even in the presence of c-ART induced viral suppression, the three determinants of dysfunction are still present, because of the residual HIV replication and microbial burden, both of which maintain a persistent antigen load, induce continuous expression of inhibitory receptors and the presence of inflammatory mediators. Tissue reservoirs promote persistent antigenic burden during HIV infection even with c-ART. In addition, loss of integrity of gut mucosa is also responsible for a high antigenic burden, which drives immune dysfunction. This model of CD8 T cell activation, exhaustion and dysfunction during treated HIV infection is depicted in the figure 32A (Perdomo-Celis et al., 2019).

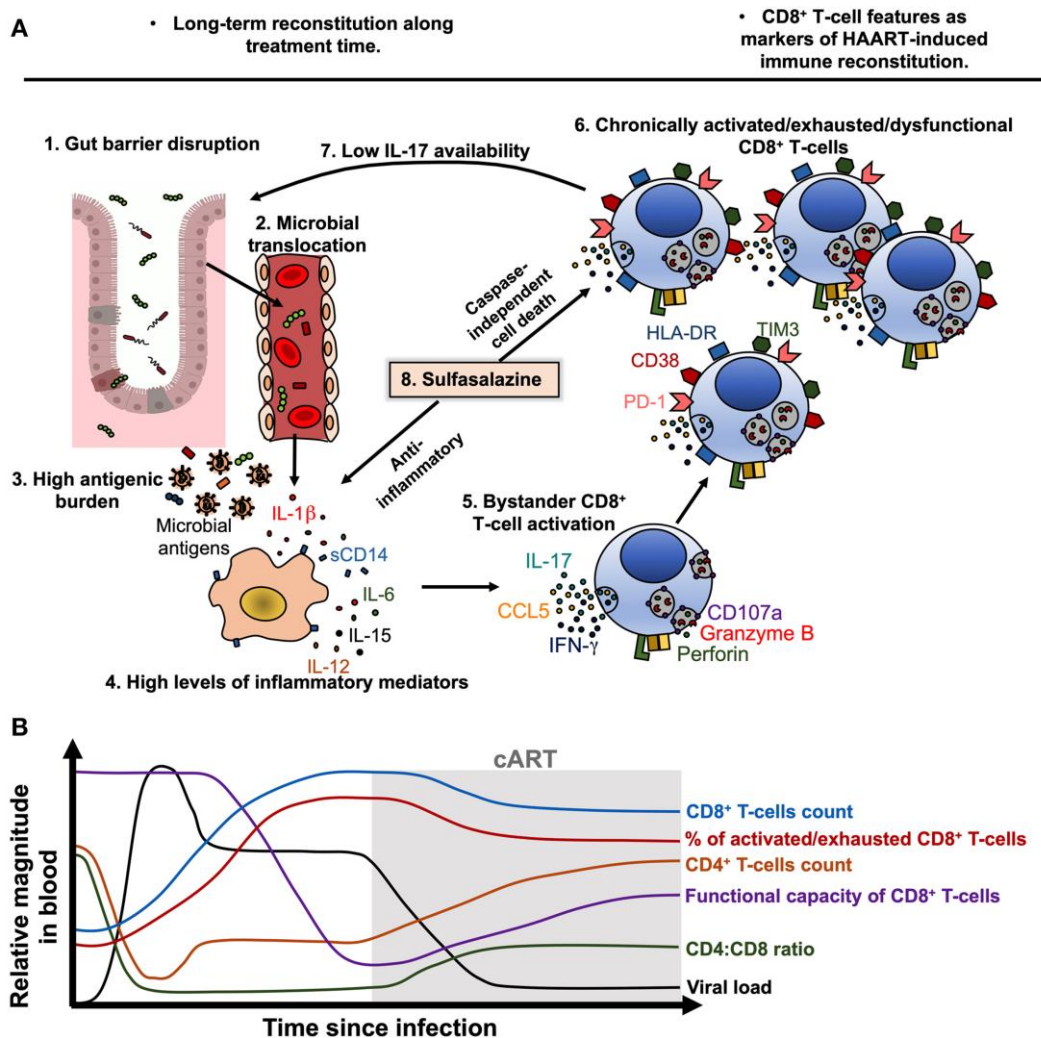


Figure 32. Model of CD8 T cell exhaustion and dysfunction during treated HIV infection (Perdomo-Celis et al., 2019)

## 6.5. Role of IL-33 on CD8+ T cells

Two independent studies have shown that activated CD8 T cells can express ST2 and that exposure to mature IL-33 (mtrIL-33), in synergy with TCR and/or IL-12 – enhanced IFN- $\gamma$  production in effector CD8 T cells (Ngoi et al., 2012; Yang et al., 2011). Consistent with these findings, Bonilla et al. showed that following LCMV infection in mice, roughly 20% of activated antigen (Ag)-specific CD8+ T cells expressed ST2 (Bonilla et al., 2012, p. 33). This study provided evidence for the first time that IL-33 can drive protective antiviral CD8+ T cell responses in vivo. However, it remained to be answered why only 20% of the activated CD8+ T Cells expressed ST2. Whether they are a unique subset of CD8 that express or upregulate ST2. Interestingly, another study examining the expression of IL-33 in cervical tissue of patients with different stages of HPV cervical disease showed that lower levels of IL-33 in cervical tissue were associated with more severe stages of HPV-induced dysplastic change, suggesting that this cytokine can have a protective Th1-type, anti-HPV response (Wang et al., 2014).

Viral infections can trigger the release of the alarmin IL-33 from non-hematopoietic cells. Given its ability to direct Th1-type immune responses, IL-33's potential role in inducing protective immunity against viral infections was studied by several groups. Bonilla et al. revealed that IL-33 is necessary for potent CD8+ T cell responses to replicating prototypic RNA and DNA viruses in mice (Bonilla et al., 2012), suggesting that IL-33 has the potential to boost CD8+ T cell immune responses. This study provided evidence for the first time that IL-33 can drive protective antiviral CD8+ T cell responses in vivo.

Bonilla et al, described the role of IL-33 for controlling lymphocytic choriomeningitis virus (LCMV). It was shown that IL-33 signals through its receptor ST2, on effector CTLs, to enhance their activation and antiviral functionality. Its function during memory formation, maintenance, and recall responses, was studied by another group, by adoptive transfers of LCMV-specific CTLs into either IL-33-deficient or wild-type (WT) hosts (Baumann et al., 2019, p. 8). IL-33 was not required for the formation and maintenance of memory CTLs. However, it is critical for memory CTL re-expansion, efficient effector differentiation, and viral control during secondary infection.

IL-33 indirectly supports the differentiation of T cells towards fully competent effector and memory cells by promoting their proliferation. In LCMV infection, IL-33 enhances the clonal expansion and functional maturation of CTLs and Th1 cells in a T cell-intrinsic fashion. ST2-deficient CTLs show an impaired capacity to kill virus-infected cells and IL-33 signals are necessary for the control of chronic LCMV infections (Peine et al., 2016). The regulation of CTL functions by IL-33 and the mechanisms of ST2 expression in these cells are not yet fully understood. Primary CD8<sup>+</sup> effector T cells that were generated *in vitro* in the presence of IL-12 were reported to express ST2 in a T-bet-dependent but Eomesodermin-independent manner. In addition, it is unknown how CTLs generated *in vivo* in the context of infections control ST2 expression at the molecular level. A major difference between the GATA-3/STAT5- and T-bet/STAT4-dependent pathways to access the *Il1rl1* gene lies in the stability and magnitude of the resulting ST2 expression. GATA-3-mediated ST2 expression in Th2 and Treg cells occurs constitutively and in high amounts. By contrast, ST2 expression in Th1 cells, where it is driven by T-bet/STAT4, occurs only transiently during the acute effector phase and at significantly lower levels than in Th2 or Treg cells (Peine et al., 2016).

In a murine coxsackie B virus model of experimental pancreatitis, IL-33 induced the production of IFN- $\gamma$  and TNF- $\alpha$  by CD8<sup>+</sup> T and by NK cells, resulting in viral clearance. Production of IL-33 in the pancreas post infection was markedly elevated, whereas the production of sST2 was reduced (Sesti-Costa et al., 2013).

These pleiotropic actions of IL-33 implicate it in diverse phases of the immune inflammatory process. This opens new avenues for harnessing the power of IL-33 as an immunostimulatory adjuvant for improving prototype vaccines against diseases that require Th1 protective immunity, such as TB, malaria, HIV and even cancer (Villarreal and Weiner, 2015). Villarreal et al. were the first to demonstrate that the delivery of IL-33 as a molecular adjuvant in a DNA vaccine setting could induce protective immunity by further enhancing, both arms of the adaptive immune responses (Villarreal et al., 2014). It was shown using a murine model for HPV-associated cancer, that IL-33 can orchestrate protective antiviral CTL responses, expand the magnitude of Ag-specific CD8<sup>+</sup> T cells responses, and enhance the production of TNF $\alpha$  and IFN $\gamma$  (Villarreal et al., 2014). The demonstration that IL-33 can induce both Ag-specific Th1 and CD8<sup>+</sup> T cell responses in preclinical settings makes IL-33 an ideal adjuvant candidate

for enhancing prophylactic and therapeutic vaccines in the treatment of chronic viral infections and cancer vaccine therapies (Figure33).

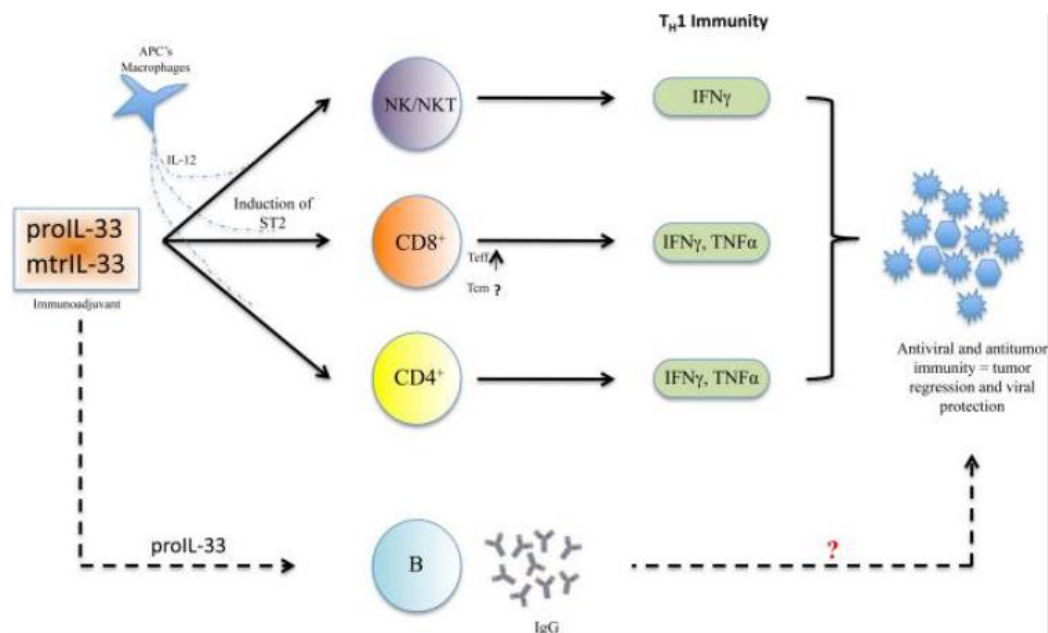


Figure 33. Immunoadjuvant properties of IL-33 for antiviral/anti tumour immunity mediated by Th1 response. (Villarreal and Weiner, 2014): Administration of either proIL-33 or mtrIL-33, upregulates the ST2 receptor and cellular activities on NK/NKT, CD4+, and effector CD8+ T cells which produce Th1-associated protective immunity. Concomitantly, M1 macrophages and APCs produce and secrete IL-12, which then induces expression of ST2 on NK/NKT cells and CD8+ T cells, permitting IL-33 to induce Th1-associated cytokine production. It is unclear whether Th1 CD4+ T cells are able to upregulate ST2, however, IFN $\gamma$  production by other activated immune cells likely leads to their amplification which then can help activate antiviral and tumoricidal immunity (Villarreal and Weiner, 2014).

## 7. Dynamics of IL-33/sST2 axis in HIV

The soluble form of ST2 functions as a decoy receptor, by binding free IL-33 and acting as a negative regulator. In 2011, Miyagaki et al. were the first to report elevated sST2 levels in HIV-infected patients on ART and in uninfected patients with skin barrier damage due to atopic dermatitis, compared to healthy controls. sST2 levels were elevated in HIV infected patients, while serum IL-33 levels were significantly lower independently of the severity of their infection, than in healthy controls. High sera sST2 levels have been reported in HIV infected patients as compared with HIV negative controls, with the highest in those at early stage of infection (Mehraj et al., 2016a). In contrast, another study reported that long term ART treated HIV infected patients with high rates of cardiovascular diseases, had similar sST2 levels compared to age and sex matched controls. Despite non-elevated levels of ST2, this marker remained an independent predictor of cardiovascular dysfunction and all-cause mortality



(Secemsky et al., 2015). It was also the only factor predictive of diastolic dysfunction (Secemsky et al., 2015). Furthermore, Fitch et al. reported elevated ST2 levels as a marker of myocardial fibrosis in asymptomatic HIV-infected patients (Fitch et al., 2016).

We have shown that in a large cohort of 1800 HIV-1 infected patients on HAART, sST2 was shown to be a significant predictor of all-cause mortality. In addition, the risk of death increased gradually with baseline sST2 serum levels, reaching a 2.3- fold increase in patients with highest values (Thiébaud et al., 2017b).

During anti-retroviral therapy, both IL-33 and sST2 levels were decreased (Khaitan and Unutmaz, 2011). While there was no significant reduction in the sST2 levels in patients initiating ART during early phase of infection, long term treatment started during chronic phase normalized sST2 (Mehraj et al., 2016a)

It has also been showed that, during acute HIV infection, sST2 levels correlated with the levels of intestinal fatty acid binding protein (I-FABP) and of sCD14 markers of gut epithelial damage and of microbial translocation, establishing a link between sST2 and gut tissue damage. These associations were lost during chronic HIV infection, suggesting a phase dependent and transient role of this axis in disease progression (Mehraj et al., 2016a).

Mehraj et al determined the alarmin contribution of the IL-33/ST2 axis to the immune response by reporting a correlation of elevated sST2 levels with the CD8 T-cell count and with T-cell activation in the absence of a correlation with the plasma viral load. Thus, the immune response following acute infection characterized by increased numbers of CD8 T-cells and increased activation of these cells was associated with elevated sST2 levels, in response to ‘the alarm’. The elevated sST2 levels correlated with indoleamine 2,3-dioxygenase enzyme activity and IFN- $\gamma$  levels, both of which are implicated in Th1/Th2 regulation. On the contrary, a recent study assessing the regulation of T cell function by IL-33/ST2 axis, reported the elevated sST2 levels as well as IL-33 in early HIV-infected patients (treatment naïve) with progressive disease. This elevated sST2 was suggested to weaken the IL-33 induced HIV-specific CD4 and CD8 T cell immune responses, indirectly contributing to the dysfunction of T cells and rapid disease progression. This was demonstrated in vitro, by the upregulation of IFN $\gamma$  by both GAG- as well as CEF-stimulated CD4 and CD8 T cells in HIV infected patients. This enhanced response was shown to be attenuated by the addition of anti-ST2 antibodies (Wu et al., 2018).

The role of this axis in the gut in acute and chronic phase of HIV infection is depicted in figure 34. In acute phase, sST2, is released into the peripheral blood and correlates with markers of gut damage and immune activation. Whereas, during chronic infection, the epithelial gut damage extends with persistent IL-33 release and immune activation. IL-33, along with TGF- $\beta$  may contribute also to tissue fibrosis. IL-33, in conjunction with long-term TGF- $\beta$  stimulation, has been shown to be associated with the development of fibrosis in a murine model of bleomycin-induced fibrosis (Li et al., 2014). During the chronic phase of HIV infection, collagen deposition in lymphoid tissues including Peyer's patches in the gut disrupts the T-cell zone and limits the number of resident CD4 T-cells (Schacker et al., 2002). CD4 T cell levels decrease in the periphery and the sST2 levels in the plasma decline in comparison to the acute phase of infection. Thus, after sounding the alarm, the IL-33/ST2 axis may contribute to the development of fibrosis during the chronic phase of HIV infection (Mehraj et al., 2016b)

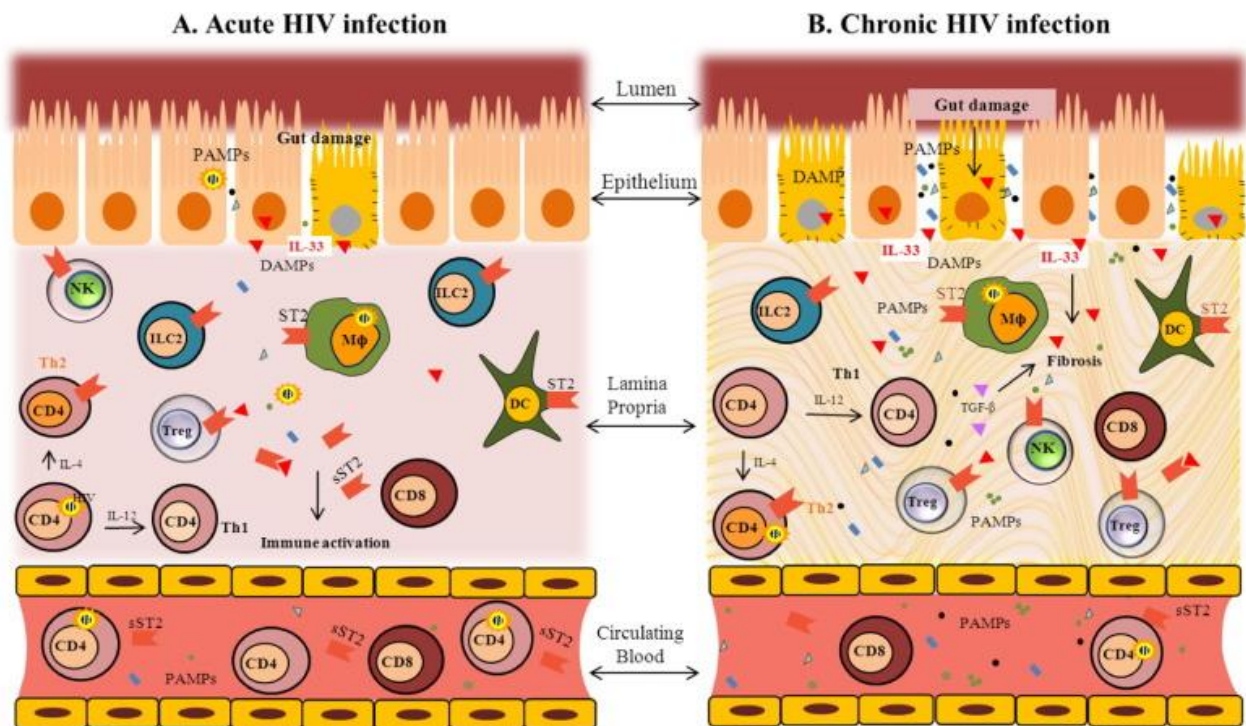


Figure 34. The Dynamic Role of the IL-33/ST2 Axis in gut epithelial homeostasis and immune activation in chronic viral-infection (Mehraj et al., 2016b): During acute phase, PAMPs and epithelial gut damage lead to release of IL-33, acting as DAMP alerting the immune system. Expression of the receptor for IL-33, ST2, is increased on the immune cells in the lamina propria. The immune activation contributes to the imbalance in Th1/Th2/Treg ratios. sST2, the decoy receptor for IL-33 is released into the peripheral blood, which correlates with the markers of gut damage and immune activation. During chronic infection, the epithelial gut damage extends with persistent IL-33 release and immune activation. IL-33, along with TGF- $\beta$  contribute also to tissue fibrosis. CD4 T cell levels decrease in the periphery and the sST2 levels in the plasma decline in comparison to the acute phase of infection (Mehraj et al., 2016b)

# AIMS AND OBJECTIVES OF THE STUDY

Despite antiretroviral therapy, the gut mucosal epithelial barrier and immune dysfunctions are not reversed. The exact mechanism is still not known as to why the gut barrier is not repaired. Several factors are responsible for the so-called leaky gut, leading to microbial translocation, which persists despite suppressed viremia, including direct damage by the virus, proinflammatory cytokines, decreased epithelial repair genes, increased enterocyte apoptosis, imbalance of Th17/Treg ratio, as well as development of fibrosis (Klatt et al., 2013b)(Tincati et al., 2016)(Sandler and Douek, 2012).

The role of Treg is controversial during HIV infection. Treg cells have been shown to perform essential functions in maintenance of tissue tolerance by promoting tissue homeostasis and regeneration in nonlymphoid tissues. These nonlymphoid tissue Treg (tTreg) cells are thought to promote tissue repair by sensing tissue damage in situ via soluble factors including the proinflammatory cytokine IL-18 and the alarmin IL-33, at least in part independent of TCR signaling. A major component of this tissue regenerative pathway in Treg cells is the production of amphiregulin, a growth factor that plays an important role in healing. Amphiregulin activates locally latent TGF $\beta$  and contributes to both the local suppression of inflammation and the local differentiation of tissue stem cells, and in this way to the process of wound healing and restoration of tissue homeostasis

The persisting gut barrier disruption with microbial translocation also adds to the high antigenic burden. This causes increase in inflammatory mediators subsequently leading to chronically activated, exhausted and dysfunctional CD8 T cells (Perdomo-Celis et al., 2019). IL-33 role in directing Th1-type anti-viral immune responses, signalling through its receptor ST2 on immune cells has been demonstrated by some groups already (Bonilla et al., 2012, p. 33) (Sesti-Costa et al., 2013) (Villarreal et al., 2014). It has also been shown that IL-33 can act as an immunoadjuvant to induce viral-specific Th1 response (Villarreal et al., 2014).

During my thesis, I wondered the dual role of IL-33 during HIV infection on the function of tTreg and on CD8<sup>+</sup>T cells. The question that arises is whether a defect in tissue repair involving IL-33/ST2 and tissue Tregs/Amphiregulin pathways are the cause of the leaky gut and systemic inflammation. The second question is whether IL-33 can enhance antiviral immune response in HIV infected patients on c-ART.

For this purpose, we enrolled 31 infected patients who had the following characteristics: i) Duration of HIV disease > 5 years, ii) CD4 T cells > 200/mm<sup>3</sup>, iii) Undetectable plasma HIV

RNA (below <20 copies/ml for at least 1 year). The healthy donor participants were age matched. We obtained from these patients PBMC and rectal biopsies. Unfortunately, due the sanitary crisis, we were not able to study the function of gut CD8+T cells because we could not enroll patients during this period.

- 1- We investigated whether the persistence of gut mucosal injury during HIV infection might be explained in part by a lack of mechanisms involved in tissue repair. We analyzed the level of IL-33 and fibrosis in the gut of HIV infected patients. We determined the phenotype and function of intestinal Treg population from HIV infected patients. Our data point out a new mechanism where the absence of amphiregulin secretion by Treg might contribute to the leaky gut and persistence of chronic inflammation.
- 2- We analyzed the phenotype and function of CD8+ST2+ T cells in healthy donor and in HIV infected patients. We determined the role of IL-33 on antigen specific CD8+ T response. Our results suggested that IL33 may serve as adjuvant for a therapeutic vaccine in order to boost the specific CD8 T cells response.

# **ARTICLE 1.**

**SUBMITTED**

**Profound defect of Regulatory T cells producing amphiregulin in the gut of HIV treated patients**

# **Profound defect of Regulatory T cells producing amphiregulin in the gut of HIV treated patients**

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**Abstract**

The persistence of a leaky gut in HIV treated patients leads to chronic inflammation with increased rates of cardiovascular, liver, kidney and neurological diseases. Tissue Treg (tTreg) are involved in the maintenance of intestinal homeostasis and wound repair through IL-33 pathway. In this study, we investigated whether the persistence of gut mucosal injury during HIV infection might be explained in part by a lack of mechanisms involved in tissue repair. We observed an increased level of IL-33 in the gut of HIV infected patients, which is associated with an increased level of fibrosis and a low peripheral reconstitution of CD4<sup>+</sup> T cells. Our results showed that intestinal Treg population from HIV infected patients is enriched in tTreg prone to support tissue repair. However, we observed a functional defect in tTreg due to the lack of amphiregulin secretion, which could contribute to the maintenance of intestinal damage. Our data points out a new mechanism where the absence of amphiregulin secretion by Treg might contribute to the leaky gut and persistence of chronic inflammation.

## **Introduction**

Human immunodeficiency virus 1 (HIV-1) infection is lifelong and to date, none of the available treatments eliminates the virus from infected patients. Indeed, effective antiretroviral therapy (ART) suppresses HIV-1 replication to undetectable levels but is unable to cure infection in people living with HIV (PLWH) because the virus integrates its genetic information into host cell's own DNA. Moreover, PLWH on treatment have chronic inflammation with increased rates of cardiovascular, liver, kidney and neurological diseases (Deeks, 2011). Microbial translocation was described as a mechanism for immune activation and an underlying cause of T-cell activation in HIV infection (Brenchley et al., 2006b) which persists in ART treated patients at significant higher levels as compared to HIV-uninfected controls. Impairment of the gut barrier is a prerequisite for microbial translocation and cross-sectional studies have shown the presence of gut mucosal injury and microbial translocation in long-term ART-treated individuals (Mudd and Brenchley, 2016b).

A novel concept in immunology is that tissue-resident immune cells not only mediate immune homeostasis and host defense but also critically contribute to the maintenance of organismal physiology (Panduro et al., 2016a). In this regard, Treg cells have been shown to perform essential functions in maintenance of tissue tolerance by promoting tissue homeostasis and regeneration in nonlymphoid tissues. These nonlymphoid tissue Treg (tTreg) cells are thought to promote tissue repair by sensing tissue damage in situ via soluble factors including the proinflammatory cytokine IL-18 and the alarmin IL-33, at least in part independent of TCR signaling (Arpaia et al., 2015b; Burzyn et al., 2013b). A major component of this tissue regenerative pathway in Treg cells is the production of amphiregulin (Arpaia et al., 2015b; Burzyn et al., 2013b; Ito et al., 2019), a growth factor that plays an important role in healing. Amphiregulin activates locally latent TGF $\beta$  (Minutti et al., 2019) and contributes to both the



local suppression of inflammation and the local differentiation of tissue stem cells, and in this way to the process of wound healing and restoration of tissue homeostasis (Zaiss et al., 2019).

IL-33 is a tissue-derived nuclear cytokine principally produced by endothelial cells, epithelial cells, fibroblast-like cells and myofibroblasts, rather than CD45<sup>+</sup> hematopoietic cells, during both steady state and inflammation, which regulates also the homeostasis of regulatory T cells in damaged tissues. Indeed, IL-33 signaling in tTregs provides a critical signal for Treg accumulation and maintenance in inflamed tissues (Schiering et al., 2014b; Sefik et al., 2015b). Thus, the prevailing model for ST2<sup>+</sup> tTreg cell function is that they specifically home to sites of damage in the intestine and mediate repair (Siede et al., 2016).

In this study, we investigated whether the persistence of gut mucosal injury during HIV infection might be explained in part by a lack of tissue repair, namely the IL-33/ST2 and the tTreg/amphiregulin pathways.

## **MATERIAL ET METHODS**

### **Study Population and sample collection**

Our study population included patients infected with HIV on c-ART. Rectal tissue and blood samples were collected during clinics for anal dysplasia screening from 31 patients recruited at the Clinical Immunology and Infectious diseases department (Henri Mondor hospital, Créteil, France). Tissue samples were also collected from 14 healthy donors (HD) during colonoscopy for colorectal cancer screening (Clinique Paris-Bercy, Charenton le Pont, France). For HIV infected patient's inclusion criteria include: i) HIV-1 infection ii) Age >18 years iii) Duration of HIV disease > 5 years iv) CD4 T cells > 200/mm<sup>3</sup> v) Undetectable plasma HIV RNA (below <20 copies/ml for at least 1 year). Median age of the 31 HIV patients was 44.3 years; 83.9% were men. None of the study participants were suffering from HBV or HCV co-infection. The healthy donor participants were within the same age range. Characteristics of HIV-1 infected patients are summarized in table 1.

For HIV patients, peripheral blood mononuclear cells and rectal biopsies were collected on the same day. All rectal biopsies (around 8 punches for each patient) were performed at the same site (i.e. at 10-15 cm from the anal margin) to avoid potential regional variation among patients and were collected in cold RPMI medium (supplemented with antibiotics). About 30-50 ml of blood was drawn by sterile venipuncture on the same day from HIV patients. Samples were transported at room temperature for immediate processing.

### **Ethics statements**

All study participants provided written informed consent to participation in the study. The study protocol was reviewed and approved by the Sud-Méditerranée II ethics committee (Comité de Protection des Personnes), was conducted in accordance with good clinical practice and the Declaration of Helsinki and was registered at ClinicalTrials.gov (NCT03622177).

### **Blood and tissue processing**

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll (Lymphoprep ProteoGenix) density gradient centrifugation and used immediately as well as the following day after overnight resting in culture medium at 37°C, simultaneously with the recovered LPL from biopsies. To isolate Lamina Propria lymphocytes (LPL) from biopsies, the tissue was first subjected to vigorous shaking incubation at room temperature for 25 minutes in HBSS medium (without Ca<sup>2+</sup> and Mg<sup>2+</sup>) (gibco) supplemented with 1mM DTT (Sigma) and 1mM EDTA, to release the Intraepithelial lymphocytes (IEL), which were discarded. This mechanical disruption is followed by transfer of the tissue specimen to R-10 medium supplemented with Penicillin (100 U/ml), Streptomycin (100 µg/ml), Amphotericin B (1.25µg/ml) and Gentamicin (50µM) (gibco), and rested overnight at 37°C, 5% CO<sub>2</sub> in a 6 well plate. The following day, the culture supernatants are collected to recover released cells.

### **Immunophenotype Analysis**

The phenotype of regulatory T cells (Tregs) was assessed using FACS staining with the following Abs: CD3 Alexa700 (UCHT1, BD Pharmingen), CD4 BV605 (RPA-T4, BD Horizon), CD8 efluor780 (SK1, eBioscience), CD25 PeCy7 (M-A251, BD), IL-18Ra APC (H44, eBioscience), ST2L FITC (B4E6 MD Biosciences), CD161 BV421 (Biolegend). For intracellular staining, cells were fixed and permeabilized using the FoxP3 staining buffer set (eBioscience, Thermo Fisher Scientific), washed, and incubated with FoxP3 Alexa488/PE-CF594 (259D/C7, BD) and amphiregulin-PE (AREG559). For all cell stainings, dead cells were excluded from the gating by using the LIVE/DEAD fixable dead cell stain kit (Molecular Probes, Invitrogen). Cells were acquired on an LSRII flow cytometer (BD Biosciences) and analyzed with FlowJo software, version 10 (FlowJo LLC).

### **ST2 and Amphiregulin staining**

To study Amphiregulin expression, isolated LPL and PBMCs suspended in R10 medium supplemented with antibiotics in a 24 well plate, were stimulated for 4 hours at 37°C, 5% CO<sub>2</sub> with αCD3-CD28 dynabeads (Thermofisher) (cell to bead ratio of 1:1) in the presence of GolgiStop (BD Biosciences) and Brefeldin A. To study Tregs, the cells were stimulated with medium alone in the presence of Golgistop and Brefeldin A. For ST2L staining, the antibody was added to all the wells for the 4hrs of incubation.

### **Immunohistochemistry**

Deparaffinized rectal tissue sections were stained with polyclonal goat anti-human IL-33 (cat. AF3625, R&D Systems) at a 1:100 dilution at room temperature for 1 hour. Enzymatic activity was blocked using BLOXALL™ Blocking Solution (SP-6000, VectorLabs) (phosphatase and peroxidase block). ImmPRESS HRP REAGENT KIT Anti-GOAT IgG (MP-7405, VectorLabs) was used containing horse blocking serum and Peroxidase Polymer anti-goat IgG reagent. For non-specific blockage prior to primary antibody staining, horse blocking serum was used for 30 minutes(ImmPRESS kit). Following the primary antibody wash, the Secondary anti-goat antibody was used for 30-40 minutes (ImmPRESS kit). Immunoreactive cells were visualized by addition of a diaminobenzidine substrate (DAB Substrate Kit for peroxidase) and were counterstained with hematoxylin.

### **Collection of rectal biopsy specimens for RNA extraction**

One or more fragments of rectal biopsies were collected in a small volume of RNAlater (QIAGEN Inc.) and kept at 4°C for 24 hours followed by transfer to -20°C.

### **RNA extraction and reverse transcriptase reaction**

The rectal biopsy specimens were cleared from the RNAlater, transferred to an aliquot containing a metallic bead and lysing solution constituting a mix of beta-mercaptoethanol and RLT plus buffer (QIAGEN Inc.) (volume ratio of 1:100), and disrupted in a cold mechanical disruptor (TissueLyser QIAGEN). RNA extraction was performed according to the manufacturer's protocol (RNeasy Plus Mini Kit, QIAGEN Inc.). RNA was converted to cDNA with High-Capacity RNA-to-cDNA Kit (Applied Biosystems).

### **Real time Quantitative PCR**

Quantitative PCRs were performed using the Brilliant II SYBR GREEN QPCR Master Mix kit (Agilent Technologies) for the expression of IL-33, EGFR and TGF $\beta$ . OAZ-1 mRNA was used as a control for sample normalization. Expression of the gene OAZ-1 was used as a reference and the relative levels of each gene were calculated using the  $2^{-\Delta\Delta CT}$  method.

### **Primers**

The following primers were used in this study:

*IL-33* (Forward CAAATGAATCAGGTGACGGTGT; Reverse TTGTTGGCATGCAACCAGAAG), *OAZ1* (Forward ACTTATTCTACTCCGATGATC; Reverse GAGAATCCTCGTCTTGTC), *EGFR* (Forward GGTGGCATTAGGGGTGACT; Reverse TGTGATAATTCAGCTCAAACCTGTG), *TGF $\beta$*  (Forward GCAAGTGGACATCAACGGGT; Reverse TCCGTGGAGCTGAAGCAATA).

### **Human Cytokine 6-plex assay**

Cytokine concentrations in the plasma of HIV patients were measured using Simoa Human Cytokine 6-plex assay, which simultaneously measures six cytokines with high sensitivity (~

fg/mL). The assay measured human IFN- $\gamma$ , IL-6, IL-10, IL-12p70, IL-17A and TNF $\alpha$  using the Simoa SR-X benchtop biomarker detection system. The plasma samples that were stored at -80°C were thawed at room temperature prior to the assay. The assay was performed in triplicates for each sample, in 96-well plate, following the manufacturer's instructions.

### **Quantification of ST2 and IL-33 protein levels**

Human sST2 protein concentrations in patients' plasma were measured by luminex assay (Luminex Human Discovery Assay (1-Plex) LXSAHM-01, R&D Systems). Human IL-33 protein concentrations in patients' plasma were measured using Bio-Plex 200 system (Bio Rad). Plasma samples were stored at -80°C degrees and thawed at room temperature for the assay. Luminex assays were performed according to manufacturers' instructions.

### **Quantification of fibrosis using semi-automated image analysis**

Paraffin embedded rectal tissue sections were stained with Sirius Red staining using Gemini AS Slide stainer. Images were taken at 4X magnification and subsequently analyzed using the batch mode of the ImageJ macro. The ImageJ macro was written to quantify the percentage of fibrosis within an image, using the example in the following link ; <https://imagej.nih.gov/ij/docs/examples/stained-sections/index.html>.

Briefly, for all images to be analyzed, the macro first splits the image into red, green and blue channels and color transformation to grayscale. The green channel is selected for thresholding, followed by analyzing to obtain % area of fibrosis.

### **Statistics.**

Groups were compared using the 2-sided nonparametric Mann-Whitney U test. P value below 0.05 was considered significant. All statistical analyses were performed using Prism (version 6.0, GraphPad Software).

## Results

### **Gut biopsies from HIV-1 infected patients expressed high level of IL-33 mRNA and protein**

We previously reported an increased level of sST2 in the serum of a large cohort of HIV-1 infected patients (Thiébaud et al., 2017a). Since sST2 is an indirect measure of IL-33 activity, we sought to measure IL-33 levels in the plasma and in gut biopsies from a cohort of 31 HIV-1 infected individuals under long-term cART (table 1) and HD. Previous studies have shown that plasma levels of IL-33 are usually very low and/or undetectable (Sun et al., 2019). Accordingly, we found that IL-33 levels in the plasma of HIV-1 and HD were below the limit of detection (data not shown). In contrast, IL-33 mRNA relative expression was significantly increased in gut biopsies from HIV-1 infected patients compared to HD ( $1.68 \pm 0.3\%$  versus  $0.58 \pm 0.1\%$ ;  $P = 0.0003$ ; **Figure 1A**). Immunohistochemistry study confirmed that high IL-33 gene expression was consistent with a significant increase of IL-33 protein expression in gut biopsies from HIV-1 infected individuals (**Figure 1B**). Remarkably, IL-33 protein was expressed by epithelial cells, endothelial cells, fibroblasts, fibroblast-like cells, as well as in the extracellular matrix in samples from HIV-1 infected patients whereas this expression was weak, limited to cell nuclei, or absent in biopsies from HD controls (**Figure 1B**). Since IL-33 is primarily produced by intestinal epithelial cells upon local damage (Griesenauer and Paczesny, 2017b, p. 33), we analyzed precisely biopsy samples based upon the level of IL-33 protein expression specifically in epithelial cells. Analysis revealed two groups of HIV-1 infected patients characterized by either a high level of IL-33 protein expressed predominantly in epithelial cells or a weak or no expression of IL-33 protein like HD (see **Figure 1C** for a representative staining in two different patients). The IL-33 expression pattern for 27 HIV-1 infected patients is depicted in Supplementary Figure 1.



## **IL-33 protein expression is associated with gut fibrosis and low peripheral CD4<sup>+</sup>T cells reconstitution**

IL-33 is an alarmin released after mucosal injury leading to control inflammation and fibrosis as mechanism of repair. Therefore, we performed Sirius red staining to analyze collagen deposition in gut biopsies as a marker of fibrosis (Dao et al., 2020). As shown in **Figure 2A**, we found an increased deposition of collagen in gut biopsies from HIV-1 infected patients than those from HD. Moreover, the frequency of areas exhibiting fibrosis was higher in biopsies from patients with high expression of IL-33 protein in epithelial cells than in patients with low or no IL-33 expression ( $P = 0.02$ ; **Figure 2B**).

Fibrosis within the parenchyma could have a profound impact on immunological function and lymphocyte population dynamics (Jacob D. Estes et al., 2008). Accordingly, we found a significant lower blood CD4<sup>+</sup> T cell counts in patients with high IL-33 expression than with low IL-33 expression ( $470.5 \pm 75.8$  vs  $694.0 \pm 81.9$ , respectively;  $P = 0.03$ ; **Figure 2C**). This distinct profile of patients was underscored by the measurement of several inflammatory markers using a SIMOA technology. Among several factors measured (**Figure 2D** and **Supplementary figure 2**), we observed a trend to an increase of IFN- $\gamma$  plasma levels in patients with high expression of IL-33 as compared to those with low levels ( $0.28 \text{ pg/ml} \pm 0.1$  vs  $0.11 \pm 0.01$ , respectively;  $P = 0.08$ ) whereas IL-12p70 was significantly increased ( $0.27 \pm 0.05$  vs  $0.17 \pm 0.01$  respectively;  $P = 0.04$ ; **Figure 2D**). IL-6, TNF $\alpha$ , IL-17 and IL-10 concentrations were similar in both groups (**Supplementary figure 2**).

## **A sub population of CD161<sup>+</sup> IL-18R $\alpha$ <sup>+</sup> Treg is specifically increased in gut biopsies from HIV-1 infected individuals**

IL-33-ST2 axis mediates repair of intestinal integrity through mucosal regulatory T cells (Whibley et al., 2019b). ST2<sup>+</sup>Treg are characterized by the capacity to produce amphiregulin and mediate wound healing and tissue repair. Among mucosal Treg cells, CD161<sup>+</sup> Treg cells,

a distinct highly suppressive population of Treg cells were described to be enriched in intestinal lamina propria (G. A. M. Povoleri et al., 2018). We investigated the frequency of tissue Treg (tTreg) in gut biopsies from patients and HD. In a first attempt, we did not find differences between groups in the frequency of total Treg cells, defined as Foxp3<sup>+</sup>CD25<sup>hi</sup>T cells (**Figure 3A**). Expression of IL-18R $\alpha$ , CD161 and ST2 led us to identify two populations of Treg in the lamina propria, i.e CD161<sup>+</sup>IL-18R $\alpha$ <sup>+</sup> Treg and ST2<sup>+</sup> Treg (**Figure 3B**). The frequency of Treg CD161<sup>+</sup>IL-18R $\alpha$ <sup>+</sup> was increased in the gut of HIV-1+ patients as compared to HD ( $11.52 \pm 2.9\%$  vs  $3.20 \pm 0.9\%$ ;  $P = 0.007$ ; **Figure 3D**). For the ST2<sup>+</sup>Treg population, its frequency was also increased in the gut of HIV patients compared to healthy donors without reaching significance ( $12.60 \pm 4.787\%$  vs  $4.77 \pm 0.19\%$ ,  $P = 0.25$ ; respectively; **Figure 3C**). The CD161<sup>+</sup>IL-18R $\alpha$ <sup>+</sup> Treg cell population was present only in the gut, but not in the blood, of HIV-1+ infected patients suggesting that these cells are tissue resident cells (**Figure 3D**).

#### **Amphiregulin production is decreased in the intestine of HIV infected patients**

We looked at three well-defined molecules involved in tissue repair and wound healing process, i.e TGF $\beta$ , Amphiregulin and its receptor, the EGF receptor (EGFR). We showed an increase of TGF $\beta$  mRNA in gut biopsies from HIV-1 infected individuals as compared to HD ( $8.14 \pm 1.9\%$  vs  $1.4 \pm 0.13\%$ , respectively;  $P = 0.06$ ; **Figure 4A**). Interestingly, IL-33 and TGF $\beta$  mRNA had a significant negative correlation ( $r = -0.88$ ;  $P < 0.0001$ ; **Figure 4B**). Amphiregulin, a molecule specifically produced by tTreg cells has a key role in protecting against tissue damage and maintaining barrier integrity during virus-induced inflammatory responses in mucosa (Arpaia et al., 2015b). We looked first at the capacity of total CD4<sup>+</sup> T cells isolated from gut biopsies to produce AREG following TCR stimulation. We found a decreased frequency of amphiregulin producing CD4<sup>+</sup> T cells in gut biopsies from HIV-1 infected individuals as compared to HD ( $1.90 \pm 0.3\%$  vs  $4.15 \pm 0.8\%$ ;  $P = 0.06$ ; **Figure 4C**). Among Treg cells secreting amphiregulin in the lamina propria from gut HD biopsies, as expected, ST2<sup>+</sup>Tregs

secrete more amphiregulin than their IL-18R $\alpha$ <sup>+</sup>CD161<sup>+</sup> counterparts do (**Figure 4D** and **Supplementary data 3**). Interestingly, we found a profound defect of Foxp3<sup>high</sup>CD25<sup>high</sup> T<sub>reg</sub> cells producing amphiregulin in gut biopsies from HIV-1 infected individuals with virtually no cell as compared to HD in whom 7.3 % of Foxp3<sup>high</sup>CD25<sup>high</sup> Treg cells produce amphiregulin (P = 0.02; **Figure 4E**). The receptor of amphiregulin, the EGF receptor (EGFR) also contributes to wound healing, mainly by inducing proliferation of epithelial cells within the wound. We found an upregulation of EGFR mRNA in HIV-1 infected individuals as compared to HDs (0.07  $\pm$  0.01 vs 0.03  $\pm$  0.0; P = 0.009; **Figure 4F**). Altogether, these data demonstrate a profound defect of AREG production by Tregs in the gut of HIV-1 infected patients despite the presence of Treg expressing ST2.

## Discussion

In this report, we showed that IL-33 expression is increased in the gut of HIV infected patients compared to non-HIV infected healthy individuals. When IL-33 secretion participates to fibrosis, mucosal Treg population from HIV infected patients is enriched in tTreg prone to support tissue repair. However, we observed a functional defect in tTreg due to the lack of amphiregulin production, which could contribute to the persistence of local damage and release of IL-33. Our data points out a new mechanism where the absence of amphiregulin secretion by Treg might contribute to the leaky gut and persistence of chronic inflammation.

We have previously reported in a large cohort of patients followed longitudinally that sST2 is a significant predictor of all-cause mortality (Thiébaud et al., 2017a). During acute HIV infection, sST2 levels correlated with the levels of intestinal fatty acid binding protein (I-FABP) and of sCD14, markers of gut epithelial damage and of microbial translocation, respectively (Jenabian et al., 2015). sST2 acts as a decoy receptor and, by sequestering IL-33, sST2 antagonizes the effects of ST2L/IL-33 interaction. These observations establish a link between IL-33/sST2 and gut tissue damage and the role of this axis in maintaining normal gut homeostasis particularly in promoting mucosal wound healing and repair (Whibley et al., 2019b). We show here that these axes are dysregulated in the gut from HIV infected patients even under long term ART and control of viral replication.

Our results have significant implication in the understanding of the role of the gut in HIV physiopathology since when dysregulated, IL-33 pathway can also play a critical role in the progression of chronic inflammation and fibrosis. Our results showed an increased area of fibrosis in patients who displayed high level of gut IL-33 expression. Increased expression/production of collagen in mucosal, GALT and lymph node has been reported previously in the setting of HIV-1 infection (Jacob D. Estes et al., 2008). GALT fibrosis is a contributor to incomplete CD4<sup>+</sup> T lymphocyte cell restoration in chronic HIV infection despite

suppressive ART and is associated with a decreased frequency of lymphoid aggregate CD4<sup>+</sup> T lymphocytes. During the chronic phase of HIV infection, collagen deposition in lymphoid tissues including Peyer's patches in the gut disrupts the T-cell zone and limits the number of resident CD4 T-cells (Schacker et al., 2005). The development of fibrosis in lymphoid tissue has a profound impact on immunological function and lymphocyte population dynamics. According to this, we show that patients with high expression of IL-33 displayed lower blood CD4<sup>+</sup>T count and longer duration of HIV infection than patients with low expression of IL-33 (table 2) but did not reach significance due to the small number of patients.

IL-33 stimulation of human subepithelial myofibroblasts induced the expression of extracellular matrix components and several growth factors, known to promote epithelial cell proliferation (Maywald et al., 2015). Previous studies reported that intestinal myofibroblasts displayed an activated phenotype and contributed to the profibrotic changes in HIV patients under c-ART (Asmuth et al., 2015). Other study has also reported that flagellin produced by AIEC (adherent-invasive *Escherichia Coli*) induces the induction of ST2 expression in IECs, which in turn augments IL-33 signaling and promotes the development of intestinal fibrosis (Imai et al., 2019a). Therefore, it is tempting to speculate that dysbiosis and microbial translocation observed in HIV infection contribute to intestinal fibrosis through the IL-33 pathway activation.

Chronic immune activation elicits a T regulatory counter response with the adverse consequence of increased collagen production and deposition. Therefore, IL-33 may contribute to the development of fibrosis by promoting Treg accumulation during the chronic phase of HIV infection. We investigated the phenotype of mucosal Treg because IL-33 promotes both tTreg accumulation and maintenance in the gut, enhancing their protective function. We did not observe any difference in the frequency of total tTregs between HIV-infected subjects and

healthy individuals but rather a shift towards the phenotype of tTregs prone to tissue repair. Our data are consistent with two previous studies showing that ART normalizes Treg frequency in the gut (Epple et al., 2006b; Favre et al., 2010b). However, we extend significantly these results by deciphering the phenotype of these tTreg. We show that the frequencies of both CD161<sup>+</sup>IL-18R $\alpha$ <sup>+</sup> Treg and ST2<sup>+</sup>Treg were increased in the gut of PLWH compared to HD. CD161<sup>+</sup> Treg cells have been described to be a distinct, highly suppressive population of Treg cells that mediate wound healing (G. A. M. Povolero et al., 2018) as well as ST2<sup>+</sup>Treg cells (Schiering et al., 2014b). Both populations were detected in tissues and not in blood from healthy individuals, suggesting their function of tissue-resident Tregs. Therefore, our results suggest that the higher secretion of IL-33 in the gut of HIV infected patients is associated with an accumulation of tTreg.

Next, we were interested to investigate the functional profile of these tTreg since these cells contribute to the maintenance of tissue homeostasis, by promoting wound healing and repair processes, beyond their classical role in the suppression of pro-inflammatory responses (Panduro et al., 2016a). The leaky gut is one of the main mechanisms linked to systemic activation and is characterized by epithelial barrier disruption. Therefore, we reasoned that tTregs may have a functional defect linked to wound healing process. One of the underlying mechanisms associated with Treg-mediated wound healing is assumed to be the suppression of pro-inflammatory stimuli. Our data showed an increased level of TGF- $\beta$  mRNA in the gut of PLWH compared to HD and an inverse correlation between mRNA-IL-33 and TGF- $\beta$  mRNA. The inverse correlation between IL-33 and TGF- $\beta$  levels is surprising and suggests that Treg are not able to control local inflammation, which is an obstacle for epithelial barrier repair. The resolution of local inflammation is clearly a key function of Treg during wound healing. As reported, Tregs exhibited reduced immunosuppressive capacity during HIV infection (López-

Abente et al., 2016a). A direct role of amphiregulin during wound healing has been described. The Rudensky group (Arpaia et al., 2015b) using a mouse strain with a Treg-specific deficiency of amphiregulin, showed a substantially more severe form of symptoms following influenza infection than wild-type mice. During infections, the injection of recombinant amphiregulin has demonstrated the amelioration of symptoms in several different experimental settings, such as during Influenza infection or following viral-bacterial co-infections (6)(Monticelli et al., 2011). Our report extends significantly these data showing for the first time to our knowledge a profound defect of tTreg producing amphiregulin in the gut of HIV infected patients.

The exact mechanism by which amphiregulin contributes to wound healing had remained unresolved. Amphiregulin, a low-affinity EGFR ligand is a poor mitogen for epithelial cells and recent data show that one major function of amphiregulin is the local induction of TGF- $\beta$  (Minutti et al., 2019). During wound healing, amphiregulin induced the TGF- $\beta$  mediated differentiation of tissue stem cells and in this way critically contributed to the restoration of tissue homeostasis. Zaiss and al showed that Treg express EGFR under inflammatory conditions and amphiregulin is critical for efficient Treg cell function (Dietmar M. W. Zaiss et al., 2013) and enhances the suppressive capacity of Treg (Wang et al., 2016). We show here a higher expression of EGFR in the gut of HIV infected patients pointing out that a specific defect of amphiregulin secretion by tTreg might contributes to the absence of epithelial wound healing during the chronic phase of HIV infection.

In conclusion, we observed an increased level of IL-33 in the gut of HIV infected patients, which is associated with an increased level of fibrosis and a low peripheral reconstitution of CD4<sup>+</sup> T cells. Our results showed that IL-33 promotes proliferation and maintenance of ST2<sup>+</sup>Treg cells but IL-33 did not induce secretion of amphiregulin by these cells. It remains to determine the reasons for this deficit. Because rAREG enhances wound healing(Burzyn et al., 2013b; Monticelli et al., 2015b), it will be interesting to test whether amphiregulin may enhance

epithelial barrier function and then decrease the symptoms of the leaky gut observed during HIV infection.

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### **Author contributions**

SH, SG, JDL and YL conceived and supervised the study.

SH, SG, JDL, YL, and MT designed the experiments and analyzed the data.

MT, MS, AW, CL and FJL performed the experiments.

SG, JLPZ, JDZ, SYD recruited the participants and collected the samples.

SH, YL, JDL and MT wrote the manuscript, with contributions from all authors.





**Table I. Main characteristics of HIV-1-infected patients**

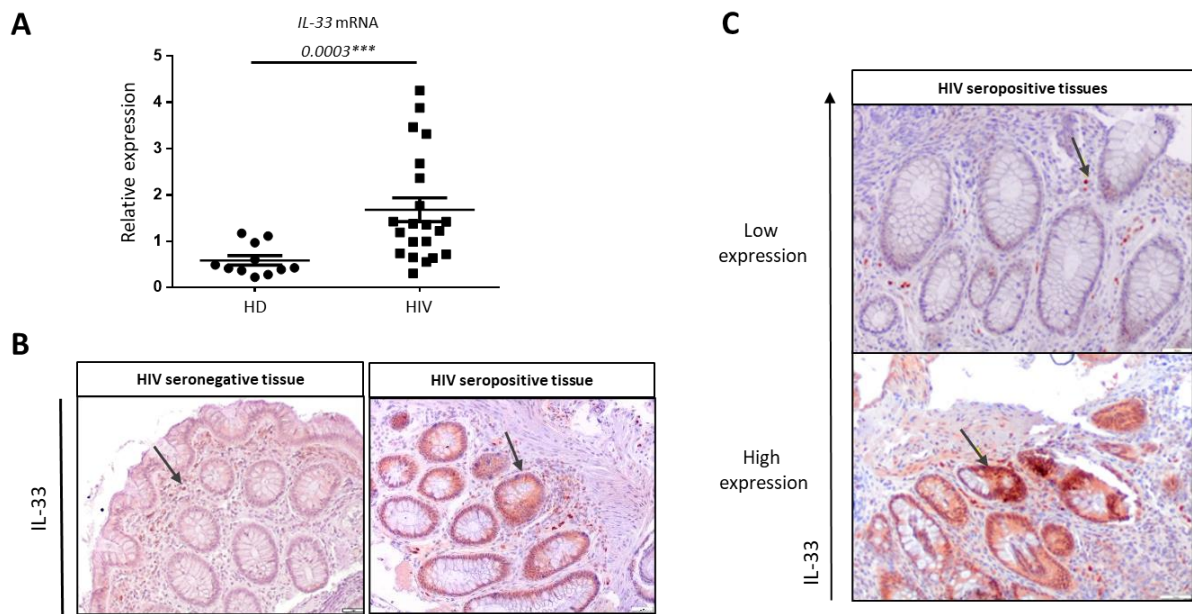
Median (Interquartile Range) or %	HIV+ cART (n = 31)
Demography	
Men (%)	83.9%
Ethnicity (%)	
Caucasian	80.6%
Sub-Saharan African	19.4%
Year of HIV diagnosis	2013 (2001 - 2014)
Pre-ART	
CD4+ T cell nadir, cells/ml (n=28)	266 (62.75 - 346.25)
CD4+ T cell nadir, <math>.500</math> cells/ml, % (n=28)	3.60%
PVL log <sub>10</sub> copies/ml (n=28)	77970 (29586.25 - 362686)
At inclusion	
Age, years	44.3 (37.9 - 46.9)
Duration with HIV, years	4.8 (3.2 - 17.6)
Overall cART exposure, years (n=29)	4.4 (2.9 - 11.7)
CD4 <sup>+</sup> T cells ml <sup>-1</sup> (n=30)	594 (453.5 - 751)
CD4 <sup>+</sup> : CD8 <sup>+</sup> ratio (n=29)	1 (0.55 - 1.28)
Coinfection <sup>a</sup> (%)	
None	100%

cART, combined antiretroviral therapy; PVL, plasma viral load; <sup>a</sup>Coinfection (HBV/HCV, hepatitis B/C virus)

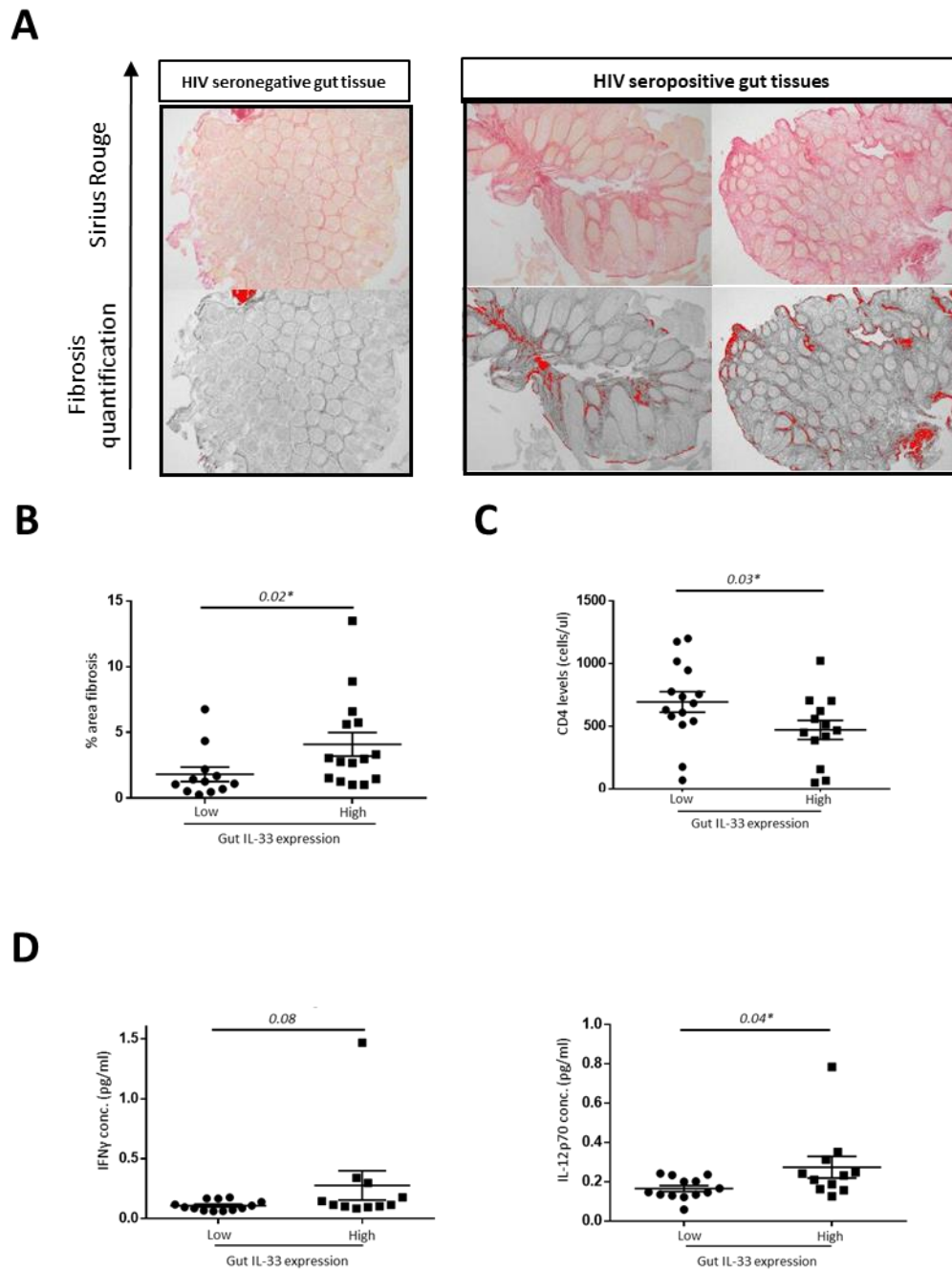
**Table II. Comparing main characteristics between two groups of HIV-1-infected patients**

	low IL33	High IL33	
Median (Interquartile Range) or %	(n = 15)	(n = 14)	p value
Demography			
Men (%)	73.3%	92.9%	0.3
Ethnicity (%)			0.65
Caucasian	73.3%	85.71%	
Sub-Saharan African	26.7%	14.29%	
Year of HIV diagnosis	2014 (2002 - 2015)	2007 (1999 - 2013)	0.09
Pre-ART			
CD4+ T cell nadir, cells/ml (n=13)	291 (72 - 371)	199 (35 - 338)	0.9
CD4+ T cell nadir, <math>.500</math> cells/ml, % (n=13)	0.00%	7.70%	
PVL log <sub>10</sub> copies/ml (n=13)	65895 (25414 - 452362)	128397 (48385 - 332794)	0.6
At inclusion			
Age, years	44.3 (41.5 - 46)	40.7 (35.4 - 50)	0.8
Duration with HIV, years	4.4 (3.1 - 17.06)	9.1 (3.5 - 19.3)	0.4
Overall cART exposure, years	3.3 (2.8 - 10.24) (n=13)	7.0 (3.1 - 19.3) (n=14)	0.2
CD4+ T cells ml <sup>-1</sup>	683 (560 - 862) (n=15)	467 (387 - 620) (n=13)	0.03
CD4+: CD8+ ratio	1.1 (0.6 - 1.35) (n=14)	0.8 (0.4 - 1.23) (n=13)	0.2
Coinfection <sup>a</sup> (%)			
None	100%	100%	

cART, combined antiretroviral therapy; PVL, plasma viral load; <sup>a</sup>Coinfection (HBV/HCV, hepatitis B/C virus)

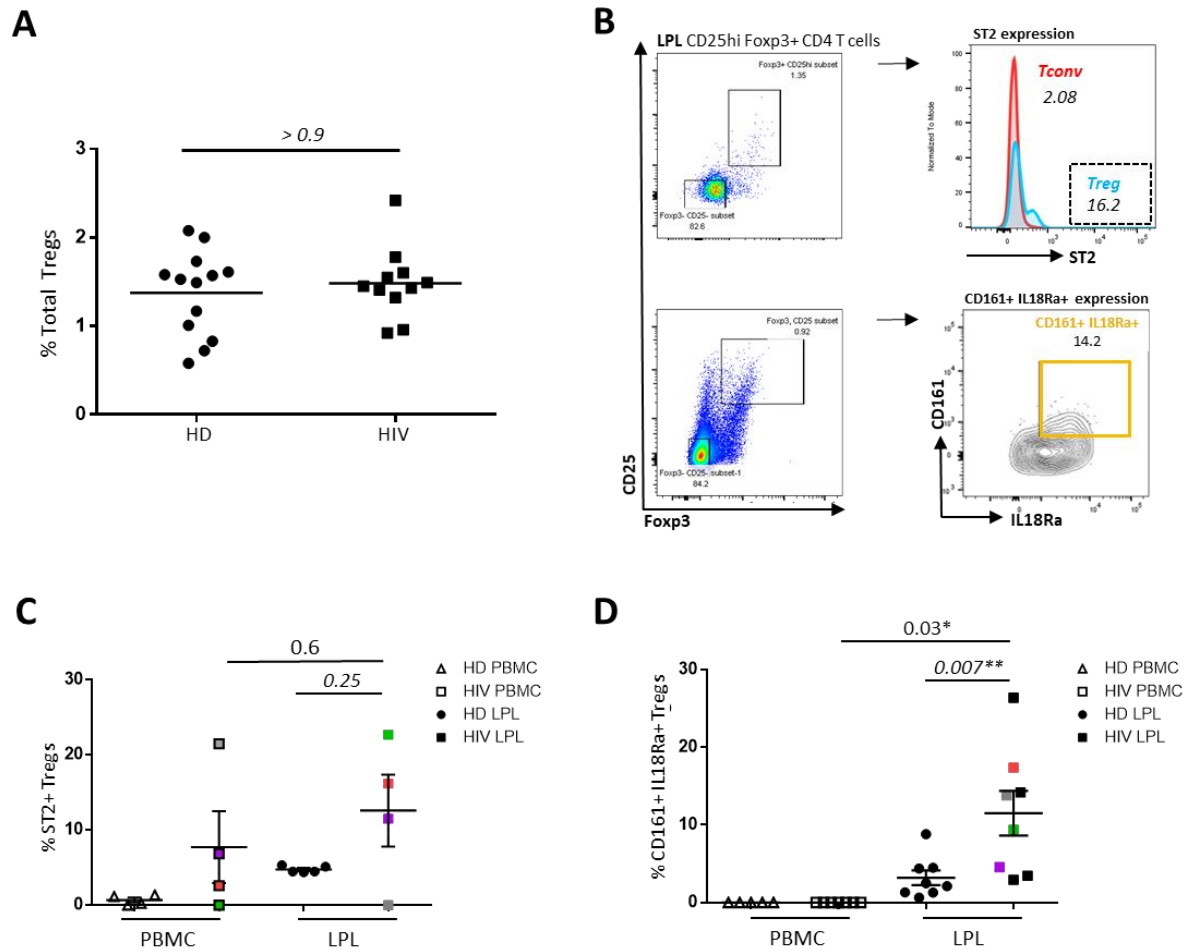


**FIGURE 1** | Increased IL-33 levels in the intestine of chronic HIV infection on c-ART. **(A)** Comparison of gut mucosal tissue *IL-33* mRNA relative expression in HIV infected patients on c-ART (n = 21) and healthy donors (HD, n = 11), using the non-parametric Mann-Whitney test. Data represents mean  $\pm$  SEM. **(B)** IL-33 protein expression by immunohistochemistry (IHC) on sections from healthy mucosal tissue with weak nuclear staining of mononuclear cells and no epithelial cell staining (left), and strong IL-33 expression on sections from HIV infected mucosal tissue (right) **(C)** Representative example of IL-33 protein expression by IHC on sections of mucosal tissue from two different HIV seropositive patients by immunohistochemistry. Low expressing tissue sections with weak staining and no epithelial expression (top) and high expressing tissue section with epithelial staining (bottom). \*P < 0.05; \*\*P < 0.01; \*\*\*P < 0.001

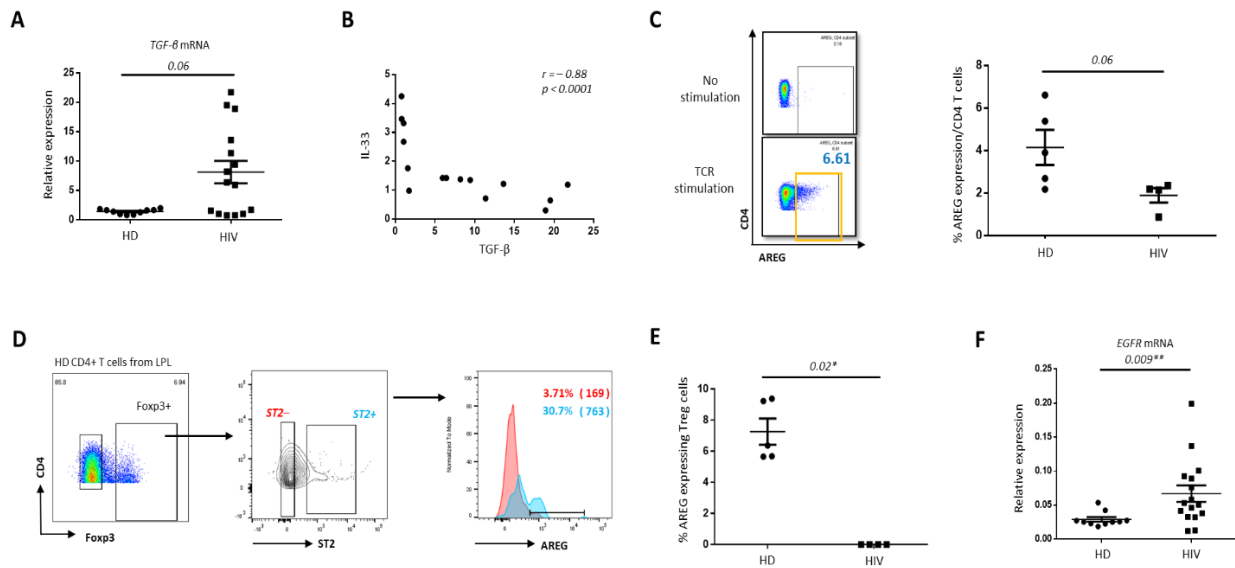


**FIGURE 2** | Fibrosis and inflammation linked to the pattern of IL-33 expression in the gut mucosa of HIV infected patients. **(A)** Sirius red staining on gut mucosal tissue sections (top), from HIV seronegative donor (left) and HIV infected patients on c-ART (two on right). Lower three images showing the sirius red staining quantification by imagej software for the respective images on top. **(B)** Graph showing the correlation between % tissue fibrosis quantified by Imagej macros and IL-33 protein expression levels in the gut by IHC. HIV seropositive patients

on c-ART (HIV, n=27). **(C)** Graphs representing the correlation of CD4 reconstitution in the peripheral blood with gut IL-33 protein expression levels. **(D)** Graphs showing association of gut IL-33 protein expression levels with inflammatory markers, IFN $\gamma$  (left) and IL-12p70 (right). Data represents mean  $\pm$  SEM. Statistical analyses were performed using non-parametric Mann-Whitney test.



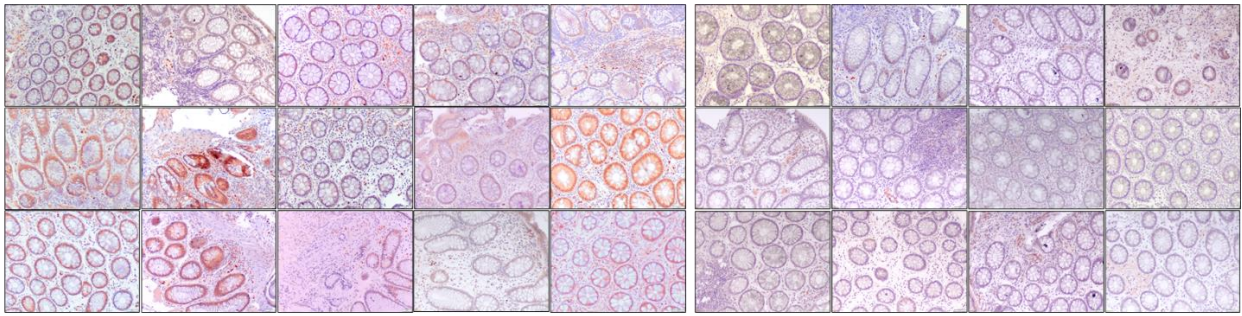
**FIGURE 3** | Repair Tregs in the intestinal mucosa in chronic HIV infection. **(A)** Comparison of frequency of Total Treg population within CD4 T cells in HIV infected patients on c-ART (n = 11) and healthy donors (HD, n = 13), using the non-parametric Mann-Whitney test. Data represents mean. **(B)** Characterization of tissue repair Treg subsets in the gut mucosal LPL. Representative FACS plots of ST2 expression (upper right) and CD161 IL18Ra co-expression (lower right) on FoxP3<sup>+</sup>CD25<sup>+</sup> CD4 T cells (in blue) or conventional T cells (in red). **(C)** Individual data showing ST2 expressing Treg frequencies in PBMC and LPL of HD and HIV infected patients. **(D)** CD161 IL18Ra co-expressing Treg frequencies are compared in the summary plot. Statistical analyses of paired PBMC and LPL of HIV patients are performed by Wilcoxon matched-pairs sign rank test. Comparison of HD and HIV LPL is performed using the non-parametric Mann-Whitney test.



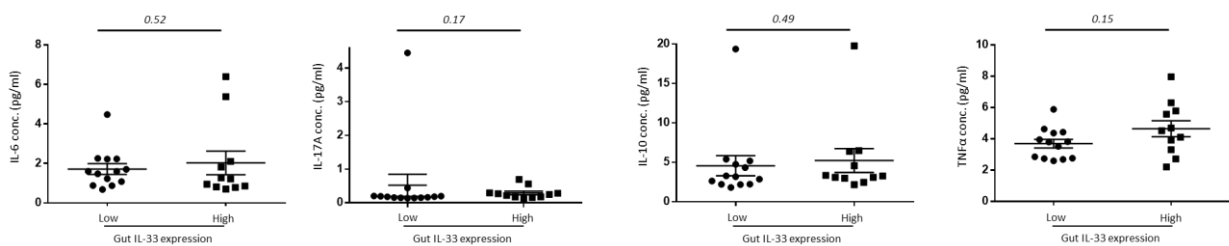
**FIGURE 4** | Factors involved in repair of the intestinal mucosa in chronic HIV infection. **(A)** Comparison of mucosal tissue *TGFβ* mRNA relative expression in HIV infected patients on c-ART (n = 16) and healthy donors (HD, n = 10), using the non-parametric Mann-Whitney test. Data represents mean ± SEM. **(B)** Graph representing *IL-33* correlation with *TGFβ*, in HIV infected patients on c-ART (n = 16), by Spearman correlation. **(C)** Expression of AREG on CD4 T cells, was assessed by flow cytometry. Representative FACS plots of AREG expression gated on CD4+ lymphocytes (left plot) in healthy LPL before and after TCR stimulation. Summary plot (right), showing AREG expression on CD4+ lamina propria lymphocytes (LPL) in healthy donor (HD, n = 5) in comparison to HIV infected samples (HIV, n = 4). **(D)** FoxP3<sup>+</sup> Tregs expressing AREG, after TCR stimulation in the Healthy donor (HD) lamina propria lymphocytes (LPL). Representative FACS plots of ST2<sup>+</sup> and ST2<sup>-</sup> cells gated on FoxP3<sup>high</sup> CD4 T cells and the expression of AREG by ST2<sup>+</sup> (blue) and ST2<sup>-</sup> (red) Treg subsets, was assessed by flow cytometry. Values represent % and MFI (in brackets). **(E)** Summary plot, comparing AREG expression on Tregs (CD25<sup>high</sup> Foxp3<sup>high</sup>) of lamina propria lymphocytes between healthy donors (HD) and HIV infected (HIV), after TCR stimulation. **(F)** Comparison of mucosal tissue



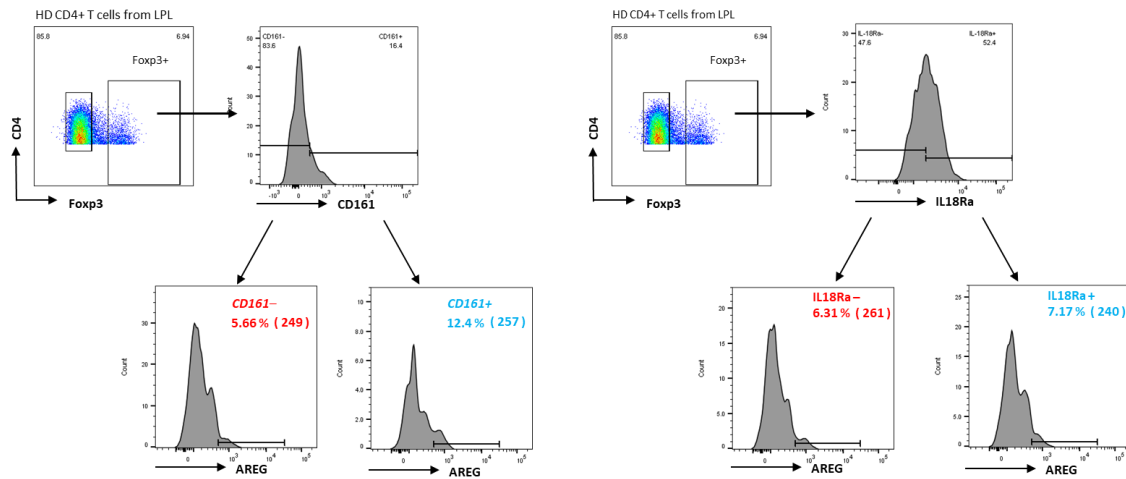
*EGFR* mRNA relative expression in HIV infected patients on c-ART (n = 16) and healthy donors (HD, n = 10), using the non-parametric Mann-Whitney test. Data represents mean  $\pm$  SEM.



**Supplementary 1** | Patterns of IL-33 protein expression on sections of mucosal tissue from HIV seropositive patients by immunohistochemistry (IHC) (n = 27). Epithelial cells IL-33 expression differentiating the IL-33 high group (left, n = 15) from IL-33 low group (right, n = 12) with no epithelial cell staining



**Supplementary 2** | Graphs showing association of gut IL-33 protein expression levels with inflammatory markers, IL-6, IL-12p70, IL-10 and TNFα (from left to right). Data represents mean ± SEM. Statistical analyses were performed using non-parametric Mann-Whitney test.



**Supplementary 3** |  $\text{Foxp3}^+$  Tregs expressing AREG, after TCR stimulation in the Healthy donor (HD) lamina propria lymphocytes (LPL). Representative FACS plots of  $\text{CD161}^+$  and  $\text{CD161}^-$  cells (left) and  $\text{IL18Ra}^+$  and  $\text{IL18Ra}^-$  (right), gated on  $\text{Foxp3}^{\text{high}}$   $\text{CD4}^+$  T cells and the expression of AREG by these subsets, was assessed by flow cytometry. Values represent % and MFI (in brackets).

# **ARTICLE 2.**

IN PREPARATION FOR SUBMISSION

**IL-33 mediated enhancement of antigen specific CD8 response in HIV infection**

## **ARTICLE 2. IN PREPARATION**

### **IL-33 mediated enhancement of antigen specific CD8 response in HIV infection**

#### **INTRODUCTION**

sST2, is the decoy receptor of the alarmin IL-33, a member of the IL-1 family. It is a significant predictor of all-cause of mortality in HIV patients on HAART (Thiébaud et al., 2017b). sST2 levels are elevated in HIV infected patients, while serum IL-33 levels are significantly lower and this is independent of the severity of the infection (Miyagaki et al., 2011). It is also demonstrated in in vitro settings, the upregulation of IFN $\gamma$  by both GAG- as well as CEF-stimulated CD4 and CD8 T cells in early HIV infected patients (treatment naïve). This enhanced response is attenuated by the addition of anti-ST2 antibodies (Wu et al., 2018).

ST2 is an interleukin (IL)-1 receptor family member with transmembrane (ST2L) and soluble (sST2) isoforms. In humans and in mice, the alternative promoter activation and splicing produce ST2L, the longest transcript, and sST2, the truncated soluble isoform. IL-33 is the functional ligand of ST2L. Once secreted, IL-33 binds the ST2L receptor, and IL-33/ST2L signalling leads ultimately to the production of inflammatory cytokines/chemokines and an immune response. sST2 avidly binds to IL-33 in competition with ST2L and thus functions as a decoy receptor for IL-33. Therefore, the interaction of sST2 with IL-33 blocks the IL-33/ST2L system (Pusceddu et al., 2019). ST2L is present on a wide variety of haematopoietic cells.

IL-33 is a pleotropic cytokine, modulating its functions according to the environment. It has the potential to induce protective immunity against viral infections owing to its ability to direct Th1- type immune responses, signaling through its membrane receptor ST2L. It enhances the activation and antiviral functionality of ST2L- expressing effector cytotoxic lymphocytes (Bonilla et al., 2012). ST2L expression is upregulated on CD8 T cells

IL-33 has also been used as a molecular adjuvant in DNA vaccine setting in a murine model for HPV-associated cancer. This is achieved by its ability to boost protective antiviral CTL responses, expand the magnitude of Ag-specific CD8 T cell responses and enhance the production of TNF $\alpha$  and IFN $\gamma$  (Villarreal et al., 2014).

HIV-specific CD8 T cells are able to control the virus during acute HIV/SIV infection, but their cytotoxic potential decreases with disease progression. In addition, persistent antigen exposure, negative co-stimulation and chronic inflammation despite ART induced viral suppression, leads to their dysfunction. This is characterized by an alteration in CD8 memory subsets and functional capacity and a high level of activation and exhaustion markers.

Two independent studies had shown that activated CD8 T cells can express ST2 (Ngoi et al., 2012; Yang et al., 2011). Moreover, following LCMV infection in mice, roughly 20% of activated antigen specific CD8 T cells expressed ST2 (Bonilla et al., 2012, p. 33). These cells have not been characterized in HIV infection.

sST2 and IL-33 are elevated in HIV infection, while ART reduces their levels (Wu et al., 2018). IL-33 signals through its receptor ST2, on effector CTLs, to enhance their activation and antiviral functionality (Bonilla et al., 2012, p. 33).

In this study, we characterized the ST2L expressing subset of CD8 T cells and assessed the role of IL-33/ST2 axis on specific CD8 immune response in HIV infected patients on c-ART.

## **MATERIAL ET METHODS**

### **Study Population**

Our study population included patients infected with HIV on c-ART. 17 patients were recruited at the Clinical Immunology department, CHU Henri Mondor. 10 HIV seronegative individuals were included from EFS. Inclusion criteria includes: i) HIV-1 infection; ii) Age >18 years; iii) CD4 T cells > 200/mm<sup>3</sup>; iv) Undetectable plasma HIV RNA (below <20 copies/ml for at least 1 year). Median age of the HIV patients was 44.3 years; 76.5% were men. None of the study participants were suffering from HBV or HCV co-infection. Characteristics of HIV-1 infected patients are summarized in table 1.

### **Ethics statements**

All study participants provided written informed consent to participate in the study. This study was approved by our local ethics committee. The study protocol was reviewed and approved by the Sud-Méditerranée II ethics committee (Comité de Protection des Personnes), was conducted in accordance with good clinical practice and the Declaration of Helsinki and was registered at ClinicalTrials.gov (NCT03622177).

### **Sample collection and processing**

Blood samples were collected from HIV patients and HIV seronegative individuals. About 30-50 ml of blood was drawn by sterile venipuncture from HIV patients and 20-30 ml from HIV seronegative individuals. Samples were transported at room temperature for immediate processing. Peripheral blood mononuclear cells (PBMC) were isolated using Ficoll density gradient (Lymphoprep ProteoGenix). PBMCs were used the following day for ST2 surface staining, and the rest of the cells were immediately cryopreserved at -80°C followed by storage in liquid nitrogen until needed for the rest of the experiments.

### **Immunophenotype Analysis**

Blood CD8 T cell phenotype was assessed using FACS staining with the following Abs: CD3 Alexa700 (UCHT1, BD Pharmingen), CD4 BV605 (RPA-T4, BD Horizon), CD8 efluor780 (SK1, eBioscience), CCR7 Alexa647 (3D12, BD Pharmingen), CD45Ra efluor610 (HI100, eBioscience), CD27 PeCy7 (M-T271, BD Pharmingen), PD-1 BV421 (EH12.2H7, Biolegend), LAG3 PE (3DS223H, Thermofisher), anti HLA-DR PerCP (AC122, Miltenyi) and ST2L FITC (B4E6, MD Biosciences). For all cell stainings, dead cells were excluded from the gating by using the LIVE/DEAD fixable dead cell stain kit (Molecular Probes, Invitrogen). Cells were acquired on an LSRII flow cytometer (BD Biosciences) and analyzed with FlowJo software, version 10 (FlowJo LLC).

### **Functional Analysis**

Functional characterization of CD8 T cells is done by staining of CD3 Alexa700 (UCHT1, BD Pharmingen), CD4 BV605 (RPA-T4, BD Horizon), CD8 efluor780 (SK1, eBioscience), ST2L FITC (B4E6 MD Biosciences), during 30 minutes at 4°C. Followed by wash and permeabilisation with BD Cytotfix/cytoperm kit during 30 min 4°C. Finally, ICS was performed with anti-granzyme B V450 (GB11, BD Horizon), and anti-perforin PCF 594 (δG9, BD Horizon) and for the expression of Interferon gamma (IFN $\gamma$ ) PerCp5.5 (B27, BD Pharmingen) following 4 hours of stimulation with PMA (100 ng/ml) and ionomycin (1  $\mu$ g/ml) in the presence of Brefeldin A (5  $\mu$ g/ml) and GolgiStop (5  $\mu$ g/ml) (added for the last 3 hours). For degranulation study, CD107a PeCy7 (H4A3, BD Pharmingen) was added to those cells in the well-plate at the start of the experiment for 4 hours in the presence of Brefeldin A and GolgiStop, added for the last 3 hours of incubation at 37°C, 5% CO<sub>2</sub>.



### **Quantification of ST2 protein levels**

Human ST2 protein concentrations in patients' plasma were measured by luminex assay (Luminex Human Discovery Assay (1-Plex) LXSAHM-01, R&D Systems). Plasma samples were stored at -80°C and thawed at room temperature for the assay. Luminex assays were performed according to manufacturers' instructions.

### **Proliferation Assay**

The proliferative capacity of non-specific and HIV-specific CD8 T cells was evaluated by flow cytometry. PBMC were stained with 5  $\mu$ M of CellTrace Violet (Thermofisher Scientific) for 15 min at 37°C. After subsequent washings, they were resuspended in culture medium, RPMI 1640 GlutaMAX HEPES (72400047, Thermofisher) supplemented with 10% Fetal Calf Serum and 100 U/mL penicillin and 100  $\mu$ g/mL streptomycin for distribution into a 48 well plate. Cells were stimulated for 5 days with  $\alpha$ CD3-CD28 dynabeads (11131D, Thermofisher) (cell to bead ratio of 1:1), or 4  $\mu$ g/ml CEF (Cytomegalovirus (CMV), Epstein-Barr virus (EBV), Flu), or 2  $\mu$ g/ml of GAG pool of peptides, or medium alone, in the presence or absence of 40 ng/ml rh IL-33 (RnD). The respective wells were supplemented with 40 ng/ml rh IL-33 on Day 2. On Day 2 and Day 5, supernatants were collected and conserved at -80°C for protein detection.

At day 5, cells were collected and stained. Cells were acquired on an LSRII flow cytometer (BD Biosciences) and analyzed with FlowJo software, version 10 (FlowJo LLC). All data was presented after subtraction of the background.

### **Protein Analyses**

Protein concentrations in the proliferation assay supernatants were analysed using the LEGENDplex Human CD8/NK Panel (13-plex) (Biolegend), a bead-based immunoassay that quantifies multiple soluble analytes in biological samples simultaneously by flow cytometry.

Human CD8/NK Panel was employed to measure the concentrations of IL-2, IL-4, IL-6, IL-10, IL-17A, TNF- $\alpha$ , sFas, sFasL, IFN- $\gamma$ , Granzyme A, Granzyme B, Perforin, and Granulysin.

The assays were performed in 96-well plates, following the manufacturer's instructions. Analysis was performed by flow cytometry using LSR II (BD Biosciences), and data was evaluated with the LEGENDplex Data Analysis software (Biolegend).

### **Statistics.**

Groups were compared using the 2-sided nonparametric Mann-Whitney U test. Samples with or without treatment with IL-33 were compared using Wilcoxon matched pairs signed rank test. A P value below 0.05 was considered significant. All statistical analyses were performed using Prism (version 6.0, GraphPad Software).

## RESULTS

### **ST2 expressing CD8 T cells are elevated and maintained in the blood of HIV infected individuals**

IL-33 acts on CD8<sup>+</sup>T cells that express ST2 and increase the cytotoxic potential of these cells during acute viral infection in mouse models. We wondered whether ST2<sup>+</sup>CD8<sup>+</sup> T cells are present in humans during a chronic viral infection. We then analyzed ST2 expression on CD8<sup>+</sup> T cells by FACS on fresh blood samples from healthy donor (HD) and HIV infected patients under c-ART. We observed an increased frequency of ST2<sup>+</sup>CD8<sup>+</sup> T cells in the fresh blood of HIV infected patients compared to HD ( $3.18 \pm 0.42$  % vs  $0.63 \pm 0.24$  %;  $P = 0.006$ ; **Figure 1A**). We noticed that freezing has an impact on ST2 expression on CD8<sup>+</sup> T cells. Therefore, we looked at ST2 expression on CD8<sup>+</sup> T cells after 5 days of culture without stimulation. We observed that ST2 expression on CD8<sup>+</sup> T cells was maintained whereas almost no ST2<sup>+</sup>CD8<sup>+</sup> T cells were detected in HD ( $3.28 \pm 0.87$  % vs  $0.74 \pm 0.08$  %;  $P = 0.0045$ ; **Figure 1B**). We have described that sST2 is increased in HIV infected patients and independent maker of all causes of mortality. IL-33/ST2L signaling pathway is downregulated in the presence of sST2. In line with this, we found the ST2 expression on CD8 T cells was inversely correlated with sST2 levels in the plasma ( $r=-0.71$ ;  $p=0.012$ ; **Figure 1C**). These results suggest that the frequency of ST2<sup>+</sup>CD8<sup>+</sup> T cells is increased during HIV infection and is linked to the level of sST2.

### **ST2 expressing CD8 T cells displayed an effector phenotype and a high proliferative and cytotoxic profile**

Because ST2 expressing CD8<sup>+</sup> T cells are increased in the blood of HIV infected patients under c-ART, we analyzed the phenotype of these ST2<sup>+</sup>CD8<sup>+</sup> T cells. Several CD8<sup>+</sup> T cell subsets have been described based on the expression of the differentiation markers CD45RA, CCR7 and CD27 among others. The classification divides them into naïve

(CD45RA<sup>+</sup>/CD27<sup>+</sup>/CCR7<sup>+</sup>), central memory (CM) (CD45RA<sup>-</sup>/CD27<sup>+</sup>/CCR7<sup>+</sup>), effector memory EM1/EM2 (CD45RA<sup>-</sup>/CD27<sup>+</sup>/CCR7<sup>-</sup>), EM3/ EM4 (CD45RA<sup>-</sup>/CD27<sup>-</sup>/CCR7<sup>-</sup>), and terminal effector (TE) (CD45RA<sup>+</sup>/CCR7<sup>-</sup>/CD27<sup>-/+</sup>) CD8 T cells. As shown in Figure 2A, we observed that ST2 expressing CD8 T cells displayed predominantly an effector phenotype (TE) in HD and the memory effector CD8<sup>+</sup> T cells are enriched in ST2<sup>-</sup>CD8<sup>+</sup> T cells. The phenotype of ST2 expressing CD8<sup>+</sup> T cells did not differ in HIV infected patients (**Figure 2A**).

We then studied expression of activation and exhaustion markers including PD-1, LAG-3 and HLA-DR on CD8<sup>+</sup> T cells. ST2 expressing CD8<sup>+</sup> T cells have a significantly lower expression of PD-1 compared to ST2<sup>-</sup>CD8<sup>+</sup> T cells in HDs ( $29.13 \pm 6.93$  % vs  $59.30 \pm 11.29$  %;  $P = 0.038$ , respectively; **Figure 2A**). LAG3 expression is not different between ST2<sup>+</sup>CD8<sup>+</sup> T cells and ST2<sup>-</sup>CD8<sup>+</sup> T cells as well as HLA-DR expression. For HIV infected patients under c-ART, we did not observe any significant difference between ST2<sup>+</sup>CD8<sup>+</sup> T cells and ST2<sup>-</sup>CD8<sup>+</sup> T cells for PD1, LAG-3 and HLA-DR expression (**Figure 2A**).

Next, we assessed the functionality of ST2<sup>+</sup>CD8<sup>+</sup> T cells. After 5 days of polyclonal activation, we observed the majority of proliferating CD8<sup>+</sup> T cells express ST2 (**Figure 2B**). ST2<sup>+</sup> CD8<sup>+</sup> T cells have a high proliferation potential compared to ST2<sup>-</sup> CD8<sup>+</sup> T cells in both HD and HIV infected patients ( $67.10 \pm 5.30$  % vs  $11.38 \pm 1.68$ ,  $P < 0.0001$ ;  $65.12 \pm 5.29$  % vs  $14.49 \pm 3.25$  %,  $P = 0.0001$ , respectively; **Figure 2B**). Analysis of granzyme B, perforin revealed that ST2<sup>+</sup>CD8<sup>+</sup>T cells express more cytotoxic molecules compared to ST2<sup>-</sup>CD8<sup>+</sup>T cells in HIV infected patients under c-ART. (**Figure 2C**). We did not observe these differences in HD. Degranulation was assessed by CD107a levels, revealing significantly high expression in ST2<sup>+</sup>CD8<sup>+</sup> compared to ST2<sup>-</sup> CD8<sup>+</sup> T cells in both HD and HIV. By contrast, IFN- $\gamma$  expression did not differ significantly between ST2<sup>+</sup> CD8<sup>+</sup> and ST2<sup>-</sup> CD8<sup>+</sup> T cells in either HD or HIV infected patients (**Figure 2C**). Altogether, these results suggest that ST2 expressing CD8<sup>+</sup>T

cells are effector cells with high capacity to proliferate and to kill via perforin/granzyme B pathway in HIV infected patients.

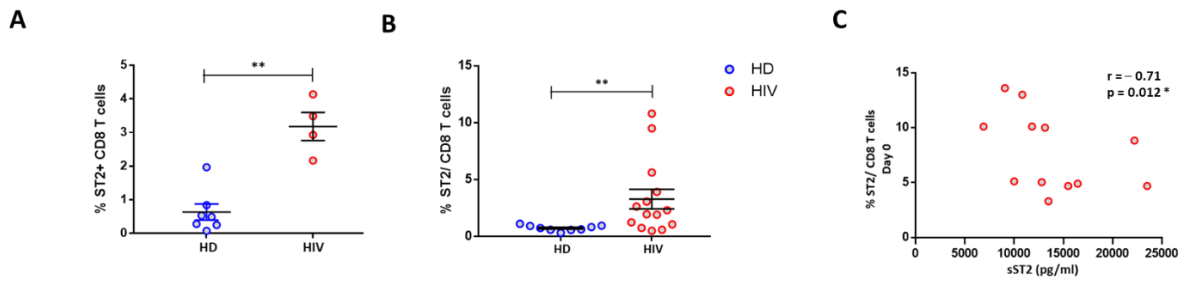
### **IL-33 mediated upregulation of antigen specific T cell responses in HIV infection**

Exogenous IL-33 boosts vaccine-induced memory T cell responses (Mc Laren, 2019, JI). Therefore, we assessed the effects of IL-33 on antigen specific immune responses in HIV infected patients under c-ART. PBMC were stimulated with a pool of GAG or CEF peptides in the presence or absence of IL-33 during 5 days. We did not observe an effect of IL-33 on antigen-specific CD8<sup>+</sup> T cell proliferation in either healthy donors or HIV-infected patients. (**Figure 3 A**). We then analyzed cytokines production in the supernatant of culture at day 5. As shown in figure 3B, IL-33 induces increased secretion of IFN- $\gamma$ , granzyme A, granzyme B, and soluble FasL in GAG and CEF peptide-stimulated PBMC from HIV-infected patients. IL-33 can also enhance Th2 response and promotes Treg function. Interestingly, we did not observe any significant difference in the concentrations of IL-2, IL-4, IL-10, IL-17A (data not shown). In healthy donors, we observed an increased secretion of IFN- $\gamma$ , granzyme A, granzyme B and soluble FasL in CEF peptide-stimulated PBMC in the presence of IL-33 without reaching significance (**Figure 3B**).

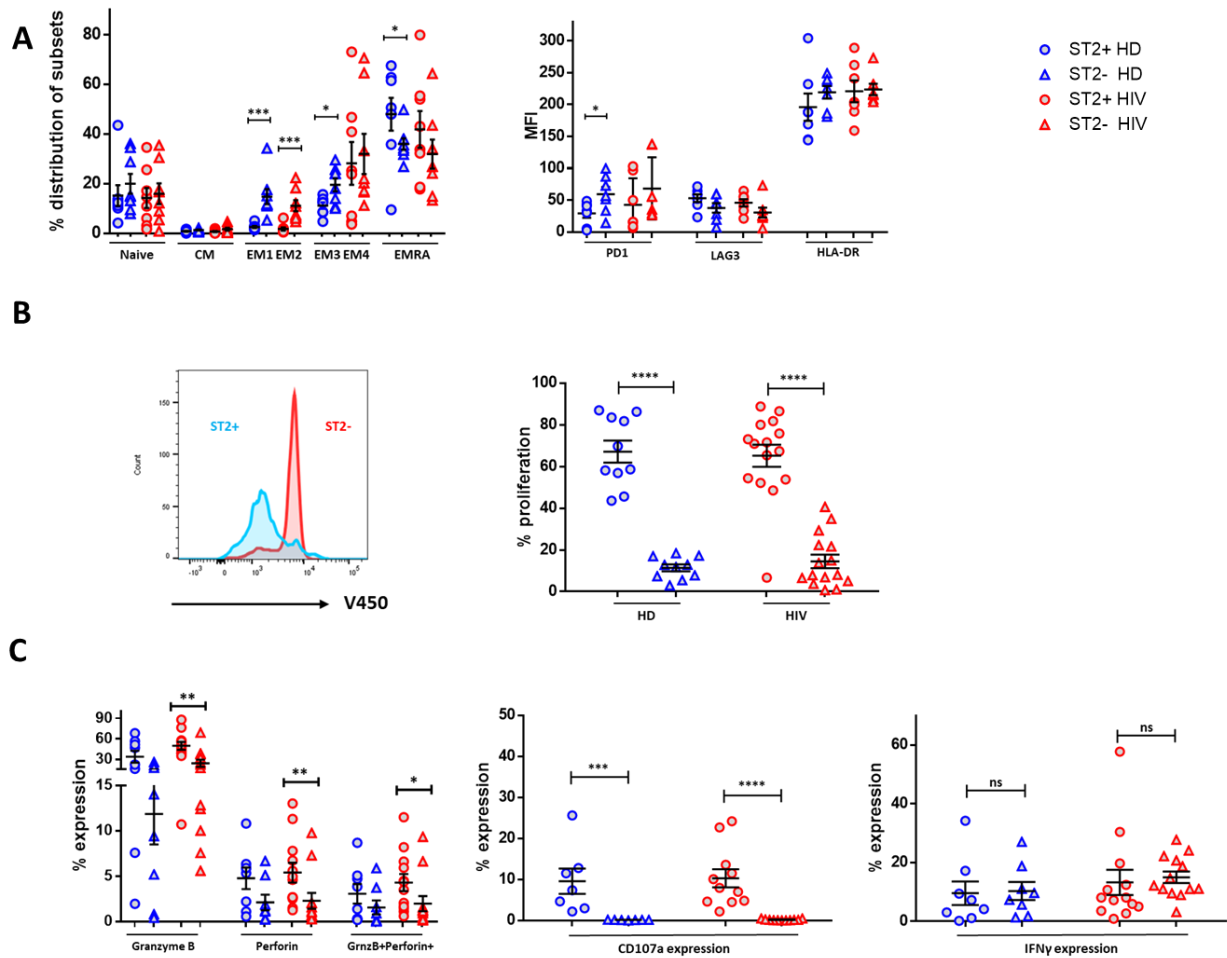
**Table I. Main characteristics of HIV-1-infected patients**

	HIV <sup>a</sup> cART
Median (Interquartile Range) or %	(n = 17)
Demography	
Men (%)	76.47%
Ethnicity (%)	
Caucasian	82.35%
Sub-Saharan African	17.65%
Year of HIV diagnosis	2004 (1993 - 2013)
Pre-ART	
CD4 <sup>+</sup> T cell nadir, cells/ml (n=14)	220 (78 - 295.5)
At inclusion	
Age, years	44.3 (41.5 - 51.6)
Duration with HIV, years	12.3 (4.4 - 18.9)
Overall cART exposure, years (n=15)	9.64 (4.38 - 17.91)
CD4 <sup>+</sup> T cells ml <sup>-1</sup> (n=13)	579 (449 - 756)
CD4 <sup>+</sup> : CD8 <sup>+</sup> ratio (n=13)	0.69 (0.57 - 1.03)
Coinfection <sup>a</sup> (%)	
None	100%

cART, combined antiretroviral therapy; PVL, plasma viral load; <sup>a</sup>Coinfection (HBV/HCV, hepatitis B/C virus)



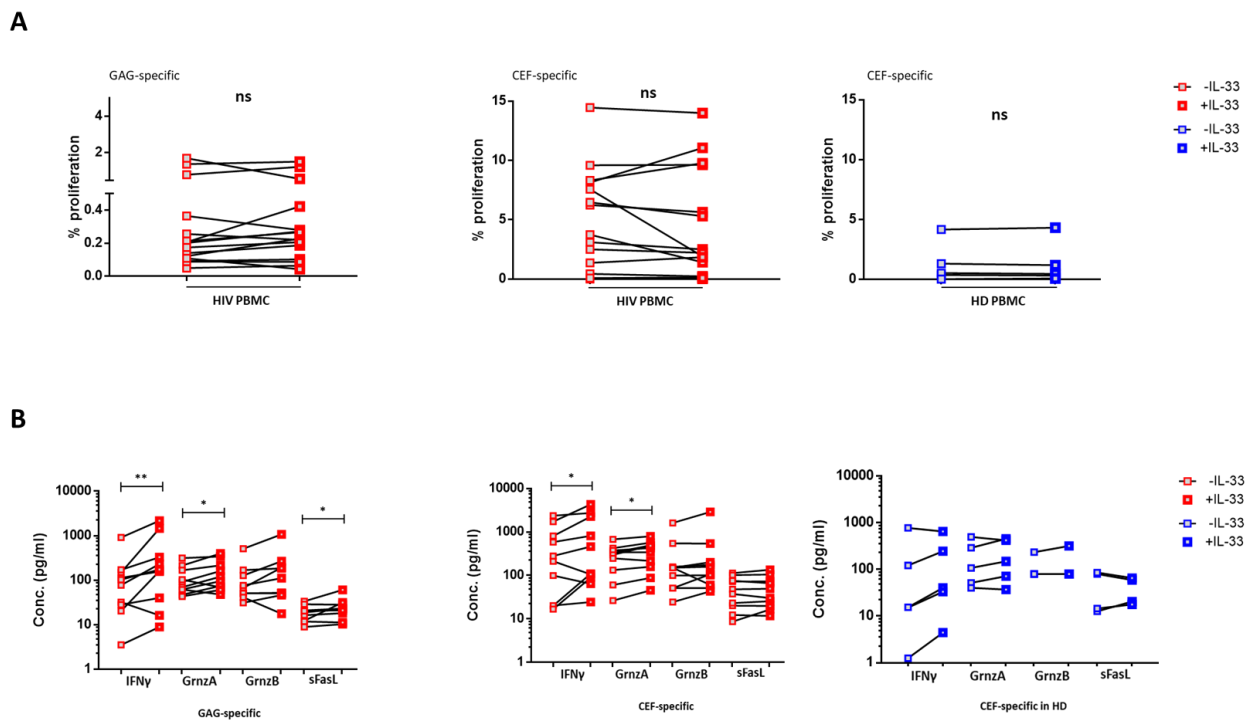
**FIGURE 1** | ST2 expression on CD8 T cells in HIV infection on c-ART. (A) Comparison of frequency of ST2 expressing CD8 T cell population in PBMCs in HIV infected patients on c-ART (n = 4) and healthy donors (HD, n = 7), using the non-parametric Mann-Whitney test. Data represents mean  $\pm$  SEM. (B) Comparison of frequency of ST2 expressing CD8 T cell population in PBMCs on thawed samples after 5 days of culture without stimulation, in HIV infected patients on c-ART (n = 14) and healthy donors (HD, n = 9), using the non-parametric Mann-Whitney test. Data represents mean  $\pm$  SEM. (C) Graph representing ST2 expression on CD8 T cells in PBMCs correlation with sST2 levels in the plasma, in HIV infected patients on c-ART (n = 12), by Spearman correlation



**FIGURE 2** | ST2 expressing CD8 T cells characterisation in healthy donors and HIV infected patients on c-ART. (A) Comparison of frequencies of phenotypic subsets (left) and of activation and exhaustion markers (right) of ST2+ and ST2- CD8 T cell population in PBMCs in healthy donors (HD, n = , blue) and HIV infected patients on c-ART (n = , red). Data represents mean  $\pm$  SEM for phenotypic subsets (left) and MFI for activation and activation markers (right). (B) Histogram showing proliferating ST2+ (blue) and ST2- (red) CD8 T cells in PBMCs. Comparison of percentage proliferation within ST2+ and ST2- CD8 T cell population in PBMCs in healthy donors (HD, n = , blue) and HIV infected patients on c-ART (n = , red). (C) Functional characterization of ST2+ CD8 T cell population in comparison with ST2- CD8 T cells in PBMCs in healthy donors (HD, n = , blue) and HIV infected patients on c-ART (n = ,

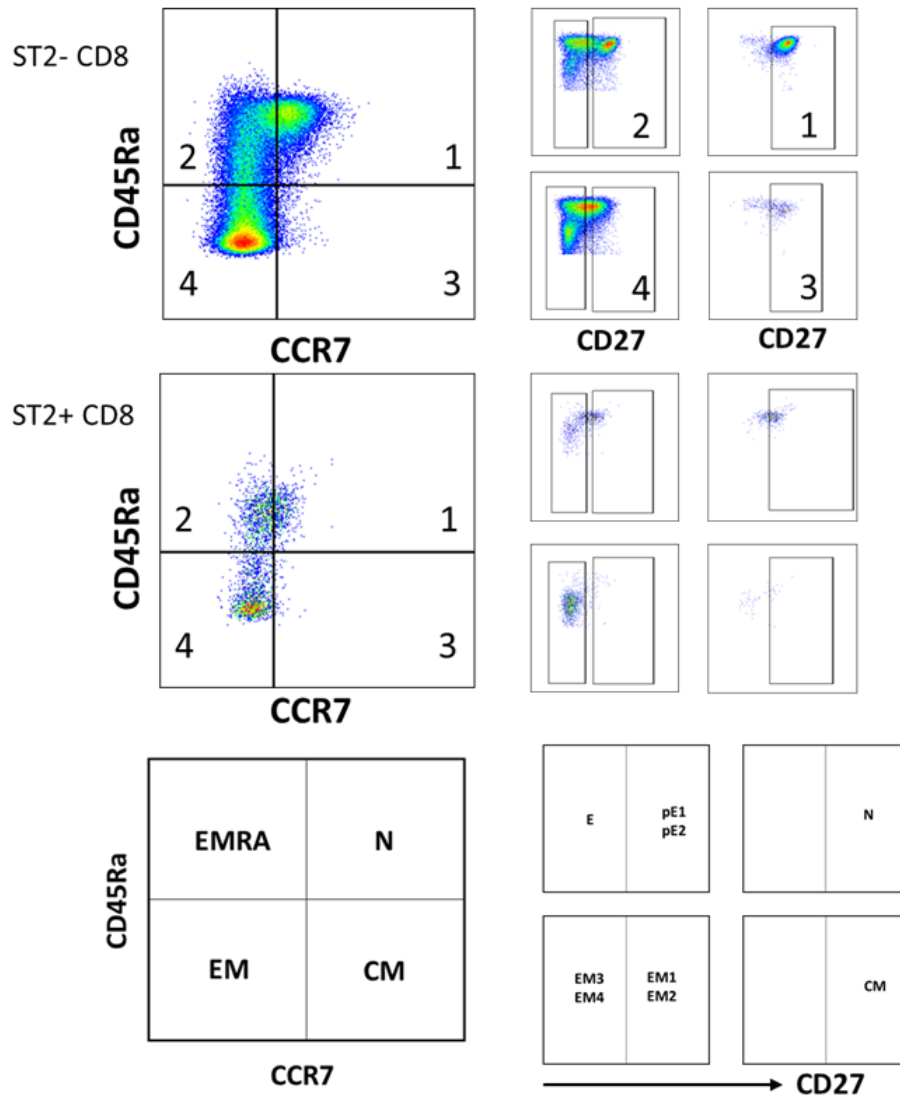


red) by assessing Granzyme B, perforin and double positive (left), CD107a (middle) and IFN $\gamma$  (right). Data represents mean  $\pm$  SEM. Statistical analyses were performed using non-parametric Mann-Whitney test.



**FIGURE 3** | IL-33 mediated antigen-specific T cell responses. (A) Effects of IL-33 on GAG-specific (left) and CEF-specific (middle) CD8<sup>+</sup> T cell proliferation in PBMCs of HIV infected patients (n = ). Effects of IL-33 on CEF-specific CD8<sup>+</sup> T cell proliferation in PBMCs of healthy donors (right, n = 5). (B) IL-33 induced cytokines production in the supernatant of GAG peptide- (left) and CEF peptide- (middle) stimulated PBMCs at day 5 from HIV infected patients (n = ) and CEF peptide- stimulated PBMCs from healthy donors (HD, right, n = 5). Statistical analyses were performed using Wilcoxon matched pairs signed rank test.

# Supplementary



**SUPPLEMENTARY FIGURE** | Gating strategy and classification for phenotype of ST2 expressing and non-expressing CD8 T cell subsets in PBMCs.

# DISCUSSION

The GI (gastrointestinal tract) is the most important target organ in the pathogenesis of SIV and HIV infection. The intestinal mucosa serves as a ‘feeding ground’ for HIV amplification in early infection and the gut associated lymphoid tissue (GALT) has the largest and most important site of persistence for virus in chronic infection (Xu et al., 2013).

Persistent inflammation exacerbates tissue damage in the patients with treated chronic HIV infection (PLWH), particularly in the gastrointestinal tract, allowing microbial components to enter into circulatory system, further increasing inflammation (Brenchley et al., 2006a; Zevin et al., 2016; Zicari et al., 2019). sST2 levels correlated with the levels of intestinal fatty acid binding protein (I-FABP) and of sCD14 markers of gut epithelial damage and of microbial translocation, establishing a link between sST2 and gut tissue damage (Mehraj et al., 2016a).

IL-33 is released from damaged cells functioning as an alarmin to promote tissue homeostasis and repair. IL-33 functions are skewed towards pathogen removal through Type 1 immune responses, followed by Innate Lymphoid Cells (ILC)- and Treg- driven repair process. This repair process is mediated in part by, EGF-like growth factor, Amphiregulin (Bonilla et al., 2012) (Burzyn et al., 2013a; Monticelli et al., 2011). Amphiregulin plays a central role in contributing to host resistance as well as induces tolerance by promoting the restoration of tissue integrity following damage associated with acute or chronic inflammation (Burzyn et al., 2013; Monticelli et al., 2011).

With persistent tissue damage and chronic unresolved inflammation, as is the case of chronic HIV infection, it may be conjectured that the tissue IL-33 pools will increase. In this work we explored the role of IL-33/ST2 axis in two ways: i) the role of IL-33 on repair process and ii) the role of IL-33 as an adjuvant for CD8<sup>+</sup> T cells response. We showed that IL-33 expression is increased in the gut of HIV infected patients compared to non-HIV infected healthy individuals. When IL-33 secretion participates to fibrosis, mucosal Treg population from HIV infected patients is enriched in tTreg prone to support tissue repair. However, we observed a functional defect in tTreg due to the lack of amphiregulin secretion, which could contribute to the persistent of local damage and release of IL-33. Our data point out a new mechanism where the absence of amphiregulin secretion by Treg might contribute to the leaky gut and persistence of chronic inflammation. In the other hands, our data showed that IL-33 can increase the

cytotoxic capacity of CD8<sup>+</sup> T cells. These results suggest that IL-33 could be used as an adjuvant for a therapeutic vaccine in PLWH.

## **1. IL-33 expression in the gut of PLWH**

Our team has previously reported in the serum of a large cohort of HIV-1 infected patients on HAART, followed longitudinally that sST2 is a significant predictor of all-cause mortality (Thiébaud et al., 2017b). Since sST2 is an indirect measure of IL-33 activity, we sought to measure IL-33 levels in the plasma and in gut biopsies of HIV-1 infected individuals under long-term cART. In our study, we found that IL-33 levels in the plasma of HIV-1 infected patients were below the limit of detection (data not shown). This result is in line with previous studies which showed that plasma levels of IL-33 were very low and/or undetectable (Miyagaki et al., 2011). We were able to observe a range of sST2 plasma levels (11917; 5528 – 24702; median; minimum – maximum)). It could be speculated that the undetectable IL-33 plasma levels were due to the high plasma sST2 buffering effects. Whereas, another possibility could be due to the anti-retroviral therapy, as has been described in literature that during anti-retroviral therapy, both IL-33 and sST2 levels were decreased (Khaitan and Unutmaz, 2011). Moreover, it has been reported that there was no significant reduction in the sST2 levels in patients initiating ART during early phase of infection, while long term treatment started during chronic phase normalized sST2 (Mehraj et al., 2016a). Therefore, we may conclude that sST2 levels are not normalized in our treated patients and they are able to downregulate the plasma IL-33 to undetectable levels. IL-33 has key roles in host immunity against various infections as well as in reducing atherosclerosis. Therefore, it was suggested that HIV infected patients with high sST2 and low IL-33 in sera may contribute to high risks of opportunistic infections and a high risk of atherosclerosis (Miyagaki et al., 2011).

In contrast, IL-33 mRNA expression was significantly increased in the gut tissue biopsies from the HIV-1 infected patients compared to HIV seronegative donor biopsies who had undergone colonic surgery (See Article 1, Figure 1A). We confirmed these findings, with IHC, showing that high IL-33 gene expression was consistent with a significant increase of IL-33 protein expression in gut biopsies from HIV-1 infected individuals compared to HIV seronegative biopsy tissue (See Article 1, Figure 1B). Consistent with the literature, IL-33 expression in the biopsies was detected in the epithelial cells, endothelial cells, fibroblasts, fibroblast-like cells,

as well as in the ECM in samples from HIV-1 infected individuals whereas this expression was weak, limited to cell nuclei, or absent in biopsies from HD controls (Article 1, Figure 1B).

Endothelial cells are major source of IL-33 in human body during steady state as well as during human disease. IL-33 expression can be further increased during inflammatory states. Although, epithelial cells are not a major source during homeostasis, but high levels of nuclear IL-33 are observed in these cells at barrier sites exposed to environment, like the respiratory and digestive tracts (Moussion et al., 2008). Fibroblastic reticular cells are other major sources of IL-33 protein in humans and mice. In addition, activated fibroblasts, fibroblast-like cells and myofibroblasts are also important sources of IL-33 during inflammation, especially in diseases associated with fibrosis (Manetti et al., 2010; Marvie et al., 2010; Masamune et al., 2010), mucosal healing and wound repair (Sponheim et al., 2010).

Similarly, in chronic inflammatory disease, such as active ulcerative colitis (UC), IL-33 expression is markedly increased in intestinal epithelial cells and infiltrating lamina propria mononuclear cells (Beltrán et al., 2010; Pastorelli et al., 2010). However, IL-33 mRNA has been associated with UC disease activity and sST2 also is known to be a marker of IBD severity, found to be significantly increased in both the gut mucosa and the serum (Kotsiou et al., 2018).

We observed a remarkable finding in IL-33 protein expression, as depicted in Figure 1C for representative staining in two different patients, revealing two groups of HIV-1 infected patients characterized by either a high level of IL-33 protein expression predominantly in epithelial cells or a pattern of weak or no expression which was not distinguishable from HD. As mentioned earlier, epithelial cells are not a major source of IL-33 during homeostasis and IL-33 is primarily produced by intestinal epithelial cells upon local damage. Therefore, we used this criterion as defining our qualitative IL-33 expression levels of high or low. This was a very interesting and novel finding, since all our patients from our HIV-1 infected cohort had similar clinical characteristics based on which they were selected. This gives rise to more questions, some of which will be addressed ahead.

**1- Whether the high gut tissue IL-33 protein expressing group also had high sST2 levels in their plasma was the first question that came forward.** Since our IL-33 protein expression was qualitative, we tried to establish as best as possible an association revealing that most of the patients (8 out of 11) with sST2 levels below the median, defined as sST2low

displayed a low IL-33 protein expression in the gut tissue. In contrast, 8 out of 12 patients with sST2 above the median, defined as sST2<sup>high</sup> exhibited high expression of IL33 (See Figure 35A below).

**2- Previous studies have already established a link between sST2 and gut mucosal damage. Whether this reciprocates to IL-33 as well, is the second question.**

Similar to previous reports, we confirmed in our HIV infected patients that there was no association between plasma sST2 levels and intestinal mucosal damage assessed by I-FABP levels in the plasma (See Figure 35B below). Similarly, we did not observe any association between gut IL-33 protein expression and I-FABP (Figure 35C).

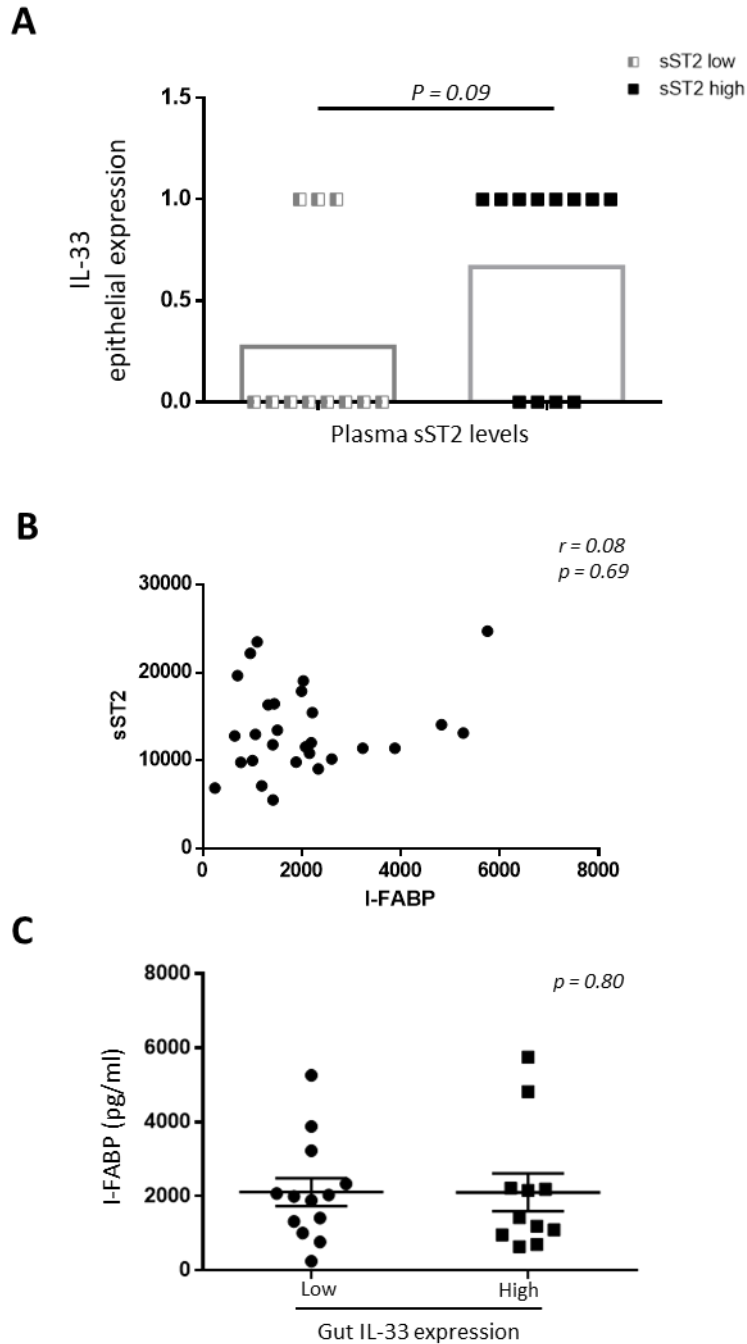


Figure 35. Graph representing correlations. A) IL-33 gut epithelial expression correlation with plasma sST2 levels, B) Plasma sST2 levels correlation with plasma I-FABP, C) IL-33 gut epithelial expression level correlation with plasma I-FABP.

This could be explained in a number of ways. I-FABP is present in the tops of the epithelial cells and released on epithelial cell damage. The levels rise rapidly after episodes of acute intestinal ischemia and inflammation. Therefore, circulating I-FABP has been a potentially useful plasma marker in early stages of intestinal diseases (Derikx et al., 2010). Moreover,

owing to its short half-life, it does not persist in the blood stream at high levels for long time. Another explanation may be that the damage following early infection could be reduced because of lower viral load and less vigorous cytotoxic T cell response after c-ART. Thus, the alarmin response may wane over time because of tissue fibrosis.

During acute HIV infection, sST2 levels correlated with the levels of intestinal fatty acid binding protein (I-FABP) and of sCD14 markers of gut epithelial damage and of microbial translocation, establishing a link between sST2 and gut tissue damage. These associations were lost during chronic HIV infection, suggesting a phase dependent and transient role of this axis in disease progression (Mehraj et al., 2016a).

3- On one hand, IL-33 has emerged as an important cytokine for the induction of mucosal healing and restoration of intestinal homeostasis. While on the other hand, it also plays a central role in wound repair which is dysregulated in case of chronic and persistent tissue damage leading to fibrosis (Lopetuso et al., 2012). For this reason, **we sought to investigate its contribution to gut fibrosis in chronic HIV infected patients.** Sirius Red staining was used to stain collagen fibers in the paraffin embedded gut tissue sections from HIV infected patients as well as HIV seronegative donors, followed by quantification of percentage of fibrosis.

We were able to demonstrate that the frequency of areas exhibiting fibrosis based on collagen deposition was higher in biopsies from patients with high expression of IL-33 protein in epithelial cells than in patients with low or no IL-33 expression (See Figure 2B). Our results suggest that in chronic HIV infection, the repair role of IL-33 would be dysregulated leading to fibrosis. These results build on existing evidence of IL-33's role in repair and fibrosis. A previous study showed that the administration of rIL-33 accelerated wound healing with enhanced re-epithelialization. IL-33 upregulation also promoted the collagen deposition and the expression of extracellular matrix (ECM)-associated genes such as fibronectin and collagen IIIa, which implies a direct effect of IL-33 on matrix synthesis. (Yin et al., 2013). IL-33 is known to induce the proliferation of colonic subepithelial myofibroblasts (SEMFs) (Lopetuso et al., 2012). Myofibroblasts are the major producers of extracellular matrix components. Previous studies have reported that intestinal myofibroblasts displayed an activated phenotype and contributed to the profibrotic changes in HIV patients under c-ART (Asmuth et al., 2015).

The alterations to the microbiome in HIV infection have also been demonstrated to result in direct effects on immunity and increased inflammation. Dysbiosis of the microbiome is



associated with increased gut and peripheral T cell activation, and plasma inflammatory soluble factors, which are independently associated with HIV pathogenesis. Furthermore, dysbiosis was associated with an increased indoleamine-2,3-dioxygenase (IDO) activity, which has been demonstrated to be associated with loss of mucosal IL-17 producing cells, also a pathogenic consequence of HIV infection. Another study also reported that flagellin produced by AIEC (adherent-invasive *Escherichia Coli*) causes the induction of ST2 expression on IECs, which in turn augments IL-33 signaling and promotes the development of intestinal fibrosis (Imai et al., 2019b). Therefore, it is tempting to speculate that dysbiosis and microbial translocation observed in HIV infection contribute to intestinal fibrosis through the IL-33 pathway activation.

4- **We wondered whether the elevated IL-33 in the gut in HIV infection contributing to the fibrosis, would also have an impact on the immunological function and CD4 lymphocyte dynamics.** According to this, we showed that patients with high expression of IL-33 displayed lower blood CD4<sup>+</sup>T count compared to patients with low IL-33 expression. In addition, these patients had a longer duration of HIV infection and c-ART treatment than patients with low expression of IL-33 (Article 1, Table 2), though this did not reach significance due to the small number of patients. At the same time, the reduction in circulating CD4<sup>+</sup> T cells may impair GALT restoration by decreasing the number of T cells that could traffic to the intestine, leading to bacterial overgrowth and increased microbial translocation. Furthermore, microbial translocation may impair CD4 recovery as well, in patients on ART by contributing to the lymphatic tissue fibrosis (Schacker et al., 2002). Thus, there is a tight interplay among low nadir CD4<sup>+</sup> T cell counts, microbial translocation, immune activation, and immunologic non-response.

This was supported by the detection of LPS in the mesenteric and axillary lymph nodes as well as the colon, in cases of chronic SIV infection (Estes et al., 2010). Similarly, studies from patients with chronic HIV infection on ART, revealed a greater density of collagen I deposition in mesenteric and axillary lymph nodes and GALT, which was associated with a smaller CD4<sup>+</sup> T cell population in the lymphatic tissue and with reduced CD4<sup>+</sup> T cell recovery. This collagen deposition was suggested to be driven by transforming growth factor  $\beta$ 1 (TGF $\beta$ 1), in response to LPS-induced local immune activation. The degree of fibrosis in the GALT correlates directly with CD4<sup>+</sup> T cell activation and inversely with CD4<sup>+</sup> T cell count (J. Estes et al., 2008; Jacob D Estes et al., 2008; Schacker et al., 2002).

5- As expected, **our subsequent investigation confirmed an association of IL-33 and plasma inflammatory and immune activation markers.** We assessed whether a correlation existed between IL-33 and plasma immune activation markers IL-12p70 and IFN $\gamma$  and between IL-33 and soluble inflammatory markers including IL-6, IL-10, TNF $\alpha$ , IL-17A measured in plasma. A positive correlation was found for IFN $\gamma$  and IL-12p70 (See Article 1, Figure 2D).

IL-12p70 is produced mainly by macrophages and DCs (APCs) causing activation and proliferation of Th1 cells, leading to the release of IFN $\gamma$  by the Th1 cells. IFN $\gamma$  in turn augments the cytotoxicity of CD8 T cells. Thus, our results may imply that IL-33 from the gut augments the Th1 and subsequently the CD8 immune response in the HIV infected patients on c-ART. In addition, IFN $\gamma$  and IL-33 are known to counter regulate activation of ILC2s, thus contributing to a shift toward Th1 response (Molofsky et al., 2015b).

Furthermore, in early non treated HIV infection, plasma sST2 was reported to be positively correlated to IFN $\gamma$  (Mehraj et al., 2016a). While this correlation was observed to be lost in our chronic HIV patients under c-ART (See Figure 36 below). This may suggest a phase-dependent and transient role of sST2. Increased sST2 level attenuates the regulation of T cells by IL-33 (Wu et al., 2018).

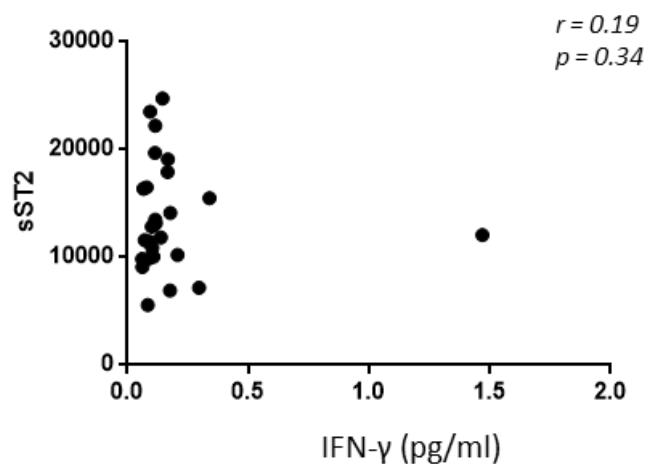


Figure 36. Graph representing the correlation of plasma sST2 and plasma IFN $\gamma$  levels

## 2. Tissue Treg in the gut of PLWH

Immune activation elicits a T regulatory counter response to limit inflammation and enhance repair. A role for Treg in chronic HIV infection had been proposed, based on the fact that the depletion of CD4<sup>+</sup>CD25<sup>+</sup> T cells from PBMC of HIV-infected donors resulted in increased anti-HIV T cell responses (Aandahl et al., 2004, p. 4; Weiss et al., 2004). Tregs secrete TGF- $\beta$ , in order to limit immunopathological immune responses. Tregs act as a double-edged sword by dampening, in addition, the antiviral immune responses as well as by triggering collagen production and deposition by fibroblasts; resulting in structural damage and fibrosis, consequently reducing CD4 reconstitution (Estes et al., 2007). IL-33 may also contribute to the development of fibrosis by promoting Treg accumulation during the chronic phase of HIV infection.

Therefore, we investigated the phenotype of Tregs in the context of cART at mucosal sites in HIV infected patients, since IL-33 promotes both tissue Treg accumulation and maintenance in the gut, enhancing their protective function. With our findings, we did not observe a significant difference in the frequency of total Tregs defined as CD4<sup>+</sup>CD25<sup>hi</sup>Foxp3<sup>+</sup> cells, in freshly isolated cells from mucosal biopsies between HIV infected patients on c-ART and healthy controls (See Figure 3A). Rather, we observed a shift towards the phenotype of Tregs prone to tissue repair. Our data is consistent with previous studies showing that ART normalizes Treg frequency in the gut and lymphoid tissue (Epple et al., 2006a)(Andersson et al., 2005; Chevalier and Weiss, 2013). However, we extend significantly these results by deciphering the phenotype of tissue Tregs in the gut. We were able to identify tissue Treg populations by characterization using the surface markers CD161, IL-18Ra and ST2 (See Figure 3B). Because we didn't get enough cells, we are unable to determine whether ST2 Treg expressed CD161 or IL18 Ra (Figure 37 below). We showed that the frequencies of both, Tregs co-expressing CD161 and IL-18R $\alpha$  and Tregs expressing ST2, were increased in the gut of HIV infected patients on c-ART compared to HD (See Article 1, Figure 3 C, D). In addition, both these populations were detected in tissues and not in blood from healthy donors, suggesting their function as tissue-residents Tregs. It is tempting to speculate that IL-33 promotes the accumulation of tissue Tregs in the gut of PLWH.

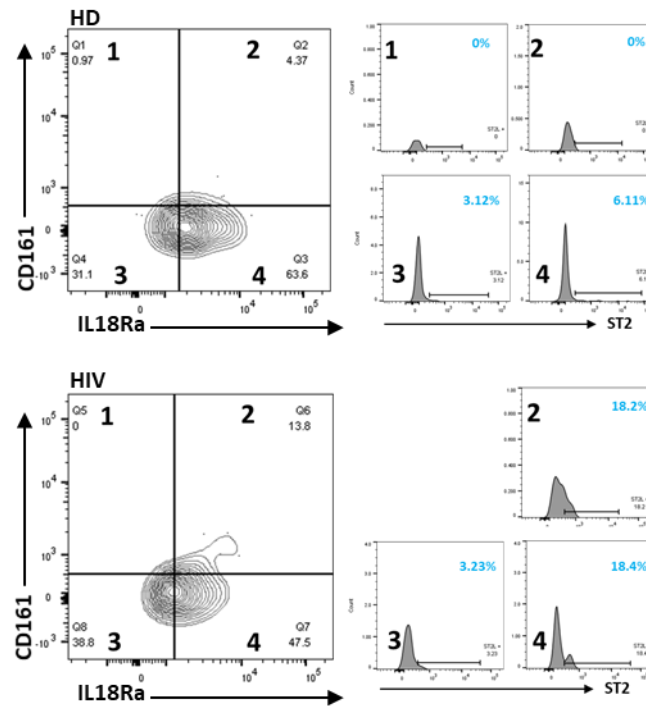


Figure 37. ST2 expression on CD161 and IL18 Ra expressing/non-expressing T regs in LPL from HD (top) and HIV infected (bottom) gut samples

Study in human intestine, described CD161<sup>+</sup> Treg cells to be a distinct, highly suppressive population of peripherally derived Treg cells that mediate wound healing. CD161<sup>+</sup> Treg cells expressed CCR9, a gut-homing chemokine receptor, subsequently the lamina propria revealed enrichment in CD161<sup>+</sup> Treg cells, particularly in IBD. The cytokines that were shown to be implicated in the healing process by CD161<sup>+</sup> Tregs included IL-17, IL-22, IL-10 and IL-4 (G. A. Povoleri et al., 2018). In our hands, we didn't get enough cells to confirm these data.

ST2<sup>+</sup>Treg cells have also been described as highly suppressive thymic-derived Treg cell populations, but mainly in the murine models. Accumulation of these cells was shown to be promoted by IL-33 upon tissue damage through selective expression of ST2 (Schiering et al., 2014a). Moreover, in another study administration of IL-33 showed an increase in the number of ST2<sup>+</sup> Tregs resulting in the amelioration of colitis in mice-model. (Siede et al., 2016). Intestinal ST2<sup>+</sup> Treg cells also have high expression of the gut homing receptors CCR9 and  $\alpha 4\beta 7$  (Whibley et al., 2019).

Once we had characterized these distinct Treg subtypes in the lamina propria lymphocytes from the intestinal tissue, we were interested to investigate the functional profile of these tissue Tregs,

since these cells contribute to the maintenance of tissue homeostasis, by promoting wound healing and repair processes, beyond their classical role in the suppression of pro-inflammatory responses (Panduro et al., 2016b; Zhang et al., 2017). Based on tissue Tregs known contribution to the repair process, we reasoned that a defect/dysfunction in their wound healing capacity may be linked to the persistence of the disrupted mucosal epithelial barrier in HIV infected individuals on c-ART.

One of the underlying mechanisms associated with  $T_{reg}$ -mediated wound healing is assumed to be the suppression of pro-inflammatory stimuli. With the assessment of TGF- $\beta$  mRNA levels, as expected, our data showed an increased level of TGF- $\beta$  in the gut of HIV infected patients on c-ART compared to HD (See Article 1, Figure 4A). However, remarkably, there was an inverse correlation observed between IL-33 mRNA and TGF- $\beta$  mRNA levels (See Article 1, Figure 4B). Although, this inverse correlation was surprising, it may suggest that Tregs are not able to control local inflammation, which is an obstacle for epithelial barrier repair. Indeed, the resolution of local inflammation is clearly a key function of Tregs during wound healing (Zhang et al., 2017). This result is in line with previous reports showing the reduced immunosuppressive capacity of Tregs during HIV infection (López-Abente et al., 2016b).

In addition, tissue Tregs are also induced by IL-33 signaling, to mediate their function of repair in part by the release of Amphiregulin, which plays an important role in healing and regeneration in the intestinal mucosa, lung epithelium and skeletal muscle (Zhang et al., 2017).

Our next results investigated the expression of this important growth factor in the gut of our patients. Characterization of the Treg subsets that produced AREG, revealed that 30% of the ST2<sup>+</sup>Foxp3<sup>+</sup> T cells expressed Amphiregulin compared to 3% of ST2<sup>-</sup>Foxp3<sup>+</sup> T cells (See Article 1, Figure 4D). Moreover, the expression of AREG by CD161<sup>+</sup> and IL18Ra<sup>+</sup> Foxp3<sup>+</sup> T cells was quite low and not significantly different from their CD161 and IL18Ra negative counterparts (See Article 1, supplementary Figure 2). Thus, we identified the tissue Treg subset expressing AREG in the healthy gut tissue. More importantly, we observed that the AREG expressing Treg cells were negligible in the LPL from HIV infected gut compared to healthy donors. These results, highlighted for the first time to our knowledge a profound defect of tissue Tregs producing AREG in the gut of HIV infected patients (See Article 1, Figure 4E). Moreover, we also observed a reduction in the capacity of total CD4 T cells to produce AREG in HIV infected patients (See Article 1, Figure 4C).

The Rudensky group, using a mouse strain with a Treg-specific deficiency of amphiregulin, showed a substantially more severe form of symptoms following influenza infection than wild-type mice. These mice displayed diminished lung function, indicating an essential role of Treg cell derived AREG in lung repair. The alarmin IL-33 and the pro-inflammatory cytokine IL-18 were able to induce AREG expression by Treg cells. Moreover, the population of tissue Tregs identified in the lungs expressed high levels of IL-18R and ST2 (Arpaia et al., 2015). During infections or acute injury, the injection of recombinant Amphiregulin (rAREG) has, in several different experimental settings, demonstrated the amelioration of symptoms, such as during Influenza infection or following viral-bacterial co-infections, as well as acute muscle injury (Burzyn et al., 2013).

AREG also has a critical role for the efficient CD4<sup>+</sup> T regulatory cell function. The function of AREG on Tregs has been described in colitis and tumour models, emphasizing its importance in suppression of local inflammation as well as promoting repair. Mice that lacked amphiregulin had insufficient numbers of Tregs to the site of inflammation, showing immune regulatory dysfunction. While the presence of amphiregulin enhanced the suppressive capacity of Tregs. A positive feedback loop has been described whereby amphiregulin produced by Tregs promoted tissue repair, and autocrine or paracrine amphiregulin signals activated Treg function by the EGFR pathway (Zaiss et al., 2015). The exact mechanism by which amphiregulin contributed to wound healing had remained unclear. Amphiregulin, a low-affinity EGFR ligand is a poor mitogen for epithelial cells and recent data show that one major function of amphiregulin is the local induction of TGF- $\beta$ . During wound healing, amphiregulin induced the TGF- $\beta$  mediated differentiation of tissue stem cells. Notably, TGF  $\beta$  can regulate AREG expression and AREG has been shown to have a critical role in the development of TGF-  $\beta$  induced pulmonary fibrosis (Zaiss et al., 2015; Zhou et al., 2012).

Surprisingly, we observed a significant inverse correlation between IL-33 and TGF- $\beta$  (See figure 4B), as well as AREG and TGF- $\beta$  levels (Shown in Figure 38 below) on mRNA expression level. This observation could be related to the defect in the production of amphiregulin and may suggest that Treg are not able to control local inflammation, which is an obstacle for epithelial barrier repair.

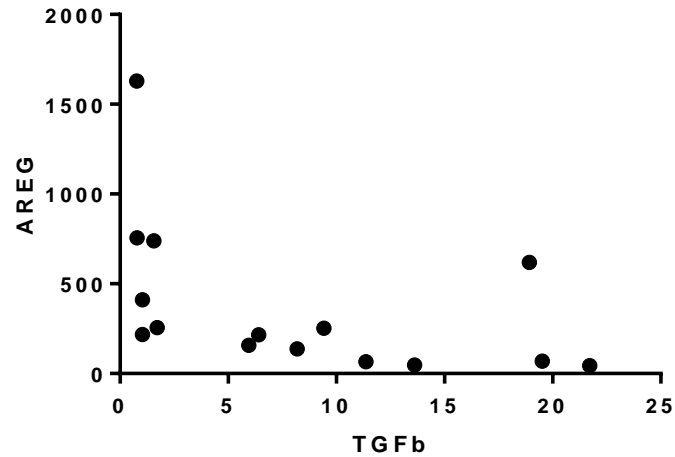


Figure 38. Correlation of amphiregulin (AREG) and TGF- $\beta$  mRNA levels in HIV infected gut biopsies

Subsequently, we assessed and observed that the EGFR relative expression in the gut of HIV infected patients was elevated compared to the healthy donors (See Article 1, Figure 4 F). This last result validates that the specific defect in amphiregulin production by tissue Tregs might be the main contributor to the defective healing of the epithelial barrier in the chronic phase of HIV infection.

Summarizing our results, we observed an increased expression of IL-33 in the gut of HIV infected patients on c-ART, associated with increased fibrosis and decreased CD4 reconstitution. It was also associated with immune activation, predominantly Th1 response. Our results point that IL-33 maintains ST2<sup>+</sup> Tregs in the gut but is unable to induce amphiregulin by these cells. The factors contributing to this deficit remain to be determined. Since rAREG has been shown to enhance wound healing, it would be interesting to test whether amphiregulin enhances the epithelial barrier function and ameliorates the symptoms of leaky gut observed in HIV infection.

### **3. Phenotype and function of CD8 T cells in the gut of PLWH**

One of the factors that could contribute to leaky gut is persistent antigen exposure by viral replication. To control this, anti-viral CD8 T cells response is needed. Even in the presence of c-ART induced viral suppression, there is residual HIV replication and microbial burden, both of which maintain a persistent antigen load, contributing further to the leaky gut and inducing CD8 T cell dysfunction and continuous expression of inhibitory receptors and the presence of inflammatory mediators. Tissue reservoirs promote persistent antigenic burden during HIV infection even with c-ART (Perdomo-Celis et al., 2019).

We characterized the phenotype and functionality of CD8 T cells in the LPL of HD and HIV infected gut biopsies of our patients. We observed an increased frequency of naïve and central memory while a decrease in effector memory (EM3 and EM4) subsets of CD8 T cells in HIV infected gut LPL compared to HD gut LPL (See Figure 39A below).

As expected, and according to literature, we observed an increased check-point inhibitor and exhaustion marker, PD-1, on CD8 T cells in HIV infected LPL compared to HD LPL (See Figure 39B). Although there was a trend towards decrease, we did not observe significant decrease of the activation markers including CD38 and HLA-DR, between HD and HIV LPL (See Figure 39D). This may probably be owing to the smaller number of samples.

The granzyme B expressing CD8 T cells were relatively increased but not significant for HIV infected LPL, whereas perforin and granzyme/perforin double positive CD8 T cells were lower in HIV infected LPL compared to HD (See Figure 39C). These results were not significant, as we did not have sufficient HD results to compare.



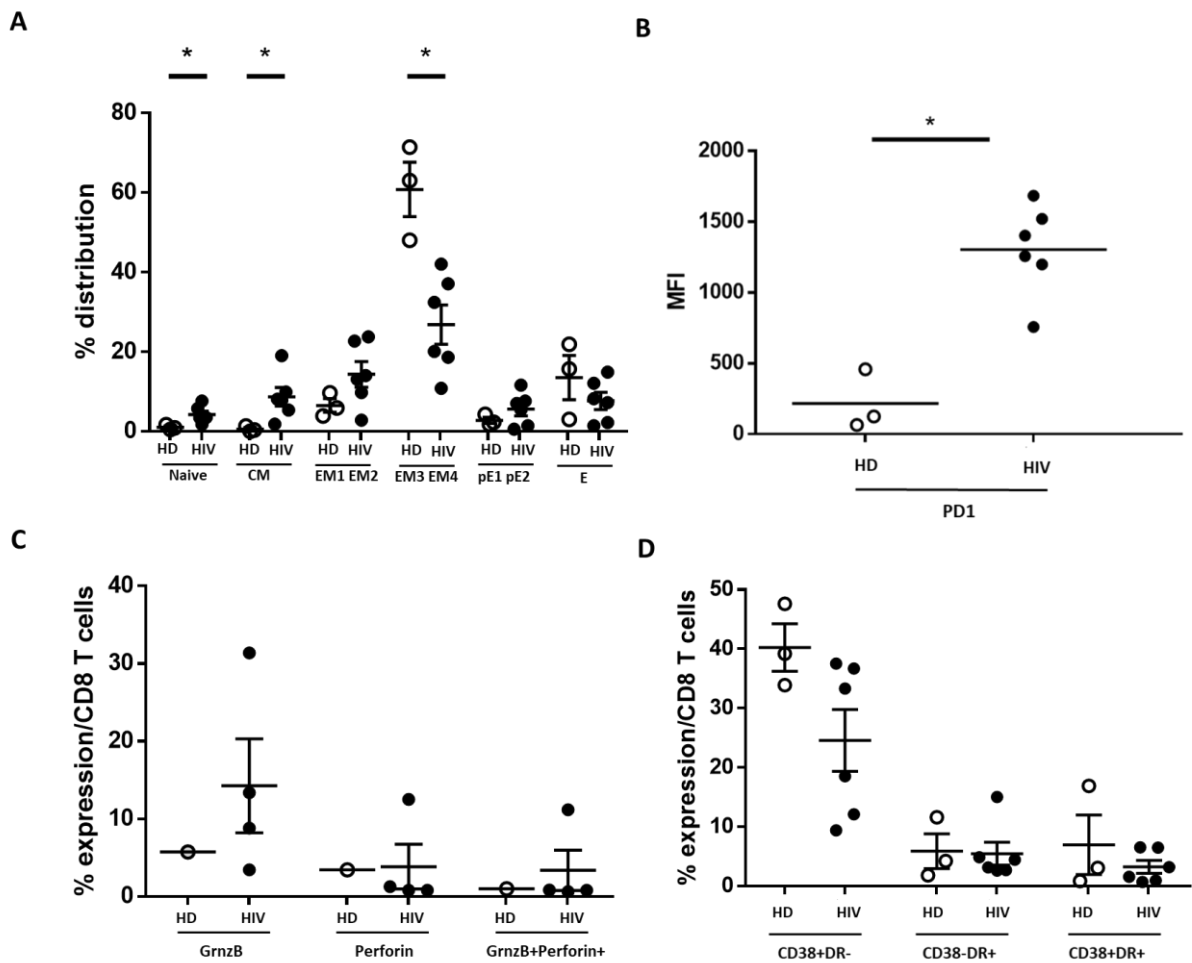


Figure 39. CD8 T cells phenotypic and functional characterisation in HD and HIV infected LPL. A) Phenotypic subsets within CD8 T cells comparison between HD and HIV infected, B) Comparison of PD1 expressing CD8 T cells between HD and HIV infected LPL, C) Comparison of granzyme B and perforin expression by CD8 T cells in HD and HIV LPL, D) Activation markers expression by CD8 T cells comparing HD and HIV infected in LPL

Then, we analyzed the phenotype of CD8 T cells in LPL and PBMC of PLWH. Figure 40A showed that the naïve subset of CD8 T cells has a greater frequency in PBMCs than LPL as well as the terminal effector subset. IFN $\gamma$  expressing CD8 T cells have a higher frequency in LPL than PBMCs, but this was not significant due to the small number of samples (See Figure 40B). Granzyme B and perforin expressing CD8 T cells are relatively lower in LPL than PBMCs (See Figure 40C). These results suggest that the functions of LPL CD8<sup>+</sup> T cells are non-cytolytic. In addition, we observed the activated phenotype of these cells by the higher frequency of CD38<sup>+</sup> (HLA-DR<sup>-</sup>) and CD38<sup>+</sup>HLA-DR<sup>+</sup> (double positive) CD8 T cells compared to PBMCs.

To conclude, we observed a functional and phenotypic dichotomy between the blood and the gut in our patients.

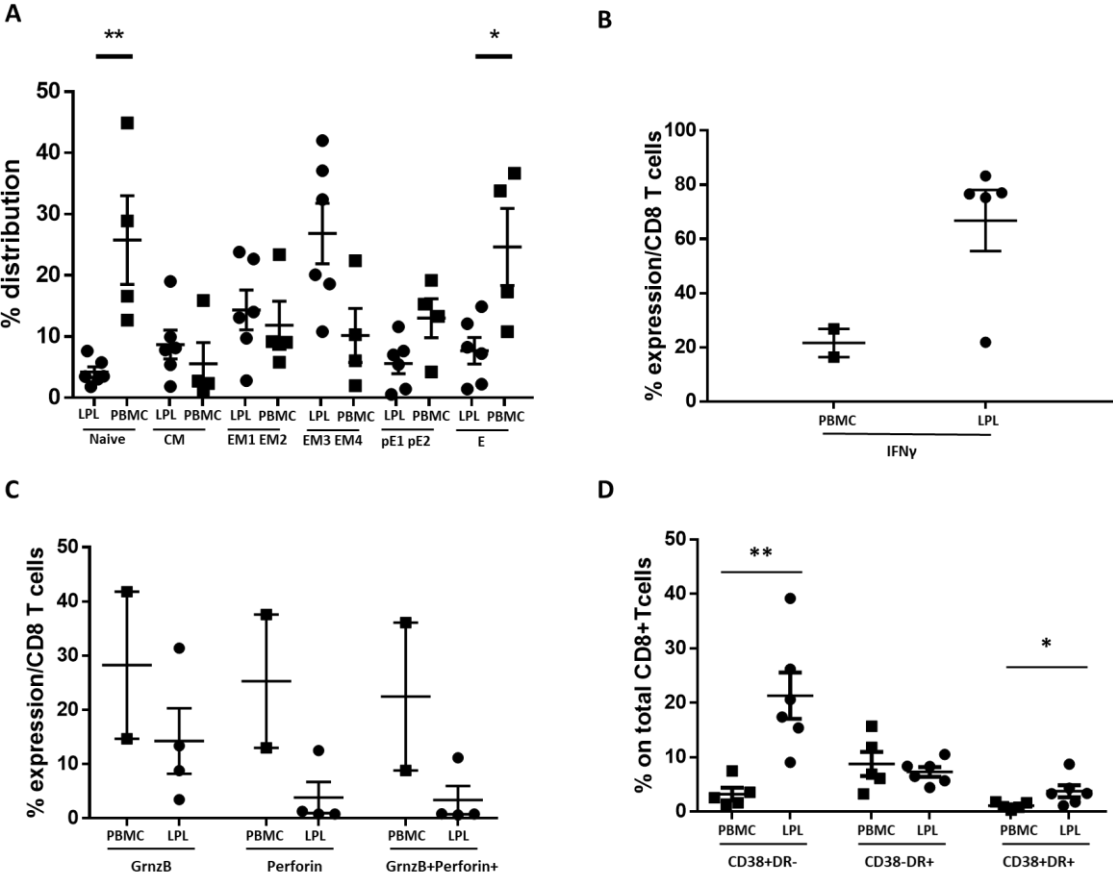


Figure 40. CD8 T cells phenotypic and functional characterisation in PBMC and LPL of HIV infected patients on c-ART. A) Phenotypic subsets within CD8 T cells comparison between PBMC and LPL, B) Comparison of IFN $\gamma$  expressing CD8 T cells between PBMC and LPL of HIV infected patients, C) Comparison of granzyme B and perforin expression by CD8 T cells in HIV PBMC and HIV LPL, D) Activation markers expression by CD8 T cells comparing HIV PBMC and HIV LPL

As mentioned, IL-33 functions are skewed towards Th1 responses following gut injury accompanied by commensals and pathogen infiltration, as in the case of HIV infection. The upregulated IL-33 induces the expression of ST2 by Th1, CD8 T cells and NK cells as a defense mechanism against intracellular pathogens, thereby increasing the capacity of these cells to produce IFN $\gamma$ . This redirects the default type 2 inducing capacity of IL-33 to effector programs in response to life threatening challenges (Liew et al., 2016). Hence, we wanted to explore the ST2<sup>+</sup> CD8<sup>+</sup> T cells in the LPL from gut biopsies. It took us quite some time to get our ST2 staining satisfactorily working. We faced some staining issues in order to achieve the ideal conditions for the staining. Figure 41 below, shows the ST2 staining on CD8 and CD4 T cells

with the good antibody on thawed PBMCs from HIV infected sample. We also observed differences in fresh and frozen samples for ST2 expression levels (data not shown).

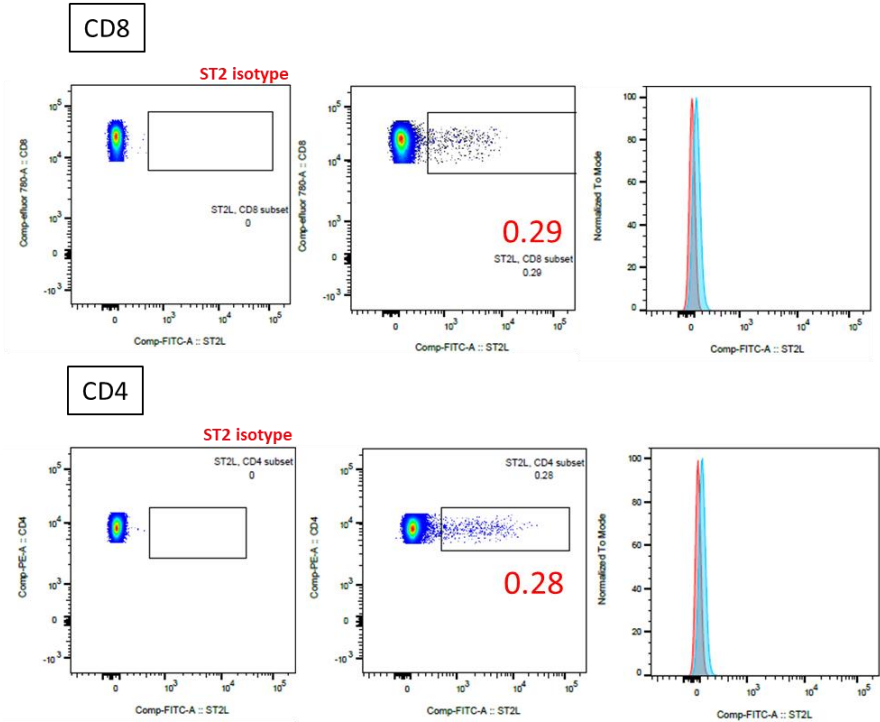


Figure 41. Extracellular surface staining on PBMCs, showing expression of ST2L on CD8 and CD4 T cells, in comparison with isotype control.

The following figure (Figure 42) depicts variation in ST2L expression under different time points. We concluded that ST2L is a recycling receptor. The ST2 expression levels are affected by stressful conditions including change of temperature as in case of addition of cold medium or with freezing/thawing or nutrient deprivation (serum free medium).

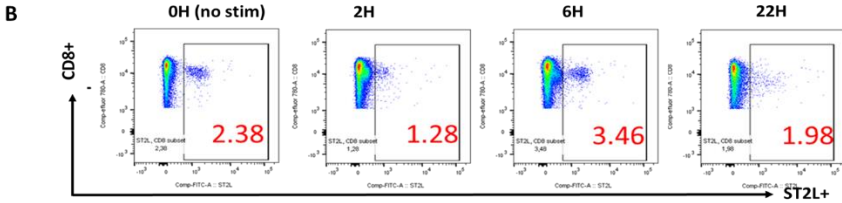


Figure 42. ST2L surface expression on CD8 T cells at different time points showing the kinetics under CD3.CD28 beads stimulation

Once our staining was functional, soon after we had the covid sanitary crisis and we were unable to perform and receive gut biopsy samples for several months, which left us no time for further experiments on this part of our study. Therefore, we decided to focus our efforts on the study of ST2<sup>+</sup> CD8<sup>+</sup> T cells in the blood of PLWH.

#### **4. Phenotype and function of ST2<sup>+</sup>CD8<sup>+</sup> T cells in the blood of PLWH**

HIV-specific CD8<sup>+</sup> T-cells are able to control the virus during acute HIV/SIV infection, but their cytotoxic potential dramatically decreases along with disease progression and are no longer capable of exerting an appropriate antiviral response (Demers et al., 2016). CD8<sup>+</sup> T-cells suffer important alterations in their frequency, differentiation, and activation profile. Moreover, persistent antigenic stimulation leads to immune exhaustion. c-ART does not fully reconstitute the CD8<sup>+</sup> T-cell counts (Tanko et al., 2018), the proportions of memory subsets, the levels of activation and exhaustion markers, and their functional capacity (See Figure 32 A and B, section 6.4) (Perdomo-Celis et al., 2019; Tanko et al., 2018). Three major determinants of CD8 T cell dysfunction during chronic infections including, persistent antigen exposure, negative co-stimulation, and chronic inflammation persist despite c-ART induced viral suppression.

The total pool of circulating CD8<sup>+</sup> T-cells is persistently increased in untreated HIV-infected patients, with higher frequency of memory subsets and reduction of naïve cells, as compared to seronegative individuals. c-ART does not fully reconstitute the CD8<sup>+</sup> T-cell counts, or the proportions of memory subsets (Tanko et al., 2018). In addition, patients exhibit higher expression of the activation markers HLA-DR, CD38, and Ki-67, and immune exhaustion markers such as programmed death (PD)-1 and T-cell immunoglobulin and mucin-domain containing-3 (TIM-3), lymphocyte activation gene protein (LAG-3), and cytotoxic T lymphocyte antigen-4 (CTLA-4) (Fenwick et al., 2019; Larbi and Fulop, 2014). Previous studies have shown that the expression of these molecules during chronic HIV infection is accompanied by the impairment of CD8<sup>+</sup> T-cell lytic and non-lytic mechanisms, as well as the proliferative ability and survival (Perdomo-Celis et al., 2019). The progressive loss of CD8 T-cell function starts with an initial loss of proliferative capacity, cytotoxic potential, and a restricted IL-2 production. Finally, by the loss of ability to produce IFN- $\gamma$ .

Previous studies have demonstrated the expression of ST2 on CD8 T cells upon activation. Bonilla et al. showed that following LCMV infection in mice, roughly 20% of activated antigen (Ag)-specific CD8<sup>+</sup> T cells expressed ST2 (Bonilla et al., 2012, p. 33). Whether this is a unique subset of CD8 T cells, needs to be addressed. We investigated and characterized this subset of CD8 T cells expressing ST2 and to assess the role of IL-33 in HIV specific response mediated by these cells.

First, we assessed the frequency of ST2 CD8 T cells in HIV infection. We observed a significant upregulation of ST2 expressing CD8 T cells in the PBMCs from HIV infected patients compared to healthy donors (See, Article 2, Figure 1A). Remarkably, in HIV infected patients, the ST2 expression on the CD8 T cells was maintained even after 5 days in culture medium, in the absence of stimulation. Whereas this was not observed for the healthy donor samples (See Figure 1 B).

Consistent with our findings, two independent studies have shown that activated CD8 T cells can express ST2 cells (Ngoi et al., 2012; Yang et al., 2011).

We observed significant negative correlation between sST2 and ST2L (See Article 2, Figure 1 C). Literature has described in mice and humans, that alternative promoter activation and splicing produces ST2L and sST2. ST2L represents the longest transcript, whereas sST2 is the truncated and soluble isoform. ST2L is the biological receptor for IL-33, and IL-33/ST2L signaling pathways is downregulated by the neutralization of IL-33 by binding with sST2, the decoy receptor of IL-33 (Pusceddu et al., 2019, p. 2). This may explain why patients with high level of sST2 displayed lower frequency of ST2<sup>+</sup>CD8<sup>+</sup> T cells.

We then characterized the phenotype of ST2<sup>+</sup>CD8<sup>+</sup> T cells in the blood of healthy donors and HIV infected patients on c-ART. Phenotypic characterization was done using the markers CD45RA, CCR7 and CD27, and four subgroups identified as naïve (CD45RA<sup>+</sup>/CD27<sup>+</sup>/CCR7<sup>+</sup>), TCM (CD45RA<sup>-</sup>/CD27<sup>+</sup>/CCR7<sup>+</sup>), TEM (including EM1/EM2; CD45RA<sup>-</sup>/CD27<sup>+</sup>/CCR7<sup>-</sup> and EM3/EM4; CD45RA<sup>-</sup>/CD27<sup>-</sup>/CCR7<sup>-</sup>), and TEMRA (including pE1/pE2/E; CD45RA<sup>+</sup>/CCR7<sup>-</sup>/CD27<sup>-</sup>) CD8 T cell (described in section 6.3). Our results showed that ST2<sup>+</sup>CD8<sup>+</sup> T cells displayed an effector phenotype and expressed less exhaustion markers (PD1 and LAG-3) than their ST2<sup>-</sup> counterpart. Our data suggests that ST2<sup>+</sup> CD8 T

cells constitute effector CD8 T cells. Whether they are a functionally intact subset in chronic HIV infection is the next question addressed.

Therefore, we next assessed the functionality of these cells. Our data showed that the ST2<sup>+</sup> CD8 T cells exhibited a high proliferating capacity (See figure 2B), compared to ST2<sup>-</sup> CD8 T cells in both healthy donors and HIV infected patients. In addition, these cells had a high cytotoxic potential, determined by the expression of Granzyme B, Perforin, GranzymeB/Perforin double positive and CD107a. We did not observe a difference in the non-cytolytic response (measured by IFN  $\gamma$ ) in either HD or HIV infected group. Based on these results, we identified ST2<sup>+</sup> CD8 T cells as a unique effector population with high cytotoxic potential.

A recent study assessing the regulation of T cell function by IL-33/ST2 axis in early HIV infected patients reported that increased sST2 weakens the IL-33 induced HIV-specific CD4 and CD8 T cell immune responses. This was demonstrated in vitro, by the attenuation of IFN $\gamma$  release by both GAG- as well as CEF-stimulated CD4 and CD8 T cells by the addition of anti-ST2 antibodies (Wu et al., 2018). Our results demonstrated an IL-33-induced upregulation of IFN $\gamma$  response by both GAG- as well as CEF-stimulated PBMCs from HIV infected patients on c-ART. In addition, we also revealed an IL-33-induced upregulation of cytotoxicity, assessed by Granzyme A, Granzyme B and sFasL expression in HIV infected patients, which did not reach significance for Granzyme B and sFasL in case of CEF-stimulated cells.

Based on our results as well as previous reports, immunoadjuvant properties of IL-33 could be highlighted. It has been a challenge to find adjuvants that can facilitate CD8<sup>+</sup> T cell responses. IL-33 might make an ideal candidate.

Villarreal et al. were the first to demonstrate that the delivery of IL-33 as a molecular adjuvant in a DNA vaccine setting could induce protective immunity by further enhancing, both arms of the adaptive immune responses (Villarreal et al., 2014). The demonstration that IL-33 can induce both Ag-specific Th1 and CD8<sup>+</sup> T cell responses in preclinical settings makes IL-33 an ideal adjuvant candidate for enhancing prophylactic and therapeutic vaccines in the treatment of chronic viral infections and cancer vaccine therapies (See Figure 33, section 6.4).

In the context of HIV infection, previous studies on mice (Sarkar et al., 2019), rabbits (Malherbe et al., 2014) and rhesus monkeys (Hessell et al., 2016) have demonstrated the ability of IL-33 in inducing protective immunity to HIV-1 infection in combination with VC10014 immunization regimen, by influencing the B cell responses.

The ability of IL-33 to enhance the formation of Ag-specific effector CD8<sup>+</sup> T cells and amplifying the effector-memory CD8<sup>+</sup> T cells responses, needs to be demonstrated in HIV-1 infected animal models. To summarize, ST2<sup>+</sup> CD8 T cells are a unique subset of CD8 T cells, with an effector phenotype, characterized by the expression of CD45RA, lack of expression of CCR7, with the presence or absence of CD27. Additionally, the effector phenotype had low PD-1 expression levels. These cells are functionally intact, cytolytic rather than non-cytolytic and show a high proliferating capacity. The ST2<sup>+</sup> CD8 T cells characterization differed neither phenotypically nor functionally between healthy donors and HIV infected patients on c-ART. However, there was an increase in frequency of these cells in HIV infected patients compared to healthy donors. Finally, we highlighted an important role of IL-33/ST2 axis in the regulation of specific CD8 T cell responses in HIV infection with suppressed viremia. The upregulation of these responses suggests that IL-33 may be used as an adjuvant for HIV vaccine.

# CONCLUSION

In our study we revealed two phenotypes of tissue Tregs in the LPL from gut mucosa, which were upregulated in the HIV infected patients compared to HIV seronegative controls. We highlighted a specific defect of AREG production by Tregs in the HIV infected gut mucosa. Tissue Tregs expressing ST2, the receptor for the alarmin IL-33, were shown as the source of the AREG producing Tregs. Our results from gut mucosa of patients also showed elevated levels of IL-33. Two groups of patients were identified based on low and high IL-33 expression. The IL-33 levels were associated with increased fibrosis, decreased CD4 restoration and elevated plasma immune activation and inflammatory markers. Our study points out the lack of amphiregulin secretion by Tregs as a mechanism of defective repair contributing to leaky gut and persistence of chronic inflammation.

In our second study, characterization of ST2 expressing CD8 T cells deciphered this subset to be a cytotoxic population of effector CD8 T cells, with a high capacity to proliferate with TCR stimulation. CD8 T cells from blood of HIV infected patients on c-ART were shown to maintain a high expression of ST2 compared to healthy donors. Our results also highlighted the role of IL-33 in enhancing GAG- and CEF specific responses in HIV patients on c-ART. This was demonstrated by the elevation of IFN $\gamma$ , Granzyme A, Granzyme B and sFas-L, which were more significant in the GAG-specific response. Our results highlight the role of IL-33 in enhancing specific immune responses in HIV infected patients indicating its potential use as an immunoadjuvant.



# PERSPECTIVES

Better understanding of the reasons of epithelial barrier dysfunction and leaky gut leading to systemic inflammation, would help us formulate therapeutic interventions to improve the morbidity of HIV infected patients.

We were limited by time and small number of cells obtained from the gut biopsies. Therefore, we were unable to carry out more than one experiment on the same donor.

There is still work that needs to be continued on this subject to further our knowledge on tissue Tregs in the human gut. The functionality of these tissue Tregs as well as the characterization of these cells needs to be continued. This future work should explore and include additional markers, revealed by analysis of gene expression. It will be imperative to carry out single cell transcriptomic analysis on the LPL from our patients' samples. The transcriptomes of Treg cells located in various non-lymphoid tissues (Tissue Tregs) diverge from those of lymphoid organ Treg cells and are also quite distinct from each other. This has been illustrated in a recent publication (Muñoz-Rojas and Mathis, 2021), where they compiled a set of robust published RNA-seq data sets from diverse tissue Treg cell populations from mice. (Illustrated below)

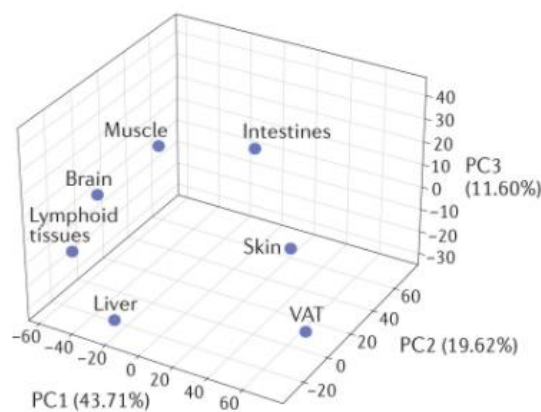


Figure 43. Analysis of tissue Treg cell transcriptomes. Compiled matrix of fold change for each of the tissue Treg population, used to perform principal component analysis (Muñoz-Rojas and Mathis, 2021)

The characterization of these cells should also include the inhibitory receptor PD-1. The PD-1hi Tregs have been recently described as non-suppressive Tregs in the oral mucosa, which respond to IL-1 $\beta$  and express AREG. They are shown to upregulate AREG expression in the

presence of IL-33 as well as IL-1 $\beta$  in HIV patients (Bhaskaran et al., 2021). It would be interesting to assess the levels of IL-1 $\beta$  in the plasma of our patients, as well as in vitro assessment with addition of IL-1 $\beta$  in culture, followed by assessment of Foxp3 and Amphiregulin expression, as has been described in this recent study on tonsillar Tregs in HIV patients (Bhaskaran et al., 2021).

The immunosuppressive capacity of these tissue Tregs as well as the repair potential needs further study. Caco-2 cell line could be used as a study model for intestinal epithelial wound repair, using supernatants of activated LPL from two different groups of patients. We identified the dysfunctional tissue Tregs by the lack of production of Amphiregulin. This raises the question whether the repressed secretion is due to elevated sST2 levels in the plasma counteracting the response. In addition, recombinant AREG (rAREG) may be used in experimental mice or in SIV-infected non-human primate (NHP) models to test for the amelioration of leaky gut and inflammation.

### **Innate lymphoid cells**

In addition to Tissue T regulatory cells, there is involvement of other immune cells in regulating the intestinal barrier and maintaining homeostasis, which are yet to be explored in the gut of HIV infection in the context of tissue repair. Innate lymphocyte cells (ILCs), a novel family of innate immune cells are considered to function as key orchestrators of immune defenses at mucosal surfaces and are crucial for maintaining an intact intestinal barrier.

While lacking antigen-specific receptors, ILCs detect changes in the microenvironment through receptors for cytokines that are released upon tissue damage, as well as a broad range of receptors for nutrient components, microbial products, lipid mediators, and neuronal transmitters. Found in both lymphoid and non-lymphoid tissues, ILCs are primarily tissue resident cells and are particularly abundant at the mucosal surfaces of the intestine and lung, whereas they are extremely rare in peripheral blood.

Depletion of ILCs is known to be involved in human immunodeficiency virus (HIV)-associated damage of the intestinal mucosa and subsequent microbial translocation. Based on the signature cytokines they produce, their phenotype, and their developmental pathways, ILCs are divided into three major groups: ILC1s, ILC2s, and ILC3s (Panda and Colonna, 2019).

Type 1 ILCs, produce IFN $\gamma$  and are implicated in tumor and viral immunity, as well as the defense of intracellular pathogens. ILC3s produce IL-22 and IL-17 in response to IL-23 and IL-1 $\beta$ , they are considered the innate counterparts of Th17.

ILC2s, the innate counterparts of Th2, secrete type-2 cytokines such as IL-5, IL-9, IL-13, and amphiregulin in response to TSLP, IL-25, and IL-33. IL-13 plays crucial roles in the expulsion of helminths. Moreover, IL-13 and amphiregulin help repair the tissue damage engendered by helminth and viral infections (Monticelli et al., 2011). In humans, ILC2s express CRTH2, KLRG1, ST2, and CD25. ILC2 development depends on the transcription factors GATA3, ROR $\alpha$ , and TCF-1 in mice. GATA3 is required for activation of human ILC2s. Although ILC2s are considered homogenous, they are classified into two groups based on their responsiveness to IL-33 and IL-25. IL-33 responsive ILC2s present in the steady state are called natural ILC2s (nILC2), whereas IL-25 responsive ILC2s elicited upon exposure to IL-25 or helminth infection are referred to as inflammatory ILC2s (iILC2). nILC2s are demarcated by elevated expression of Thy1 and ST2 along with relatively little KLRG1; in comparison, iILC2s express more KLRG1, less Thy1 and almost no ST2 on the cell surface (Panda and Colonna, 2019).

ILC2s facilitate IL-33-mediated tissue protection in DSS colitis, while Tregs seem to play an ILC2-supporting role. This could be due to the fact that ILC2s constitutively express the receptor ST2<sup>+</sup> and are therefore able to act immediately upon IL-33 treatment, whereas only a subpopulation of Tregs display ST2 expression. There is lack of information on the repair role of ST2 expressing ILC2s in the intestinal mucosa of HIV infection, which needs to be explored.

In the healthy gut, ILC3 are thought to be one of the major cell populations contributing to overall homeostasis. This is, in part, because ILC3 produce large quantities of IL-22 and IL-17, and directly interact with intestinal epithelial cells to maintain an intact barrier and modulate inflammation. IL-22 protects intestinal epithelium from inflammation and promotes wound healing by inducing STAT-3 dependent increases in production of antimicrobials by epithelial cells and epithelial cell proliferation, thus maintaining barrier integrity (Shah et al., 2017).

Frequency of colonic ILC3 was inversely correlated with serum levels of I-FABP and sCD14, surrogate markers for loss of gut barrier integrity and microbial translocation, respectively (Krämer et al., 2017). During the acute phase of lentivirus infection, early innate responders (DCs and other cells) secrete cytokines leading to apoptosis of ILC3. Subsequently, reduced IL-17 and IL-22 production leads to damage of gut epithelial barrier and an influx of microbial products, causing further inflammation (Shah et al., 2017).

Our second study paves the way for enhancing the vaccine response to HIV infection, by considering the use of IL-33 as an immunoadjuvant. To further our in-vitro study, the role of IL-33 as immunoadjuvant needs to be studied in HIV vaccine in animal models to demonstrate the antigen specific CD8 T cell response. We would be carrying this out in our VRI team on infected mice models.

IL-33 has emerged as a switch-hitting cytokine adjuvant: a multi-talented immune player that promotes both regulatory, TH2 and TH1 immune responses given the right type of immune cells activated, the tissue involved, the microenvironment, and the cytokine milieu. IL-33 may play different roles in different stages of HIV disease and its role as an immune therapeutic gene warrants further investigation.

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