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**CD4+ FOXP3+ Regulatory T cells Homeostasis :
Role of interleukin-7 and implication in HIV infection
pathophysiology**

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ABSTRACT

Regulatory T cells (Treg) represent a crucial CD4 T cells subset involved in maintenance of immune-tolerance. Since their first description important efforts have been undertaken to better understand their biology, their development and their mechanisms of action. However, little is known about factors controlling Treg peripheral homeostasis.

The aim of this thesis work was to better define mechanisms involved in governing Treg homeostasis and to investigate the eventual contribution of perturbation of Treg homeostasis in human disease.

In the first part of this thesis work we tried to define in the murine system the role played by IL-7 in governing Treg homeostasis. We showed that Treg surface expression of CD127, the IL-7 receptor alpha chain, is finely regulated as it depends on their activation as well as on their tissue localization. More importantly, we demonstrated that Treg do express functional levels of CD127, identifying these cells as potential target of IL-7. Using both genetically modified murine models of altered IL-7 signaling and adoptive transfer models, we obtained definitive evidence for a direct role of IL-7 in governing Treg cell numbers. Finally, we demonstrated that IL-7 signaling in Treg optimizes their capacity to react to IL-2 an important cytokine regulating Treg homeostasis.

In the second part of this work we investigated Treg homeostasis in the context of HIV infection. Employing for the first time in HIV infection a novel consensus Treg identification strategy and applying it to different groups of HIV infected patients, including primary infected patients and HIV controllers, we showed that HIV infection is characterized by an early and long lasting alteration of Treg homeostasis. In particular we demonstrated that effector rather than naive Treg are affected by HIV infection. Moreover, we showed that effector Treg numbers inversely correlated with HIV specific CD8 T cells responses, providing *ex vivo* evidence of Treg involvement in HIV immunity.

RESUME

Les cellules T régulatrices Foxp3⁺ (Treg) représentent une sous-population T CD4 cruciale pour le maintien de l'immuno-tolérance. Mieux comprendre la biologie des Treg, leur hétérogénéité, leur développement, leur mécanisme d'action et les facteurs assurant leur survie en périphérie reste un objectif majeur. L'objectif de ce travail de thèse était de mieux définir les mécanismes impliqués dans le contrôle de l'homéostasie Treg et d'évaluer l'éventuelle contribution des perturbations de l'homéostasie Treg en pathologie humaine.

Dans la première partie de ce travail de thèse nous avons essayé de finalement définir dans le modèle murin le rôle joué par l'IL-7 dans le contrôle de l'homéostasie Treg. Nous avons montré que l'expression de CD127, la chaîne alpha du récepteur à l'IL-7, est finement régulée à la surface des Treg et qu'elle dépend de leur activation ainsi que de leur localisation tissulaire. Nous avons démontré que l'expression de CD127 par les Treg activées est fonctionnelle, identifiant ces cellules comme cibles potentiels de l'IL-7. En utilisant des modèles murins présentant une altération de la voie de signalisation IL-7/IL-7R et des modèles de transfert adoptif, nous avons obtenu une démonstration définitive du rôle direct de l'IL-7 dans la régulation du nombre de cellules Treg. Enfin, nous avons démontré que la signalisation IL-7 optimise la capacité de ces cellules de réagir à l'IL-2, une cytokine importante dans la régulation de l'homéostasie Treg.

Dans la deuxième partie de ce travail, nous avons étudié l'homéostasie Treg dans le contexte de l'infection par le VIH. Cette étude a bénéficié de l'accès à des patients au cours de la primo infection et des HIV contrôleurs. Nous avons montré que les Treg effecteurs plus que les Treg naïves sont affectés par l'infection par le VIH. De plus, nous avons montré que le nombre des effecteurs Treg corrélant inversement avec les réponses T CD8 spécifiques, offrant une preuve ex vivo de l'implication des Treg dans l'immunité anti-VIH.

1. INTRODUCTION

1.1 REGULATORY T CELLS

The immune system has evolved to protect the host from a wide range of potentially harmful elements, mainly pathogenic microorganisms. However parallel mechanisms to control excessive immune responses and prevent reactivity to components of the organism or to not harmful antigens are required to prevent host damage. The set of mechanisms involved in such a process takes the name of immune-tolerance. These mechanisms include thymic deletion of autoreactive T cells, T cell anergy and regulation of exuberant responses by specialized populations of cells, a process known as “dominant tolerance”.

Regulatory T cells (Treg) are a critical CD4 T cell subset involved in the control of immune-tolerance by regulating immune homeostasis and preventing autoimmune diseases. Treg typically represent 10-15% of peripheral CD4 T cells in mice and 2-10% of human peripheral blood CD4 T cells.

The existence of a T cell sub-population with suppressive activity has been suggested since four decades (Gershon and Kondo, 1971); Nishizuka and Sakakura showed in 1969 that neonatal thymectomy (NTx) of normal mice between day 2 and 4 after birth resulted in the destruction of ovaries, which later turned out to be of autoimmune nature (Nishizuka and Sakakura, 1969). Depending on the mouse strain used, NTx leads to the development of thyroiditis, gastritis, orchitis, prostatitis, and sialadenitis (Kojima and Prehn, 1981). Importantly, inoculation of spleen cells or thymocytes from normal syngeneic animals inhibited NTx-induced murine autoimmune disease (Sakaguchi et al., 1982).

1.1.1 Tregs identification

Early markers – CD5 is a T cell coreceptor expressed at cell surface which is strongly upregulated upon cell activation. Sakaguchi showed in 1985 that, when splenic CD4⁺ T cell suspensions from normal BALB/c mice were depleted of CD5^{high}CD4⁺ T cells *ex vivo* and the remaining CD5^{low}CD4⁺ T cells were transferred to congenitally T cell-deficient BALB/c athymic nude mice, mice developed autoimmune disease in multiple organs (stomach, thyroid, ovaries, or testes) in a few months after cell transfer (Sakaguchi et al., 1985). Co-transfer of normal untreated CD4⁺ T cells with CD5^{low}CD4⁺ T cells inhibited autoimmunity. In 1990, Powrie and Mason took advantages of CD45 isoforms discrimination among T cells. CD45^{RC} and CD45^{RB} represent isoforms of the protein tyrosine phosphatase receptor type C (PTPRC or CD45). They showed that transferred CD45^{RChigh}CD4⁺ T cells elicited graft-versus-host disease-like systemic disease and autoimmune tissue damage in multiple organs including thyroid and Langerhans' islets in a model of splenic T cell reconstitution of athymic nude rats (Powrie and Mason 1990). Powrie et al. and Morrissey et al. then independently showed that transfer of BALB/c CD4⁺ T cells with high levels of CD45^{RB}, another CD45 isoform, to T/B cell-deficient BALB/c SCID mice induced inflammatory bowel disease (IBD) (Powrie et al., 1993; Morrissey et al., 1993).

CD25 - It was not until the mid 90s that a specific CD4 T cell subset, constitutively expressing the IL-2 receptor alpha-chain (CD25) molecule, was identified as exerting regulatory activity in mice (Sakaguchi et al., 1995). In their seminal article, Shimon Sakaguchi and coworkers identified the CD25 molecule as a candidate because CD25⁺ T cells were confined in the CD5^{high} and CD45^{RBlow} fraction of CD4⁺ T cells. More importantly they showed that the inoculation of CD25-depleted CD4⁺ cell suspensions from BALB/c mice into lymphopenic mice induced autoimmune disease. Moreover, reconstitution with CD4⁺CD25⁺ cells within a limited period after transfer of CD4⁺CD25⁻ cells prevented the development of the disease.

Subsequently two studies investigated functional characteristics of CD4+CD25+ Treg *in vitro* (Thornton et al., 1998; Takahashi et al., 1998).

Although their constitutive expression of CD25 raised the possibility that the CD4+CD25+ population might be hyper-responsive to stimulation with IL-2 and/or via the TCR, Treg exhibited virtually no proliferative response to *in vitro* stimulation with high concentrations of anti-CD3 and Con A. The two studies disagree on the effect of anti-CD28 costimulation on Treg hypo-responsiveness. However it is clear that addition of IL-2 to anti-CD3 stimulation is able to restore responsiveness of Treg to soluble anti-CD3 to levels similar to those observed with the CD4+CD25- population.

Importantly the two studies showed that addition of CD4+CD25+ cells prevented the proliferation of anti-CD3 stimulated CD4+CD25- cells. This suppressive capacity was blocked by addition of anti-CD28 or IL-2 to the culture.

A CD4+ T cell subpopulation expressing high levels of CD25 has also been detected in humans. The use of *in vitro* suppressive assay allowed the demonstration of their capacity to inhibit both proliferation and cytokine production of effector CD4 T cells. (Baecher-Allan et al., 2001; Levings et al., 2001; Ng et al., 2001; Jonuleit et al., 2001; Dieckmann et al., 2001).

FOXP3 - The forkhead box P3 (FOXP3) transcription factor was first identified investigating the genetic basis for the Immunodysregulation Polyendocrinopathy Enteropathy X-linked syndrome (IPEX syndrome), an x-linked recessive disorder characterized by the neonatal onset of insulin-dependent diabetes mellitus (IDDM), infections, enteropathy, thrombocytopenia and anemia, other endocrinopathy, eczema and cachexia (Chatila et al, 2000; Bennett et al, 2001; Wildin et al, 2001;). A similar

autoimmune disease was observed in mice with the scurfy mutation in the FOXP3 gene (Brunkow et al., 2001). CD4 T cells played a major role in murine scurfy phenotype as i) CD4 but not CD8 T cell depletion in scurfy could ameliorate the disease ii) the disease could be transplanted to T cell-deficient hosts upon transfer of CD4+ T cells and iii) CD4 T cells from scurfy mice presented an activated phenotype (Blair et al., 1994). Based on these observations four groups independently showed that mutations in FOXP3 gene caused the disease as a result of the loss of Treg and consequent lymphocyte activation and lymphoproliferation (Chatila et al, 2000; Bennett et al, 2001; Wildin et al, 2001; Brunkow et al., 2001).

FOXP3 is now widely accepted as playing a crucial role in Treg development and function (Zheng & Rudensky, 2007; Campbell & Ziegler, 2007). Retroviral gene transfer of Foxp3 (Hori et al., 2003 ; Fontenot et al., 2003) or transgenic FOXP3 expression (Khattry et al., 2003) in CD4+ CD25- T cells convert them toward a regulatory T cell phenotype and function. Moreover it has been shown that Foxp3, by repressing NFAT and NF- κ B activity, inhibits proliferation and effector functions of peripheral T cells (Bettelli et al., 2005).

In vivo depletion of FOXP3 expressing cells using mice expressing a diphtheria toxin (DT) receptor-green fluorescent protein fusion protein under the control of the foxp3 gene locus, allowing selective and efficient depletion of Foxp3+ T reg cells by DT injection, rapidly resulted in the development of scurfy-like symptoms with splenomegaly, lymphadenopathy, insulinitis, and severe skin inflammation (Lahl et al., 2007 ; Kim et al., 2007).

FOXP3 is to date the most specific marker for Treg identification in mice and to a lesser extent in humans. In mice, there is a good correlation between FoxP3 and CD25 although a minor proportion (10-20%) of FoxP3+ cells in secondary lymphoid organs is CD25 low/negative. However, in humans the situation is more complex, as

the expression of FoxP3 is also observed in activated CD4 + cells (Allan et al., 2006) and therefore does not allow a precise definition of the Treg population. In addition FOXP3 staining requires fixation and permeabilisation of the cells rendering this approach not convenient for cell isolation for functional studies.

CD127 - Cozzo et al. (Cozzo et al., 2003) first reported low CD127 expression at murine Treg surface. Subsequently it has been confirmed in humans that CD4+FOXP3+CD25^{high} cells express lower levels of CD127 than the FOXP3-counterpart (Cox et al., 2005; Seddiki et al. 2006; Liu et al. 2006). Moreover the authors showed that FOXP3 expression negatively correlates with CD127 expression. The combination of the CD25 and CD127 surface markers has thereafter been widely employed to isolate viable FOXP3 enriched CD4+CD25⁺ T cells (Hartigan-O'Connor et al., 2007). However such an approach presents two major drawbacks. First, even in early reports, was evident that using this method a minor but detectable population of CD4 T cells expressing both CD25 and FOXP3 was excluded. Very recently, Becher-Allan et al., investigated and better defined the functional relevance of such population (Becher-Allan et al., 2011; See discussion section 4.1). Second, as discussed in section 1.1.2.3, non-Treg CD4 T cells downregulate CD127 expression during activation while they upregulate CD25. It is therefore not surprising that CD127 and CD25 expression cannot accurately discriminate *ex vivo* Treg cells from activated T cells in situations of immune-activation (Aerts et al., 2008; del Pozo-Balado et al., 2010).

Other Treg markers - Studies aiming to define a specific marker for Treg identification are still in progress. Numerous other molecules have been considered. Unfortunately, none appears to provide Treg specific labeling. A list of other markers that have been reported to identify or characterize Treg cells is resumed in Table 1.1.

Molecule	Species	Cellular localization	Reference
CTLA-4	Human/mouse	Extra/intracellular	Takahashi et al., 2000
OX40	mouse	extracellular	McHugh et al., 2002
GITR	mouse	extracellular	McHugh et al., 2002
LAG-3	mouse	extracellular	Huang et al., 2004
Neuropilin-1	mouse	extracellular	Bruder et al., 2004
CD39	Human/mouse	extracellular	Deaglio et al., 2007
CD73	Human/mouse	extracellular	Deaglio et al., 2007

Table 1.1 - Other Treg markers.

1.1.2 Treg heterogeneity

Similarly to other T cells compartments, Treg population presents a high degree of heterogeneity both in humans and mice. Several surface markers allow the identification of distinct Treg subsets, some of which present peculiar functional characteristics.

CD62L – CD62L or L-Selectin is expressed on naive CD4⁺ T cells and is involved in cellular recirculation and compartmentalization between blood and lymph node. In mice, CD62L is expressed on about half of FOXP3⁺ Treg cells isolated from secondary lymphoid organs (Kuniyasu et al., 2000; Thornton et al., 2000). No difference in suppressive capacity between CD62L⁺ and CD62L⁻ Treg has been detected in *in vitro* suppressive assays (Kuniyasu et al., 2000; Thornton et al., 2000). However, depending on the model employed, some difference have been demonstrated *in vivo*. CD62L⁺ Treg were superior to their CD62L⁻ counterparts in preventing the development of diabetes (Szanya et al., 2002) or of graft-versus-host disease (GVHD) (Taylor et al., 2004; Ermann et al., 2005). In contrast, CD62L⁺ and CD62L⁻ Treg cell subsets were equally protective in an adoptive transfer model of colitis (Fu et al., 2004). Given CD62L role in Treg migration (Venturi et al., 2007), disparities between results obtained using different models are most likely due to

differences in tissue localization. In human studies CD62L has been mainly employed, in conjunction with CD25 and/or CD127 expression, to differentiate Treg from activated T cells which down-regulate CD62L expression.

CD45RA/RO- Several studies have shown that CD45RA is an extremely useful marker for Treg identification when combined to CD25 (Valmori et al., 2005; Seddiki et al., 2006; Fritzsching et al., 2006) or FOXP3 (Miyara et al., 2009). CD45RA expression allows the repartition of the FOXP3+ CD4 T cell population in three subsets : i) “naive” FOXP3^{low}CD25^{low}CD45RA⁺ Treg ii) “effector” FOXP3^{high}CD25^{high}CD45RA⁻ Treg and iii) a FOXP3^{low}CD25^{low}CD45RA⁻ population which contains non-Treg activated cells (Miyara et al., 2009). Such an analysis presents two major advantages : first, it allows the exclusion from Treg analysis of the FOXP3+ non-Treg contaminating cells, which are not excluded even when CD127 is employed; second, it allows the identification of the naive CD45RA⁺FOXP3^{low}CD25^{low} Treg subset, which can be otherwise not taken into account. Indeed studies of human Treg based on the CD25^{high} gating strategy initially proposed by Bacher-Allen (Bacher-Allen 2001) inadvertently restricted the analysis to the effector Treg compartment.

HLA-DR - MHC-II expression identifies a population that represents about 20-30% of human circulating Treg cells (Baecher-Allan et al., 2001; Baecher-Allan et al., 2006). *In vitro* activation of HLA-DR⁻ Tregs converts them into HLA-DR⁺ Tregs. *Ex vivo* isolated HLA-DR⁺ Treg cells suppress responder T cell proliferation and cytokine secretion more efficiently and more rapidly than HLA-DR⁻ Treg cell. All HLA-DR⁺ Treg cells are part of the effector FOXP3^{high}CD45RA⁻ compartment (Miyara et al., 2009). HLA-DR⁺ Treg cells isolated *ex vivo* seem to constitute a terminally differentiated subset in the effector Treg cell pool (reviewed in Sakaguchi et al., 2010).

Inducible costimulatory molecule (ICOS) – ICOS is a costimulatory molecule involved in cell activation that is expressed on effector/memory T-cell subsets. In mice ICOS represent an activation marker at the Treg surface. Its expression identify *ex vivo* a sizable population which represent about 10-20% of CD4+FOXP3+ T cells isolated from secondary lymphoid organs. Whether ICOS expression exerts any function in Treg activity is still unknown. In humans differential expression of ICOS has been shown to delineate two different subsets of Treg cells in the peripheral blood (Ito et al., 2008). Interestingly these two phenotypically distinct subsets present differences in their suppressive capacity and in the mechanisms of action employed (see section 1.3.3).

CD103 – CD103 or αE integrin was initially described as a marker for intraepithelial T cells residing in mucosal compartments such as gut, skin or lung. In mice, CD103 expression identifies a subset of Treg which represents about 20% of the Treg population isolated from secondary lymphoid organs (Lehmann et al., 2002). This subset presents the phenotypic characteristics of effector/memory cells (Huehn et al., 2004). From a functional point of view CD103 expressing regulatory T cells present a higher suppressive activity both *in vitro* (Lehmann et al., 2002) and *in vivo* (Lehmann et al., 2002; Huehn et al., 2004; Zhao et al., 2008). In humans, CD103 expressing CD25^{high} Foxp3⁺ Treg cells represent less of 5% of the whole population (Iellem et al., 2003; Lim et al., 2006; Allakhverdi et al., 2006). A detectable population of human CD4 T cells expressing CD103 and presenting regulatory properties *ex vivo* has been reported only in tonsils (Allakhverdi et al., 2006).

Ki67 – Intracellular Ki67 staining identifies an actively proliferating fraction of Treg cells. In both mice and humans the percentage of Ki67 cells among FOXP3⁺ cells is higher than the percentage among conventional FOXP3⁻ CD4 T cells (Vukmanovic-Stejić et al., 2008). This is in accordance with their more activated profile. Notably all cycling Ki67⁺ Treg cells are part of the effector FOXP3^{high}CD45RA-

compartment (Miyara et al., 2009).

1.1.3 Mechanisms of Treg-cell function

Although evidence points to Treg as essential players in maintenance of immune-tolerance and immune-homeostasis, the exact mechanisms through which these cells exert their function at the molecular level still remain obscure. Determine the processes involved in Treg function is of paramount importance as their disruption can be the basis for immune-pathological diseases and they can represent potential targets for immune-interventions. The picture provided by the published literature indicates that a wide range of mechanisms are probably involved in Treg suppression.

1.1.3.1 Secretion of inhibitory molecules

Tregs have been reported to suppress target cells by the secretion of inhibitory molecules such as IL-10, TGF- β and IL-35.

IL-10 – IL-10 is a cytokine with anti-inflammatory properties which has a central role in immune-tolerance by limiting the immune response and thereby preventing damage to the host (Saraiva and O'Garra, 2010). The role of IL-10 in Treg mediated suppression has long been a matter of debate, mainly as the result of discrepancies between *in vitro* and *in vivo* results. *In vitro* reports failed to demonstrate any inhibition of Treg suppressive activity by the addition of IL-10 neutralizing antibodies (Takahashi et al., 1998 ; Thornton et al., 1998) leading to the conclusion that IL-10 was not necessary for Treg function. However, murine *in vivo* studies subsequently showed that IL-10 played indeed a role in Treg mediated suppression: adoptive transfer of IL-10 deficient CD4+CD25+ cells failed to prevent inflammation in a model of adoptive transfer induced colitis (Asseman et al., 1999; Annacker et al., 2001). More recently, Rudensky and coworkers generated mice in which the Treg cell-specific ablation of a conditional IL-10 allele was induced by Cre recombinase knocked into the *Foxp3* gene locus (Rubtson et al., 2008). These mice developed

spontaneous colitis and were more prone to allergic inflammation of the lung and the skin, thus formally demonstrating the implication of IL-10 in Treg mediated control of immune responses *in vivo*. In humans, Ito et al. showed that IL-10 participates to suppression mediated by a subset of Treg expressing ICOS but not by the ICOS- Treg (Ito et al., 2008).

TGF- β - Transforming growth factor- β (TGF- β) is a regulatory cytokine that has pleiotropic functions in numerous physiological and pathological processes such as embryogenesis, carcinogenesis, and the immune responses (Li and Flavell 2008). Conflicting results have also been reported regarding the role of TGF- β in Treg-mediated suppression *in vitro*. Some investigators reported that high levels of TGF- β 1 are produced by activated CD4+CD25+ cells and that TGF- β blockade is sufficient to abrogate CD4+CD25+ T reg cell-mediated suppression (Nakamura et al., 2001). In contrast, others have failed to find any requirement for Treg cell-derived TGF- β 1 as neither TGF- β neutralization by neutralizing antibodies nor use of Treg cells deficient in TGF- β abrogated the suppression (Piccirillo et al., 2002).

However, several *in vivo* murine studies indicate that TGF- β is essential for suppression of colitis in models of inflammatory bowel disease by Treg (Powrie et al., 1996) although even in this case conflicting results have been reported : CD4+CD25+ cells from TGF- β 1-/- and TGF- β 1+/+ mice have been shown to inhibit the induction of colitis equally well (Kullberg et al., 2005). However as TGF β -/- mice present a state of immune activation, a potential bias of all these studies was the isolation of Treg on the basis of CD25 expression. Definitive evidence of the involvement of TGF β for *in vivo* Treg suppression derived from the work of Li et al. (Li et al., 2007). By isolating Treg on the basis of RFP under the FOXP3 promoter, the authors demonstrated that TGF β -/- deficient CD4+FOXP3+ cells failed to suppress naive CD4+ induced colitis upon transfer into empty mice.

In human, the role of TGF β is still debated: some studies have shown that the natural Treg-suppression is independent of TGF- β (Levings et al. 2001; Enarsson, et al.

2007) whereas other studies have shown that TGF β is involved in the suppression (Strauss, et al. 2007). As for IL-10, a potential conciliation could derive from the results from Ito et al, who proposed that TGF β is implicated in suppression mediated by ICOS-Treg while is not involved in ICOS+Treg suppression which, as discussed above, rely more on IL-10 (Ito et al, 2008).

IL-35 - IL-35 is a heterodimeric protein composed of the IL-12 p35 chain and Ebi3 (Niedbala et al., 2007; Collison et al., 2007) highly expressed and secreted by Treg, but not by conventional T cells in mice. Treg cells lacking either p35 or Ebi3 are less suppressive *in vitro* and are unable to control IBD induced by Teff cells (Collison et al., 2007). IL-35 inhibits T-cell proliferation upon *in vitro* stimulation while Treg cultured in the presence of IL-35 proliferate and produce high levels of IL-10 (Niedbala et al., 2007). However, human Treg cells do not express detectable amounts of IL-35 (Bardel, et al. 2008).

1.1.3.2 Suppression of dendritic cells activity

Modulation of dendritic cells function is an indirect way for Treg to suppress effector T cell responses. Tregs can inhibit DC maturation and immuno-stimulatory capacity through several mechanisms.

CD4+CD25+ T cells down-regulate the expression of the co-stimulatory molecules CD80 and CD86 on both murine (Cederbom et al., 2000) and human (Misra et al., 2004) dendritic cells. Such a mechanism is mediated, at least in part, by CTLA-4 (Oderup, et al. 2006; Onishi et al., 2008; Wing et al., 2008), an important molecule for Treg mediated activity (Read et al., 2000; Read et al., 2006). The interaction between CTLA-4 and CD80/86 also mediates the ability of Treg to induce DCs to produce indolamin 2,3-dioxigenase (IDO), a tryptophan-catabolizing enzyme producing kynurenine that has been implicated in immune suppression (Fallarino, et al. 2003).

Lymphocyte activation gene-3 (LAG-3) is a CD4-related trans-membrane protein

expressed by regulatory T cells that binds MHC II on APCs. It has been shown that during Treg:DC interactions, LAG-3 engagement with MHC class II inhibits, through an ITAM-mediated inhibitory signaling pathway, DC activation and immunostimulatory capacity (Liang et al., 2008).

Neuropilin-1 (Nrp-1), which is expressed by murine Treg cells but not naive CD4 T cells (Bruder et al., 2004), plays a key role in promoting long interactions between Treg cells and immature DCs, thereby interfering with conventional T cell stimulation (Sarris et al., 2008). However in humans it has been shown that Treg do not specifically express Nrp-1 and that Nrp-1 expression is induced on peripheral blood T lymphocytes upon activation (Milpied et al., 2009).

1.1.3.3 Cytolysis

T-cells can suppress immune responses by inducing apoptosis of their target cell by two major pathways: through death receptor signaling or through a granule-dependent pathway. Treg have been shown to induce responder T cell death through Fas-Fas ligand interaction (Janssens et al., 2003 ; Strauss et al., 2009) as well as through the TRAIL-DR5 pathway (Ren et al., 2007).

Activated Treg cells express granzyme A, granzyme B and perforin (Grossman et al., 2004; Cao et al., 2007). Furthermore, Treg display perforin-dependent cytotoxicity against several target cells, including activated CD4⁺ and CD8⁺ T cells, CD14⁺ monocytes, and dendritic cells (Grossman et al., 2004). This cytotoxicity participates to *in vivo* Treg suppression of anti-tumor responses (Cao et al., 2007 ; Boissonnas et al., 2010) but not of graft versus host disease (Cai et al., 2010).

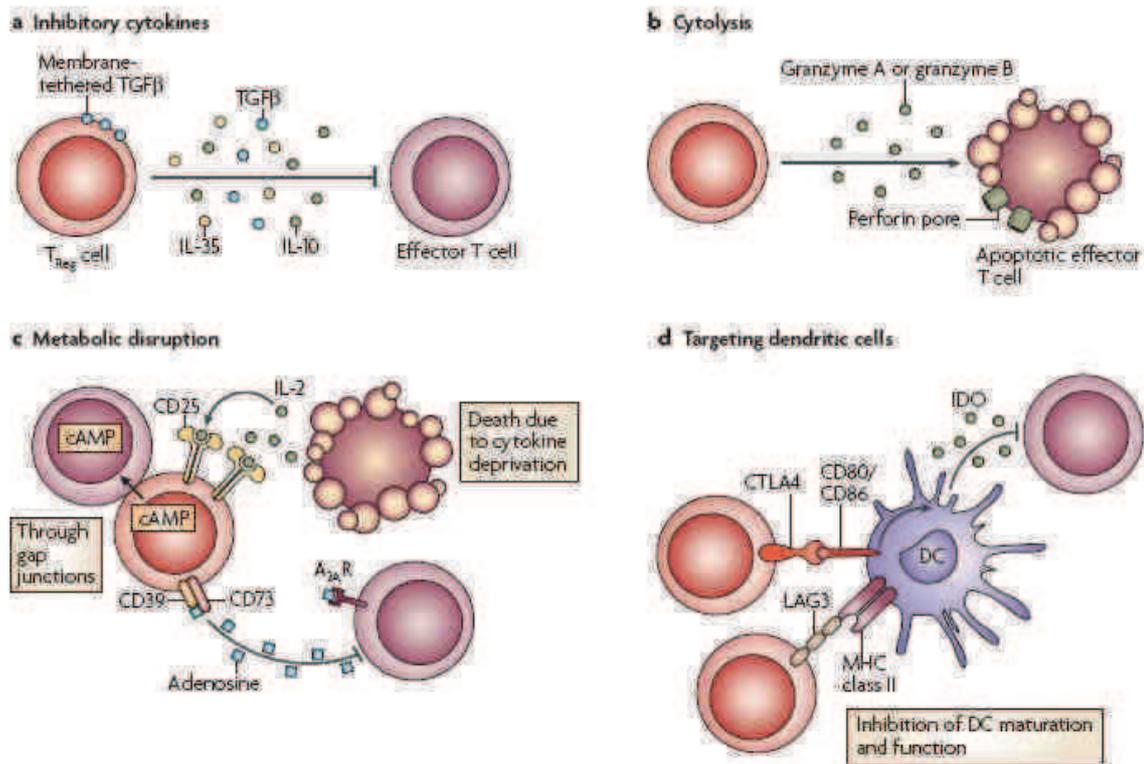
Finally, galectin-1 is a β -galactoside binding protein which is preferentially expressed in Treg cells and is up-regulated upon TCR activation. Galectin-1 induces cycle arrest and apoptosis in responder cells. Blocking of galectin-1 markedly reduced the inhibitory effects of human and mouse Treg cells and Treg cells from galectin-1-deficient mice had reduced Treg cell activity (Garin et al., 2007).

1.1.3.4 Suppression by metabolic disruption

Tregs can affect the metabolism of target cells in different ways. T reg cells inhibit conventional T cells proliferation through transfer of cyclic adenosine monophosphate (cAMP) via cell contact-dependent gap junctions (Bopp et al., 2007). By their coordinated expression of the ectozymes CD39 and CD73, Tregs can generate the inhibitory molecule adenosine which can suppress effector T-cells by binding to the adenosine receptor 2A at their surface (Deaglio et al., 2007).

Treg constitutively express CD25 and are strictly dependent on IL-2 for their *in vitro* proliferation (Papiernik et al., 1998 ; Thorthon et al., 1998 ; Takahashi et al., 1998) although they do not produce IL-2 (Papiernik et al., 1997). Thorthon et al., initially proposed that Treg could suppress conventional T cells by inhibiting IL-2 transcription (Thorthon et al., 1998). After having shown that IL-2 uptake is required for Treg suppression *in vitro*, De la Rosa et al. suggested that Treg could compete for IL-2 secreted by responder T cells (De la Rosa et al., 2004) and showed that suppression is completely abrogated by selective blocking of the IL-2 receptor on Treg during co-culture with responder T cells. Such a model was further supported by the *in vitro* and *in vivo* data from Stockinger and coworkers who further proposed that Treg could exploit IL-2 for the induction of IL-10 production (Barthlott et al., 2005). Finally, through IL-2 deprivation, Tregs have been shown to induce apoptosis in responder CD4+ T cells (Pandiyani et al., 2007).

However, some doubts on the « IL-2 competition model » of Treg mediated suppression derive from the fact that CD25^{-/-} FOXP3⁺ Treg cells have been shown to efficiently suppress conventional T cells proliferation (Fontenot et al., 2005), indicating that such mechanism if existing is dispensable for Treg function. Moreover, this model has failed to be reproduced in the human system (Oberle et al., 2007).



Scheme issued from Vignali, Nature review Immunology, 2009

Figure 1.1 Mechanisms of Treg suppression. Regulatory T (Treg)-cell mechanisms can be grouped in four basic modes of action. a) Inhibitory cytokines include interleukin-10 (IL-10), IL-35 and TGFβ. b) Cytolysis includes granzyme-A- and granzyme-B-dependent and perforin-dependent killing mechanisms. c) Metabolic disruption includes IL-2 deprivation-mediated apoptosis, cyclic AMP (cAMP)-mediated inhibition, and CD39- and/or CD73-generated immunosuppression. d) Targeting dendritic cells (DCs) includes mechanisms that modulate DC maturation and/or function or induction of indoleamine 2,3-dioxygenase (IDO), which is an immunosuppressive molecule made by DCs.

1.1.4 Treg homeostasis

Despite the importance of the Treg compartment for the maintenance of immune tolerance, factors controlling peripheral Treg homeostasis still remain ill defined.

The homeostatic mechanisms controlling Treg numbers appear to be distinct from those regulating the conventional CD4 T cell pool. Indeed, Treg seem to rely on some factors specific for this subset, such as TGF β , IL-2 and B7 costimulatory molecules. Treg dependency upon factors classically involved in naïve or memory CD4 T cells homeostasis (such as IL-7, IL-15 and TCR) still remains controversial.

1.1.4.1 TGF β

TGF- β signaling plays an essential function in the maintenance of immune tolerance. Mice deficient for TGF β 1 present a severe autoimmune multi-organ disease (Shull et al., 1992). Interestingly, the autoimmune phenotype developed in TGF β 1 $^{-/-}$ mice resembles that of Foxp3 $^{-/-}$ mice, which possess a specific defect of Treg cells (Fontenot et al., 2003). Therefore the interrelation between TGF β signaling and Treg biology has been the object of intensive investigation.

In addition to its extensively studied role for Treg suppressive function (See section 1.3.3.1) and peripheral differentiation of adaptive Treg (Shevach et al., 2008), TGF β is also required for peripheral maintenance of Treg cells. The first evidence for a role of TGF β in Treg homeostasis *in vivo* came from the observation that adoptively transferred Treg cells expressing a dominant-negative mutant of TGF β RII into WT hosts failed to proliferate in a chemically induced model of colitis (Huber et al., 2004). Similar effects on CD4 $^{+}$ CD25 $^{+}$ proliferation were obtained in a NOD model of diabetes; employing a transgenic mouse model in which expression of TGF- β in the pancreatic cells could be regulated using a tetracycline on/off system, Peng et al. showed that a short pulse of TGF β promoted the proliferation of islets infiltrating CD4 $^{+}$ CD25 $^{+}$ T cells and was sufficient to inhibit the development of type I diabetes (Peng et al., 2004). A subsequent study of CD4 $^{+}$ CD25 $^{+}$ T cell numbers in young (8-

10 day old) WT and TGF β 1^{-/-} mice revealed that spleens from TGF β 1^{-/-} mice presented reduced CD4⁺TCR⁺CD25⁺ cell numbers when compared to WT mice (Marie et al., 2005). Associated to the presence of normal Treg cell numbers in the thymus, these results suggested that TGF β plays a role in peripheral Treg homeostasis. Furthermore, the authors demonstrated that TGF β signaling in the periphery was necessary for Treg to maintain FOXP3 expression and optimal suppressive functions. Study of mice harboring a T cell-specific deletion of TGF β RII confirmed the reduced peripheral but not thymic numbers of Foxp3⁺ T-reg cells in the absence of TGF β signaling (Marie et al., 2006 ; Li et al., 2006). Moreover, using both adoptive transfer and bone marrow chimera models Li and coworkers demonstrated that TGF β responsiveness conferred competitive advantages to Treg over TGF β RII-deleted Treg (Li et al., 2006). Finally, in that same study was also shown that TGF β RII-deficient Treg were proliferating faster in the periphery than the wild-type counterparts, suggesting that TGF β signaling promotes peripheral Treg survival independently of its proliferation potential.

1.1.4.2 Gamma-chain cytokines

IL-2 – IL-2 is by far the most studied and best established factor governing Treg homeostasis. Although IL-2 was considered the prototypical T cell growing factor for conventional T cells, animals lacking IL-2 or a component of its receptor paradoxically present expanded T cells with activated memory phenotype. These animals also suffer of a lymphoproliferative syndrome characterized by severe autoimmunity (Schorle et al., 1991 ; Sadlack et al., 1993 ; Willerford et al., 1995 ; Suzuki et al., 1995) indicating an indispensable role for the IL-2/IL-2 receptor signaling in maintenance of immune tolerance. Using a bone marrow chimera and an adoptive transfer experimental system, Suzuki and coworkers demonstrated that normal T cells could regulate the abnormal activation of IL-2R β 2^{-/-} T cells (Suzuki et al., 1999) suggesting that IL-2R β is essential for the development of a regulatory TCR- α/β T cell subset

that effectively controls T cell activation. Those results were subsequently confirmed by two other groups which finally established that the critical defect in mice with impaired IL-2 signaling resulted from the absence of functional Tregs (Malek et al., 2002; Almeida et al., 2002). Moreover, these works further documented that adoptive transfer of limited numbers of CD4⁺CD25⁺ T cells prevented the expansion of IL-2 receptor deficient CD4⁺ cells and the associated lethal autoimmunity. Although these studies defined a critical role for IL-2 signaling in Treg biology, whether IL-2 affected Treg development, homeostasis, or function still remained unclear.

By analyzing mice containing the GFP-knock-in allele that were genetically deficient for either IL-2 or CD25, Fontenot et al. showed that IL-2 signaling is dispensable for the induction of Foxp3 expression in thymocytes (Fontenot et al., 2005). Similar conclusions were drawn by another group using a TCR-transgenic model (D'Cruz et al., 2005). However a two-fold reduction in thymic CD4⁺FOXP3⁺ cell absolute numbers is present in the absence of IL-2 signaling (Fontenot et al., 2005 ; Soper et al., 2007 ; Burchill et al., 2007 ; Bayer et al., 2007) indicating that, although not strictly required, IL-2 signaling participates in Treg development under physiological conditions.

The formal demonstration of a role of IL-2 on Treg homeostasis independently from its effects on thymic development came from experiment based on IL-2 neutralization. Injections of anti-IL-2 to neonatal and adult mice induced selective reduction in Treg cell numbers (Bayer et al., 2005 ; Setoguchi et al., 2005), leading to immune-pathology in disease-prone mice. Such an effect was still present when experiments were performed on thymectomized animals thus restricting the study to the peripheral effect (Setoguchi et al., 2005). At the steady state an important fraction of Treg extensively proliferates, probably as a result of chronic self antigen exposure (Fisson et al., 2003). IL-2 neutralization inhibits natural *in vivo* proliferation of peripheral CD25 cells in normal mice, though it is not effective in reducing Treg proliferation in lymphopenic hosts (Setoguchi et al., 2005). This interesting result, while in-

dicating that IL-2 is involved Treg turnover, indirectly suggests that other factors are implicated in lymphopenia-driven proliferation of Treg cells.

In the past decades IL-2 has been widely investigated as a treatment to ameliorate immune-reconstitution and/or to sustain immune-responses, mainly in the field of HIV infection and cancer. A preferential expansion of a CD4 T cell population characterized by the expression of high levels of CD25 was first identified in HIV patients treated with IL-2 in addition to HAART (Sereti et al., 2002). Subsequently, in a cohort of individuals with pediatric sarcoma enrolled in an investigational trial incorporating immune reconstitution and a tumor vaccine after cyclophosphamide-based chemotherapy-induced lymphopenia, Mackall and coworkers showed that IL-2 therapy preferentially increased the size of the suppressive CD4+CD25^{high} Treg pool (Zhang et al., 2005). Extending these results to the mouse model, the authors demonstrated that IL-2 induces the peripheral expansion of CD4+CD25⁺ Treg cells transferred into normal syngenic hosts. Remarkably, such an effect was increased when Treg were transferred into lymphopenic hosts, suggesting that lymphopenia and IL-2 are able to synergistically govern Treg homeostasis. Many studies have thereafter confirmed the beneficial role for IL-2 therapy in human Treg homeostasis (Ahmadzadeh et al., 2006 ; Weiss et al., 2010).

Other insights about the role of IL-2 in human Treg homeostasis can derive from studies addressing the effects of two non-depleting anti-CD25 antibodies Basiliximab and Daclizumab on Treg cells. Both antibodies are employed for prevention of graft rejection in transplanted patients. Moreover, Daclizumab is currently under investigation for treatment of relapsing remitting multiple sclerosis (Wynn et al., 2010). In most studies, Basiliximab administration did not induce any reduction in FOXP3+ CD4+ T cells percentages and absolute numbers (Bloom et al., 2008 ; Wang et al., 2009 ; Vondranet al., 2009 ; De Goer de Herve et al., 2010), although one study detected a transient reduction of these cells early during the treatment (Bluestone et al., 2008). Studies agrees in detecting a reduction of CD25 expression on FOXP3+ CD4+

T cells after treatment with Basiliximab although caution should be taken regarding these results as, depending on the anti-CD25 clone employed, reduction in CD25 staining could be linked to competition with the *in vivo* bound Basiliximab (Abadja et al., 2010). Globally, these results have lead some authors to directly conclude that CD25 appears non essential for human peripheral Treg maintenance *in vivo* (De Goer de Herve et al., 2010). Such a conclusion is probably excessive as no proof of the effective blockade by *in vivo* concentration of Basiliximab of IL-2 signaling capacity by Treg has been provided.

Conversely, a well conducted study in patients with relapsing remitting multiple sclerosis treated with Daclizumab allows drawing some important conclusions (Oh et al., 2009). After having confirmed that Daclizumab treatment reduced the IL-2 signal transduction in peripheral blood mononuclear cells as assessed by STAT5 phosphorylation upon *in vitro* IL-2 stimulation, Oh et al. showed that Declizumab treatment *in vivo* was associated with a reduction in the frequency of Foxp3-expressing CD4+ cells. Finally the authors showed that Treg *in vivo* proliferation and *ex vivo* suppression are impaired during anti-CD25 antibody treatment.

IL-7 – Accordingly with their low CD127 expression, Treg cells are believed to be independent from IL-7 for their peripheral biology. Very few studies assessed directly the role of IL-7 in Treg biology.

In mice, Peffault de Latour et al. showed that in IL-7 *-/-* mice a 10-fold reduction was present in both thymic and splenic Treg cells numbers, a difference similar to that present in other T cell compartments (Peffault de Latour et al., 2006). Importantly, Treg from IL-7 *-/-* mice were efficiently suppressing both *in vitro* and *in vivo*. These results led the authors to conclude that IL-7 is dispensable for ontogeny, function, and peripheral homeostasis of regulatory T cells.

Subsequently another study addressed the role of IL-7 on Treg development and homeostasis (Mazzuchelli et al., 2008). Mazzuchelli et al., showed that Treg cells

were strongly reduced in IL-7RKO mice compared to WT mice but also to IL-7^{-/-} mice. IL-7 neutralization by anti-IL-7 injection into WT mice led to a reduction in Treg cell numbers in both the thymus and spleen. Unfortunately the lack in the experiment of a thymectomized group does not allow any conclusion on the central or peripheral effect of IL-7. Finally, upon adoptive transfer of total CD4 T cells into irradiated Rag^{-/-} or Rag^{-/-}IL7^{-/-} mice the authors failed to detect any differences in Treg numbers recovered. Unfortunately, total lymph-node cells were transferred and no information was given about conventional CD4 T cell numbers and proliferation profile, impeding any conclusions on the direct effect of IL-7 on Treg cells in this setting.

In humans, three published reports analyzed the proportion of Treg cells in peripheral blood from patients treated with IL-7 (Rosenberg et al., 2006 ; Sportes et al., 2008 ; Sereti et al., 2009). Either slight reduction or no difference in the percentage of Treg cells among CD4 T cells was detected after IL-7 treatment in those studies. As total CD4 T cell count increased in IL-7 treated patients, we can imagine that, even in human studies, Treg changes in absolute numbers paralleled those detected in conventional CD4 T cells. Analysis of the Ki-67 proliferation marker during IL-7 treatment revealed that, although a strong increase in the proliferating fraction of conventional T cell was induced by IL-7 treatment, only a minor increase in Ki67⁺ cells was detected in the Treg compartment (Sportes et al., 2008 ; Sereti et al., 2009). These results suggest that IL-7 mainly affects Treg cell numbers in this setting by increasing Treg survival. However, whether IL-7 exerts this effect by directly acting on Treg cells or by affecting IL-2 production by conventional T cells is at present unknown.

In conclusion, based on published results is difficult to formally conclude about the role of IL-7 in Treg peripheral homeostasis.

IL-15 – CD122, the beta chain of the IL-2 receptor, participates, together with IL-15Ra and CD132, to the constitution of the IL-15 receptor. Discrepancies between the

phenotype of CD25^{-/-} mice, which still present functional Treg cells, and that of CD122^{-/-} mice, which are completely devoid of Treg cells, could suggest that IL-15 participates in Treg homeostasis. Study of IL15R α ^{-/-} as well of IL-15^{-/-} mice failed to reveal any defect in Treg percentages or absolute cell numbers in the absence of IL-15 signaling (Burchill et al., 2007 ; Soper et al., 2007). Burchill et al. further showed that IL-2^{-/-}IL-15^{-/-} mice present a defect in Treg thymic development which is similar to that observed in CD122^{-/-} mice. Therefore IL-15 does not seem to be involved in Treg homeostasis.

1.1.4.3 Costimulatory molecules

In addition to their constitutive expression of CD25, Treg cells are characterized by their expression at the steady state of several molecules known to be involved in costimulation of T cells, such as CTLA-4, OX40 or 4-1BB. Costimulatory pathways represent therefore perfect candidates as factors regulating Treg homeostasis.

CD28 - The first evidence for a role of B7 family costimulatory molecules in Treg peripheral homeostasis came from the study of NOD mice that were deficient for either CD28 or B7-1 (CD80) and B7-2 (CD86) (Salomon et al., 2000). B7-1/B7-2-deficient mice presented a strong reduction in Treg percentage among CD4 T cells and developed more severe diabetes as compared to control NOD mice. Similar results were obtained analyzing CD28^{-/-} NOD mice. Adoptive transfer of WT CD25⁺CD4⁺ T cells into CD28^{-/-} NOD mice prevented the development of the disease, thus formally linking the pathology to the lack of Treg. In order to distinguish between a Treg developmental defect and lack of peripheral survival, the same authors injected WT NOD mice with either CTLA-Ig (Salomon et al., 2000) or anti-B7 (Tang et al., 2003). Both treatments reduced the proportion of Treg cells and, although B7 blockade resulted in a parallel reduction in thymic Treg cellularity, the effects in the periphery were still present when thymectomized mice were employed. Collectively those res-

ults pointed for a role of CD28 and B7 molecules in both Treg development and peripheral homeostasis. Furthermore, transfer of CD4⁺CD25⁺ cells into anti-B7 treated hosts resulted in a reduction in Treg proliferation and cell recovery formally establishing the participation of B7 molecules in Treg homeostasis (Tang et al., 2003). Interestingly, CD28 also influenced Treg homeostasis indirectly, in an IL-2 dependent manner : on one side, CD28 sustains IL-2 production by activated T cells, thus influencing IL-2 availability for Treg; on the other hand, B7 dependent signals sustain CD25 expression on Treg, thus potentially increasing their capacity to react to IL-2 (Tang et al., 2003).

Cytotoxic T lymphocyte antigen 4 (CTLA-4) - CTLA-4, which as CD28 binds to CD80 and CD86, is an essential inhibitory molecule. CTLA-4-deficient mice develop a lymphoproliferative disorder as a result of uncontrolled polyclonal CD4 T cell expansion, resulting in death within three to four weeks of age. While CTLA-4 only appears at conventional T cells surface after activation, it is constitutively expressed by Treg cells (Takahashi et al., 2000) and its expression is under the control of Foxp3 (Hori et al., 2003). While its role in Treg mediated suppression has been now clearly established (see section 1.1.3.2), CTLA-4 does not seem to be essential for Treg cell homeostasis. Study of CTLA-4 deficient mice revealed a surprisingly enlarged population of Foxp3⁺ Treg resulting from their enhanced proliferation in the periphery (Schmidt et al., 2009 ; Kolar et al., 2009). However, Treg restricted CTLA-4 deficiency does not affect the survival or proliferation of Tregs when CTLA-4-deficient or -intact Tregs alone were transferred to nu/nu mice (Wing et al., 2008). Therefore the observed Treg expansion in CTLA-4 deficiency, either global or Treg restricted, is probably the result of conventional T cell deregulated activation and of the accompanying IL-2 increased production.

Similar conclusions can be drawn by murine and human studies addressing the effect of injection of anti-CTLA4 blocking antibodies. In mice anti-CTLA4 treatment did

not deplete CD4⁺Foxp3⁺ Treg cells, chronic CTLA4 blockade consistently resulted in a significant increase in both percentages and absolute numbers of Treg (Quezada et al., 2006). Similarly, anti-CTLA4 treatment in humans led to an increase not only in the number of activated effector CD4⁺ T cells, but also in the number of CD4⁺FoxP3⁺ Treg (Kavanagh et al., 2008). This expansion was associated with an increase in the fraction of actively proliferation Ki67⁺ Treg cells.

OX40 - OX40 is a costimulatory molecule which at the steady state is preferentially expressed by murine Treg, although also conventional T cells up-regulate its expression upon activation. OX40 stimulation through injection of agonist anti-OX40 has been shown to increase Treg cell numbers and to lead to augmentation in the fraction of proliferating Treg (Ruby et al., 2009). Moreover, OX40 has been shown to be a non-redundant factor shaping Treg sensitivity to IL-2 (Piconese et al., 2010). Accordingly OX40-null Treg presented defective expansion after adoptive transfer into lymphopenic hosts.

1.1.4.4 TCR signaling

TCR interaction with self-peptide/major histocompatibility complex (MHC) is a well established factor influencing homeostasis of conventional CD4 and CD8 T cells (reviewed in Boyman et al., 2009). Transfer of purified CD4⁺ CD25⁺ cells into lymphopenic RAG1-deficient hosts that were deficient or sufficient for MHC class II revealed that Treg requires MHC-II to undergo expansion in lymphopenic environment, similarly to what is reported for naive CD4⁺ CD25⁻ cells (Gavin et al., 2002). Using conditional deletion of MHC-II, Shimoda et al. showed that in the almost complete absence of MHC-II Treg levels were reduced of about two-fold (Shimoda et al., 2006). Kim et al. crossed Ox40-cre mice to mice carrying a conditional null allele of the Lck gene (Kim et al., 2009). As OX40 surface expression at the steady state is almost completely restricted to Treg cells, this model

allowed the authors to examine the importance of TCR signaling in the homeostasis of Treg cells. Regulatory T cells were about two-fold reduced in number in the thymuses and spleens of Lck mutant mice. Moreover Lck deficiency led to impaired proliferation of Treg cells upon transfer into lymphopenic recipients. BrdU labeling experiments revealed that loss of Lck expression impaired Treg cell turnover having only minor effects on their survival. Globally these results point to a similar requirement in TCR signaling for conventional and regulatory T cell. Although interesting and well conducted, it is unfortunate that this study employed OX40-cre mice instead of FOXP3-cre mice for targeting of Treg. Indeed, as OX40 is also expressed by conventional T cells upon activation, we can not exclude that the observed effects are not linked to reduction in IL-2 production derived by abrogation of TCR signaling in activated conventional T cells.

1.1.4.5 Antigen presenting cells

Dendritic cells (DCs) represent the main subset of specialized antigen presenting cells and are critically involved in the induction and regulation of T cell responses. Modifications in DCs numbers through genetic ablation or manipulation of Flt3L concentration led to parallel changes in T reg cell numbers (Swee et al., 2009; Darrasse-Jeze et al., 2009). This has led to a model of coordinated DC/T reg cell homeostasis (Darrasse-Jeze et al., 2009). DCs have been also shown to support reconstitution of the Treg pool after incomplete depletion of FOXP3⁺ CD4 T cells (Suffner et al., 2010). DCs seem to exert their role on Treg homeostasis through different mechanisms. Using two different models for MHC-II conditional ablation, Darrasse-Jeze et al. elegantly demonstrated that MHC-II expression at dendritic cell surface is required to maintain T reg cell homeostasis (Darrasse-Jeze et al, 2009). Moreover, it has been shown that DC are capable to induce preferential antigen specific Treg expansion by presenting antigens under non-inflammatory conditions (Chappert et al., 2008). More recently Bar-On et al uncovered a role for CD80/86 expression by DC in peripheral Treg maintenance (Bar-On et al. 2011). Using

CD80/CD86 mutant animals and a strategy that restricts the B7 deficiency to DC, the authors showed that CD80/CD86 deficiency on DCs did not impair thymic development of Treg, but results in about a two-fold reduction in peripheral Treg numbers. Recently, a rare human syndrome characterized by monocytopenia, B and NK lymphoid deficiency (Vinh et al., 2010) which includes a nearly complete absence of dendritic cells (Bigley et al., 2011). Interestingly, severe DC deficiency correlated with depletion of T reg cells, supporting a role for DC in human T reg cell homeostasis.

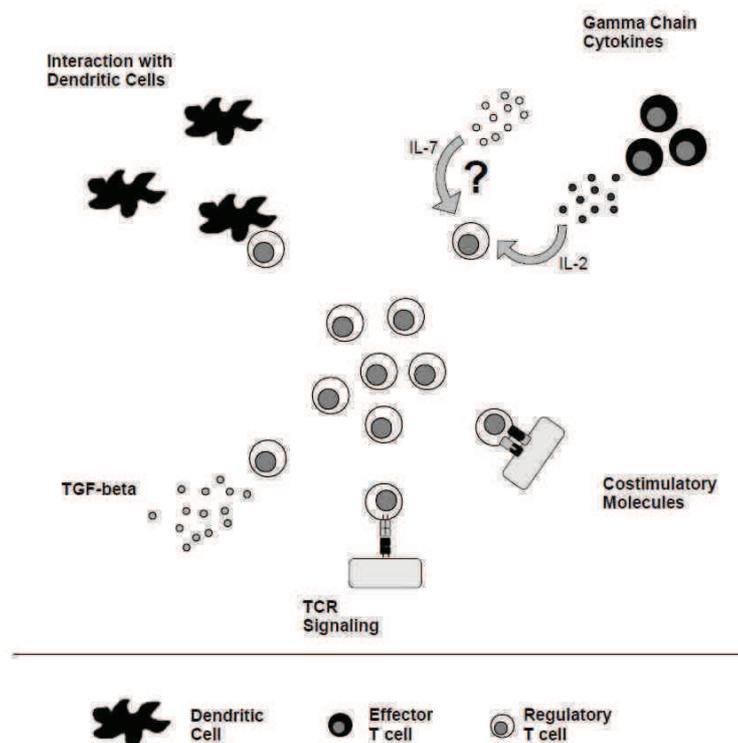


Figure 1.2 – Treg homeostasis. Schematic representation of factors regulating Treg homeostasis.

1.1.4.6 Other factors

The role of inflammatory mediators in Treg homeostasis remains to be elucidated. In a recent interesting report, Grinberg-Bleyer and coworkers showed that activated conventional effector T cells are able to boost the expansion of Tregs *in vivo* by an IL-2-independent, TNF α -dependent mechanism (Grinberg-Bleyer et al., 2011). Whether such an effect depends on a direct effect of TNF on Treg survival and proliferation or it depends on TNF α action on signaling by other homeostatic factors remains to be further investigated.

Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) is a growth factor for granulocyte, macrophage, erythroid, megakaryocyte, and eosinophil progenitors which is produced by a number of different cell types including T cells, B cells and macrophages in response to different cytokines or inflammatory stimuli. The GM-CSF receptor is composed of a specific α chain (CD116) associated with a β chain shared with the IL3 and IL5 receptors. Kared et al., reported that CD116 is constitutively expressed at murine Treg surface (Kared et al., 2008). Moreover, GM-CSF enhances anti-CD3–induced Treg *in vitro* proliferation in an IL-2-independent manner. The same authors showed that GM-CSF participates, in conjunction to the Notch signaling, to the Treg expansion induced by mobilized Lin⁻Sca-1⁺c-kit⁺ (LSK) hematopoietic progenitor cells (HPCs) (Kared et al., 2006; Kared et al., 2008).

1.1.5 Treg cells in clinical settings

1.1.5.1 Role of Treg cells in human autoimmune disease

Several human autoimmune diseases have been reported to be caused by or to be associated with alterations in CD4⁺CD25⁺ Treg cell numbers or function.

1.1.5.1.1 Monogenic autoimmune syndromes – Although rare, autoimmune syndromes due to monogenic defects offer a unique opportunity to study *in natura* the molecular mechanisms responsible for the Treg mediated immune tolerance. The first

genetically determined Treg alterations leading to human disease were linked, as discussed above, to mutations of the *Foxp3* gene (Chatila et al, 2000 ; Bennett et al, 2001 ; Wildin et al, 2001). Although stop mutations lead to the complete absence of the protein and therefore of Treg cells, several other mutations of *FOXP3* gene have been reported leading not to a lack of differentiation of Treg but rather to a dysfunction in these cells (Bacchetta et al., 2006).

As already discussed CD25 expression is important for the suppressive activity and for the peripheral survival of Treg cells. It is therefore not surprising that an IPEX-like syndrome due to deficiency in CD25 has been reported (Sharfe et al., 1997). Clinically, CD25 deficiency differs from *Foxp3* deficiency by an increased susceptibility to viral infections and variable penetrance of endocrinopathies. Interestingly, CD25 deficiency does not lead to the disappearance of *FOXP3*+ CD4 T cells (Caudy et al., 2007), confirming in humans that IL-2 signaling is not strictly required for *FOXP3*+ CD4 T cells development. However the suppressive capacity of the remaining *FOXP3*+ CD4 T cells is unknown.

Autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED) is a monogenic autoimmune syndrome characterized by the development of organ-specific autoimmunity involving endocrine glands (thyroid, parathyroid, pancreas, adrenal, gonads) as well as the liver, gut, and skin. Typically patients also suffer from chronic mucocutaneous candidiasis in infancy. APECED is caused by mutations of the autoimmune regulator (*AIRE*) gene (Nagamine et al., 1997) a gene regulating the ectopic transcription of tissue-restricted antigens in the thymus. Although implicated in the thymic negative selection of auto-reactive T cells, a role for *AIRE* expression for Treg cell development has been proposed (Aschenbrenner et al., 2007). Ryan et al., first showed that CD4+CD25+ T-regulatory cells are decreased in patients with APECED (Ryan et al., 2005). Such observation has been subsequently confirmed in larger cohorts of patients (Kekäläinen et al., 2007 ; Wolff et al., 2010). Moreover, Kekäläinen et al., showed that CD4+ CD25^{high} cells isolated from APECED patients

presented *in vitro* a reduced suppressive activity.

Wiskott-Aldrich syndrome (WAS) is a primary human X-linked immunodeficiency caused by mutations in the hematopoietic-specific cytoskeletal regulator Wiskott-Aldrich syndrome protein (WASP) and characterized by recurrent infections and thrombocytopenia (Notarangelo et al., 2008). In addition WAS patients present an increased incidence in autoimmunity and allergy. Murine data revealing defects in Treg cells function from WAS^{-/-} mice lead to investigate the potential Treg implication in WAS pathogenesis. Although WAS patients present a normal percentage of CD4⁺CD25⁺FOXP3⁺ nTreg cells in the blood, functional assays revealed defective suppressor activity of nTreg cells from WAS patient (Marangoni et al., 2007 ; Maillard et al., 2007 ; Adriani et al., 2007).

1.1.5.1.2 Autoimmune diseases - While major Treg cells alterations are clearly involved in the pathogenesis of several monogenic autoimmune syndromes, many studies have investigated whether more subtle changes in Treg cell function or numbers could play any roles in other polygenic autoimmune diseases.

Impaired Treg cell-mediated tolerance in autoimmunity can be resumed in three major mechanisms : i) Inadequate numbers of Treg cells, ii) defects in Treg cell function and iii) resistance of effector T cells to suppression.

It should be noted however that identification of any quantitative or qualitative alteration in Treg in immune-pathological diseases faces three major complications. First, in autoimmune diseases as in human studies in general, conclusions about Treg enumeration strictly depend on the cellular markers and on the identification strategy employed. As a result very conflicting results have been obtained for most of diseases. Second, Treg quantification has been usually performed in peripheral blood and not at the site of disease. Third, different disease phases and ongoing immunosuppressive treatments add further confusion.

For the majority of diseases very conflicting results regarding a quantitative defect of Treg have been reported. A condition for which a sort of consensus has been reached is Systemic Lupus Erythematosus (SLE). Indeed most, although not all, studies in SLE reported a decrease in the percentage of Treg cells in patient with active flares compared to controls (Miyara et al., 2005). Such a reduction was confirmed by employing the new Naive/Effector Treg identification strategy proposed by Miyara and Sakaguchi (Miyara et al., 2009).

No less conflicting has been the study of the functional activity of Treg in autoimmune diseases. The MS case nicely represents the disparity of conclusions that can be obtained as a consequence of different Treg identification strategies. Viglietta et al. concluded that a defect existed in the capacity CD4⁺ CD25^{high} cells from MS patients to suppress conventional CD4 T cell proliferation (Viglietta et al., 2004). Several group reported conflicting results (Putheti et al., 2004; Haas et al., 2005), until Michel et al. finally demonstrated that by removing CD127^{high} cells from the CD4⁺CD25^{high} population, MS patient and control cells equally inhibited proliferation and cytokine production of T cell activated by anti-CD3 (Michel et al., 2008). Such results have been recently confirmed although CD4⁺CD25^{high}CD127^{low} cells from MS patients have been shown to still be impaired when activated by anti-CD2 (Baecher-Allan et al., 2011).

Another interesting case is that of RA. Early studies failed to detect any functional defect of CD4⁺CD25^{high} T cells from RA patients, as assessed by their capacity to suppress the proliferation of conventional CD4 T cells (Cao et al., 2003). However Ehrenstein et al. subsequently demonstrated that Treg from RA patients presented a selective defect to suppress IFN γ and TNF α secretion from conventional T cells, while retaining the capacity to inhibit their proliferation (Ehrenstein et al. 2004). Interestingly the authors showed that the anti-TNF α therapy restored the full suppressive capacity of RA Treg. Some years later the same group further established that this defect was linked to a defect of CTLA-4 in Treg from RA patients (Flores-

Borja et al., 2008).

A third mechanism of impaired Treg-mediated suppression derives from the resistance of conventional T cells. Such a phenomenon has been reported in patients affected by T1D (Schneider et al., 2008 ; Lawson et al., 2008), SLE (Venigalla et al., 2008 ; Vargas-Rojas et al., 2008) and RA (van Amelsfort et al., 2007). The cause of such resistance remains in most cases unknown, and has been suggested to be cell intrinsic. However, as CD4⁺CD25⁻ cells were employed, we cannot exclude that differences in behavior between cells from patients and donors simply reflect differences in cell subsets contained in the cell pool studied. A different picture has been proposed for RA, in which resistance of T cells seems to derive from pro-inflammatory mediators such as co-stimulatory molecules, IL-7 and TNF α (van Amelsfort et al., 2007).

1.1.5.2 Role of Treg cells in human infectious disease

In contrast to autoimmune diseases, where a beneficial regulatory action exerted by the Treg compartment is easily understandable, a much more complex picture emerges for the role of Treg in infectious diseases. Treg mediated inhibition of antimicrobial immune-responses could lead to ineffective clearance of the pathogen contributing to the chronicization of the infection. On the other side, Treg participate to terminate immune response thus preventing exacerbated and potentially harmful responses to develop : immune activation contributes to the pathogenesis of several infectious diseases, such as viral hepatitis and HIV invention, and a limiting action exerted by Treg could eventually result beneficial (Belkaid 2008, Belkaid and Tarbell 2009).

1.1.5.2.1 HIV Infection – HIV infection is a chronic viral infection associated with progressive loss of CD4⁺ T-cells and immune deficiency. CD4 T cell depletion is believed to be the consequence of both the direct cytopathic effect of the virus and the state of

chronic immune hyper-activation (reviewed in Douek et al., 2009).

Despite the fact that many studies addressed the potential role of Treg in HIV infection pathophysiology, very few conclusions are at present consensually accepted. For a synthetic purpose, Treg studies can be re-conducted to four kinds of analysis : i) Treg quantification ii) assessment of Treg function iii) Treg susceptibility to HIV infection and iv) correlation of Treg levels to disease parameters.

Treg quantification – Dozens of studies have been published in the last few years reporting quantification of Treg percentage and/or counts in HIV patients. Table 1 resumes Treg quantification data from the published literature. It should be noted however that Treg enumeration in HIV infection and in human studies in general, is complicated by the lack of an efficient and universally accepted strategy for their identification. As already discussed, all known human Treg markers, including CD25 and FOXP3, are up-regulated during activation of conventional CD4 T cells. It is therefore difficult to find a good combination of markers to be used in a situation which is characterized by chronic immune-activation. Secondly, as CD4 depletion is the pathogenic hallmark of HIV infection and CD4 counts decline during disease progression, determining Treg percentages instead of Treg counts may also participate to the uncertainties concerning Treg quantification. Finally, a third major aspect participating to the discrepancies obtained is represented by the diversity of HIV clinical stages rendering universal conclusion difficult to be drawn.

Despite these considerations some conclusion can be drawn. During primary HIV infection (PHI), decreased Tregs percentages have been described (Kared et al., 2008; Ndhlovu et al., 2008) although results differ depending on the staining strategy (Ndhlovu et al., 2008). Treg percentages are shown to be consistently increased while Treg absolute numbers consistently decreased in chronically infected viremic patients

Reference	Studied Population	Tissue analysed	Identification Strategy	Treg Percentages	Treg count	Other Findings
Aandahl et al., 2004	HD, VIR	Blood	CD25+	CD25+ percentage among CD4 T cell unaltered in HIV patients when compared to HD		
Kinter et al., 2004	HD, VIR, ART	Blood	CD25high CD45RO+ PCR FOXP3	Small increase in CD25high CD45RO+ percentages among CD4 T cells		
Oswald-Richter et al., 2004	HD, HIV infected	Blood	CD25high CD45RO+ PCR FOXP3	Increase in CD25high CD45RO+ percentages among CD4 T cells		Decreased Foxp3 mRNA in CD25high CD45RO+ of HIV-infected vs HD
Weiss et al., 2004	HD, ART	Blood	CD25+	Increase in CD25+ among CD4 T cells		
Andersson et al., 2005	HD, VIR	Blood, Tonsils	FOXP3 (PCR and IHC)		Higher numbers of FOXP3 expressing in lymphoid tissue from untreated patients compared with ART patients.	FoxP3 mRNA expression in T cells from untreated HIV-infected donors was decreased in blood but increased in lymphoid organs.
Apoil et al., 2005	HD, ART	Blood	CD25high	No difference in percentage among CD4 T cells. Reduction in the percentage among CD3 cells.		FOXP3 mRNA levels are decreased in peripheral blood CD4+ Lymphocytes from HIV-Positive Patients
Tsunemi et al., 2005	HD, ART, VIR	Blood	CD25high	Increase in Treg percentages in VIR but not aviremic HIV patients		
Epple et al., 2006	HD, ART, VIR	Blood Duodenal mucosa	CD25high FOXP3	Blood : Higher percentages of CD25high FOXP3 in VIR but not in ART patients vs HD Mucosa ; higher percentages of FOXP3+ cells in VIR vs ART and HD	Blood : No difference between VIR and ART patients vs HD Mucosa ; Higher FOXP3+ cell counts in VIR vs ART and HD	
Kinter et al., 2006	HD, VIR, ART	Blood Lymph nodes	CD25high FOXP3	Higher percentages in LN vs PB		
Lim et al., 2006	HD, VIR, ART	Blood	CD25+ GITR+ NRP-1+ LAG-3+	Equivalent level of Treg markers expressing cells in VIR, ART and HD.		Higher levels of FOXP3 mRNA in ART but not VIR vs HD
Marziali et al., 2006	HD, ART (Immunological responders IR and Immunological non-responders InR)	Blood	CD25high CD25high CD62Lhigh CD25highCD27	Reduction in InR ART compared with IR ART and HD		
Montes et al., 2006	HD, VIR	Blood	CD25+FOXP3	Increased		Highest Treg percentages in patients with lower CD4 counts
Nilsson et al., 2006	HD, VIR, LTNP	Tonsils	FOXP3 (IHC and PCR)		Higher numbers of FOXP3+ T cells in tonsils from VIR compared with HD and LTNP	Higher levels of FOXP3 mRNA in tonsils from VIR compared with HD and LTNP
Lim et al., 2007	HD, VIR (low CD4, high CD4)	Blood	FOXP3 CD25+ CD127low	No difference in FOXP3 between high CD4 VIR and HD. Reduction in low CD4 VIR	Higher numbers of FOXP3 CD4 T cells in high CD4 vs low CD4 VIR	Higher FOXP3+ CD4-percentages and counts in low CD4 VIR vs HD and high CD4 VIR
Mozos et al., 2007	HD, VIR, ART	Blood Tonsils	FOXP3(FACS, IHC, PCR)	Higher levels of FOXP3+ cells in PB of VIR and ART compared to HD	Lower numbers of tonsillar FOXP3+ cells in ART compared to HD and VIR. No difference between HD and VIR.	Lower levels of tonsillar FOXP3 mRNA in ART and VIR compared to HD Higher of blood FOXP3 mRNA in ART and VIR compared to HD
Chase et al., 2008	HD, VIR, ART, HIC	Blood	FOXP3 FACS and PCR CD25high CD62Lhigh	Lower percentages of CD25highFOXP3+ cells in ART compared to HIC and HD. No difference between HIC and HD.		Lower levels of FOXP3 mRNA in VIR and ART compared to HD and HIC
Gaardbo et al., 2008	HD, VIR, ART	Blood	CD25high FOXP3 mRNA	Higher Treg percentages in VIR and ART than in HD. No difference between VIR and ART	Lower Treg counts in VIR and ART than in HD. Higher Treg counts in ART than in VIR	Higher levels of FOXP3 mRNA in ART and VIR than in HD
Kared et al., 2008	PHI, VIR	Blood	CD25high	Lower percentage in PHI compared to VIR. Lower percentage in ART compared to PHI.		
Ndhlovu et al., 2008	HD, PHI, VIR	Blood	CD25+ CD127low	Lower percentage in PHI and VIR compared to HD. No difference between PHI and VIR		
Rallon et al., 2009	HD, HIV infected (ART and VIR)	Blood	CD25+ FOXP3+	No difference between HIV and HD		Lower percentage of CD45RA+ CD27+ among CD25+FOXP3+ CD4 T cells in HIV vs HD. Higher levels of CD38 at CD25+FOXP3+ CD4 T cells surface in HIV vs HD.
Tenorio et al., 2008	HD, VIR, ART	Blood	CD25+ CD127low	Increased total and memory Treg percentage in HIV patients <200 CD4 vs HD. Decrease in naive Treg percentage in HIV patients <200 CD4 vs HD and HIV >500 CD4		
Zhang et al., 2008	HD, HIV infected (asymptomatic and AIDS)	Blood	CD25+FOXP3+	Higher percentages in AIDS vs asymptomatic. No difference HIV vs HD	Lower numbers in HIV (AIDS and asymptomatic) vs HD	
Bi et al., 2009	HD, VIR (low CD4, high CD4), ART	Blood	FOXP3+	Lower percentages in VIR (both low CD4 and high CD4) vs HD. Lower percentages in low CD4 vs high CD4. Reduction in Treg percentage on ART compared to VIR.	Lower numbers in VIR (both low CD4 and high CD4) vs HD. Lower numbers in low CD4 vs high CD4. Increase in Treg counts on ART compared to VIR.	Higher percentages of Ki67+ among FOXP3+ cells in Low CD4 VIR compared with high CD4 VIR and HD

continues

<i>Cao et al., 2009</i>	HD, HIV infected (slow progressor, fast progressor)	Blood	CD25+ FOXP3+	Higher percentages in HIV (both SP and FP) vs HD		
<i>Card et al., 2009</i>	HD, HIV infected, Exposed Uninfected	Blood	CD25+FOXP3+	No difference in percentage within CD4 T cells and lower percentage within CD3 cells between HIV+ and HD- Higher percentages within CD4 and CD3 in EU vs HD		
<i>Jiao et al., 2009</i>	HD, VIR, ART (responder, non responder, LTNP)	Blood	CD25+ FOXP3+	Increased percentages in VIR vs HD and LTNP. No difference between LTNP and HD. Higher percentages of Treg in non responder ART vs responder ART.	Reduced numbers in VIR vs HD and LTNP. Higher numbers in LTNP vs HD. Higher numbers in responder ART vs non responder ART	
<i>Kotte et al., 2009</i>	HD, ART	Blood	CD25+ CD127low CD25+CD45RA+	Higher percentages in ART than in HD.		Higher percentages of CD25+CD45RA+ in ART compared to HD. Positive correlation between CD25+CD45RA+ absolute numbers and TREC frequency
<i>Lim et al., 2009</i>	HD, ART, Untreated (<300 CD4, >400 CD4)	Blood	FOXP3+	Higher percentages in Untreated vs ART and HD.	Higher count in Untreated >400 CD4 vs Untreated <300 CD4 and HD.	Higher proportion of Ki67+ cells among FOXP3+ from Untreated vs ART.
<i>Seddiki et al., 2009</i>	HD, VIR, ART, IRD	Blood	CD25+ CD127low	No difference in percentage between HD, VIR, ART. Higher percentages in IRD.		
<i>Tenorio et al., 2009</i>	HD, VIR	Blood	CD25+ CD127low	Higher percentage in VIR vs HD.		
<i>Terzieva et al., 2009</i>	HD, HIV infected	Blood	CD25+ FOXP3+	Higher percentage in HIV vs HD.		
<i>del Pozo-Balado et al., 2010</i>	VIR, ART	Blood	CD25+, CD25high, CD25+ FOXP3+, CD25high FOXP3+, CD25+ CD127low, CD25high CD127low	Lower percentages of CD25+, CD25high, CD25+ FOXP3+, CD25high FOXP3+ in VIR compared to ART. No difference in CD25+ CD127low and CD25high CD127low percentages		
<i>Favre et al., 2010</i>	HD, VIR (low CD4, high CD4), ART, HIC	Blood	FOXP3	Higher percentage in low CD4 VIR vs HD, HIC, ART		Higher proportion of Ki67+ Treg in VIR vs HIC
<i>Lake et al., 2010</i>	HD, VIR, HIC	Blood, rectosigmoid mucosa	FOXP3			Positive correlation between percentage of FOXP3 cells among CD4 between blood and rectosigmoid mucosa
<i>Owen et al., 2010</i>	HD, VIR, ART, HIC	Blood	CD25high CD127low CD152+	Higher percentages in ART vs HD	Higher counts in ART vs VIR	
<i>Piconi et al., 2010</i>	ART (>500 CD4, >500 CD4)	Blood	CD25high FOXP3+ PD1 intracellular/extracellular	Higher percentage of CD25high FOXP3+ cells, CD25high FOXP3+ intracellular PD1+ cells in <500 CD4 ART vs >500 CD4 ART		
<i>Prendergast et al., 2010</i>	HD, VIR	Blood	CD25high FOXP3+	Lower percentage of CD25high FOXP3+ among CD3		
<i>Thorburn et al., 2010</i>	HD, VIR	Blood	CD25+ FOXP3+		Lower absolute numbers in VIR vs HD	
<i>Weiss et al., 2010</i>	ART	Blood	CD25+ CD127low	Increase in percentage upon ART interruption.	Decrease in absolute numbers upon ART interruption	
<i>Xing et al., 2010</i>	HD, VIR, ART (Responders and Non-responders), LTNP, AIDS	Blood	CD25high FOXP3+			Higher proportions of Ki67+, caspase3+ and annexin V+ Treg in VIR and AIDS compared with HD and LTNP. ART responders but not ART non-responders present lower percentages of Ki67+, caspase3+ and annexin V+ among Treg upon treatment
<i>Brandt et al., 2011</i>	HD, VIR, ART, HIC	Blood	CD25+ CD127low FoxP3+	Higher percentage in VIR, ART and HIC vs HD. Higher percentages in VIR vs HIC.	Lower numbers in ART and HIC vs VIR	
<i>He et al., 2011</i>	HD, VIR, ART	Blood	CD25+ FOXP3+	Higher percentages in VIR and ART vs HD. Lower percentages in ART vs VIR.		
<i>Hunt et al., 2011</i>	HD, VIR, ART, HIC	Blood	CD25+ CD127low	Lower percentages in HIC vs HD, VIR, ART		
<i>Montes et al., 2011</i>	VIR, ART	Blood	CD25+ FOXP3+	Reduction of percentage upon ART	Increase in absolute numbers upon ART	
<i>Nikolova et al., 2011</i>	HD, VIR, ART	Blood	CD25high Foxp3+ CD127low	Higher percentages in VIR and ART vs HD.		Higher proportion of CD39+ Treg in VIR and ART vs HD. Higher CD39 MFI on Treg in VIR and ART vs HD. No difference between VIR and ART.
<i>Schulze zur Wiesch et al., 2011</i>	HD, VIR, ART, LTNP, HIC	Blood	CD25high Foxp3+ CD127low	Higher percentages in VIR and ART vs HD, HIC and LTNP. Higher percentages in VIR vs ART. Higher percentages in ART vs LTNP. No differences in HD, HIC and LTNP. Decrease in percentage upon ART.	No difference in HD and HIC. Lower numbers in LTNP, VIR and ART vs HD. No difference between LTNP, VIR and ART. Higher numbers in HIC vs LTNP. Increase in counts upon ART.	Higher proportion of CD39+ Treg in LTNP, ART and VIR vs HD. No difference between HIC and HD. Higher proportion of ICOS, CTLA4 and CCR5 expressing Treg in HIC vs HD and ART. Higher proportion of ICOS+

Table 1.2 – Treg quantification in HIV infection. Healthy donors (HD), primary HIV-infected (PHI), viremic (VIR), antiretroviral treated (ART) patients, long term non progressors (LTNP), HIV controllers (HIC).

(Tsunemi et al., 2005 ; Montes et al., 2006 ; Lim et al., 2007 ; Rallon et al., 2008 ; Tenorio et al., 2008 ; Tenorio et al., 2009 ; Bi et al., 2009 ; Piconi et al., 2010 ; Favre et al., 2010). During efficacious antiretroviral therapy Treg percentages have been shown to progressively decrease to normal levels (Marziali et al., 2006 ; Bi et al., 2009 ; Jiao et al., 2009 ; Montes et al., 2011 ; Schulze zur Wiesch et al., 2011) while Treg counts increase progressively in parallel with total CD4 counts (Gaardbo et al., 2008 ; Bi et al., 2009 ; Jiao et al., 2009 ; Montes et al., 2011 ; Schulze zur Wiesch et al., 2011). Interestingly this effect is reversed upon ART interruption (Weiss et al., 2010). Studies of Treg levels in HIV controllers, a subpopulation of patients which spontaneously control viral loads (Lambotte et al., 2005), revealed alternately unchanged (Chase et al., 2008 ; Owen et al., 2010 ; Schulze zur Wiesch et al., 2011), increased (Brandt et al., 2011) or reduced (Hunt et al., 2011) percentages.

Treg susceptibility to HIV infection - Several studies have shown that Treg cells are highly susceptible to HIV infection (Oswald-Richter et al., 2004 ; Chase et al., 2008 ; Tran et al., 2008 ; Moreno-Fernandez et al., 2009). Moreover, Treg susceptibility seems to differ depending on the HIV type 1 strain, Treg being less susceptible to R5 viruses compared with effector T cells (Moreno-Fernandez et al., 2009). Interestingly, Tran et al., suggested that Treg could represent a preferential cellular reservoir of viral infection (Tran et al., 2008).

Treg function - Regarding Treg function on control of HIV-specific CD4 and CD8 responses, it has been shown that presence of CD25 cells during *in vitro* HIV specific stimulation led to decreased HIV-specific CD4 and CD8 responses (Table 2) (Aandahl et al., 2004 ; Kinter et al., 2004 ; Weiss et al., 2004 ; Eggena et al., 2005 ; Kinter et al., 2006 ; Legrand et al., 2006 ; Kinter et al., 2007 ; Chase et al., 2008 ; Kared et al., 2008 ; Tran et al., 2008 ; Elahi et al., 2011 ; Moreno-Fernandez et al., 2011 ; Nikolova et al., 2011). Importantly, Treg have been reported to suppress both cell proliferation and effector molecules production in response to HIV. Recently, Elahi and coworkers reported the

Reference	Studied Population	Experimental Procedure	Major Findings
<i>Aandahl et al., 2004</i>	HD, VIR	CD25 depleted PBMC stimulated with HIV antigens	CD25 depletion increases the percentage of IFN-g and TNF-a producing T cells among PBMC
<i>Kinter et al., 2004</i>	HD, VIR, ART	CD25 depleted PBMC stimulated with HIV antigens Bead-isolated CD25+ CD4 T cells cocultured with CD4 or CD8 T cells	Depletion of CD25 cells enhances the frequency of CD8 T cells producing IFN-g in response to HIV Gag peptides CD25+ CD4+ T cells suppress proliferation and IL-2 production by p24 stimulated CD4 T cells and proliferation of perforin-expressing effector CD8 T cells cultured in the presence of autologous HIV super-infected target cells.
<i>Weiss et al., 2004</i>	HD, ART	CD25 depleted CD4 T cells stimulated with p24 Purified CD4+CD25+ T cells cocultured with CD4+CD25- T cells stimulated with p24	CD4+ CD25+ T cells inhibit CD4+ CD25- T cell proliferation in response to p24 stimulation.
<i>Eggena et al., 2005</i>	HD, VIR	CD25 depleted PBMC stimulated with Gag peptide ELISPOT of PBMC stimulated with Gag peptide	Increased Gag-specific responses upon CD25 depletion. No significant association between ELISPOT detected Gag-specific responses and CD4+CD25highCD62Lhigh percentages of CD3 or of CD4 cells
<i>Kinter et al., 2006</i>	HD, VIR, ART	CD25 depleted LNMC stimulated with p24 coculture of CD25high CD8- cells Assays of Gag-specific Cytolytic Activity	Increased CD4 proliferation in response to HIV p24 upon CD25 depletion Inhibition of CD8 T cells Gag-specific Cytolytic Activity by PB and LN derived CD25high cells
<i>Legrand et al., 2006</i>	HIV exposed uninfected neonates	CD25 depleted cord blood MC stimulated with Gag peptides pools	Augmented Gag CD8+ IFN-g and CD4+ IL-2 immune responses in exposed-uninfected neonatal cord blood upon the removal of Treg cells
<i>Kinter et al., 2007</i>	HD, VIR, ART	CD25 depleted PBMC stimulated with Gag peptides pools Cytotoxic assay with Gag-loaded target cells	Increased proliferating TNFa/IFNg producing Gag-specific CD8 T cells and increased cytolytic activity upon CD25 depletion
<i>Chase et al., 2008</i>	HD, VIR, ART, HIC	CD25high CD62Lhigh suppressive assay with anti-CD3 and irradiated autologous PBMC stimulation	Lower suppression by Treg from VIR compared with HIC and HD. No difference between HIC and HD.
<i>Kared et al., 2008</i>	PHI, VIR, ART	CD25high cocultured with CD4+CD25- cells stimulated with PPD or p24	Comparable suppressive activity of CD25high cells from PHI and VIR. Reduced suppressive activity upon p24 but not PPD stimulation of CD25 high cells isolated from ART compared to PHI
<i>Tran et al., 2008</i>	ART	Granzyme B ELISPOT of CD25 depleted PBMC stimulated with p24, RT and Nef	Increase in Granzyme B production upon CD25 depletion
<i>Elahi et al., 2011</i>	LTNP	Beads isolated CD25 cells cocultured with PBMC or CD8 T cells in the presence of HIV-1 epitopes	Treg cells differentially suppress HIV epitope proliferation of CD8+ CTLs restricted by different HLA alleles (epitope-specific CD8+ CTLs restricted by the protective HLA allele groups HLA-B*27 and HLA-B*57 were not suppressed by Treg)
<i>Moreno-Fernandez et al., 2011</i>	HD	FACS sorted CD25high CD127low	Treg inhibit HIV replication in activated T cells through a cAMP-dependent mechanism
<i>Nikolova et al., 2011</i>	HD, VIR, ART	Beads isolated CD25high cells cocultured with CD8+ T cells stimulated overnight with a pool of whole Gag 15-mer peptides supplemented with anti-CD28 and anti-CD49d antibodies	Treg inhibit cytokine production of Gag-stimulated CD8+ T cells through a CD39 dependent mechanism.

Table 1.3 – Treg function in HIV infection. Healthy donors (HD), primary HIV-infected (PHI), viremic (VIR), antiretroviral treated (ART) patients, long term non progressors (LTNP), HIV controllers (HIC).

interesting finding that Treg cells differentially suppress HIV epitope proliferation of CD8+ CTLs restricted by different HLA alleles, epitope-specific CD8+ CTLs restricted by the protective HLA allele groups HLA-B*27 and HLA-B*57 being not susceptible to Treg mediated suppression (Elahi et al., 2011). Collectively, published results suggest that a dominant mechanism of suppression by Treg could reduce *in vivo* antiviral responses participating to the incapacity to eradicate HIV infection.

Correlation of Treg levels to disease parameters – Results derived from the published literature correlating Treg levels to disease parameters are resumed in Table 3. Most of published studies agree in reporting a positive correlation between absolute numbers of Treg and CD4 count (Kinter et al., 2004 ; Eggena et al., 2005 ; Montes et al., 2006 ; Kolte et al., 2008 ; Tenorio et al., 2008 ; Cao et al., 2009 ; Jiao et al., 2009 ; Thorborn et al., 2010 ; Schulze zur Wiesch et al., 2011) and a negative correlation between Treg percentages and CD4 count (Tsunemi et al., 2005 ; Limet al., 2007 ; Tenorio et al., 2008 ; Zhang et al., 2008 ; Jiao et al., 2009 ; Piconi et al., 2010 ; He et al., 2011 ; Schulze zur Wiesch et al., 2011). Viral load has been reported mostly to be positively correlated to Treg percentages (Limet al., 2007 ; Zhang et al., 2008 ; Loke et al., 2010 ; Schulze zur Wiesch et al., 2011) and negatively correlated with Treg numbers (Eggena et al., 2005 ; Nilsson et al., 2006 ; Tenorio et al., 2008 ; Schulze zur Wiesch et al., 2011). Immune hyperactivation as revealed by expression of CD38 and HLADR activation markers at CD4 and CD8 cell surface is a negative prognostic factor associated with disease progression in HIV infection (Bouscarat et al., 1996; Liu et al., 1997; Deeks et al., 2004). In order to investigate if Treg alteration played a role in this phenomenon, several studies have tried to identify a correlation between Treg level and lymphocytes activation. Unfortunately no accepted conclusion can be drawn as CD4 and CD8 activation as determined by expression of CD38 and HLADR activation markers as been reported to be in no or positive or inverse correlation with Treg percentages or counts (see Table 3). It is important to underline that correlations have been only performed with activation profile of total CD4 and CD8 T cells and no published study has still investigated the correlation between Treg numbers and

HIV-specific T cell activation *ex vivo*.

Globally published results suggest that Treg are a double edged sword in Treg infection. On one side they are detrimental as they inhibit HIV-specific immune response. On the other side they could participate to the maintenance of immune-homeostasis by reducing non-specific chronic immune hyper-activation.

Reference	Studied Population	Identification Strategy	Correlation with CD4 count	Correlation with Viral Load	Correlation with Immune-activation
Kinter et al., 2004	HD, VIR	CD25high CD45RO+	Positive correlation between CD25high CD45RO+ counts with CD4+CD45RO+ cell numbers		
Oswald-Richter et al., 2004	HD, HIV infected	CD25high CD45RO+	Decreased Foxp3 mRNA in CD25high CD45RO+ CD4 T cells from patients with low CD4 cell counts	No correlation	decreased Foxp3 mRNA in CD25high CD45RO+ CD4 T cells from patients with increased immune activation
Eggens et al., 2005	HD, VIR	CD25high CD62Lhigh	Positive correlation between Treg count and CD4 count	Negative correlation between Treg count and viral load	Negative correlation between Treg percentages and counts with CD4 and CD8 immune-activation
Tsunemi et al., 2005	HD, ART, VIR	CD25high	Negative correlation between CD25high frequency and CD4 count in VIR but not in aviremic HIV patients		
Marziali et al., 2006	HD, ART (immunological responders IR and immunological non-responders InR)	CD25high CD62Lhigh CD25highCD27	Positive correlation between CD4+CD25high percentages and CD4 count		Negative correlation between CD4+CD25high percentages and HLA-DR expression on CD4 T cells
Montes et al., 2006	HD, VIR	CD25+FOXP3	Positive correlation between CD4+CD25highFOXP3+ cell count and CD4 count		
Nlissonet et al., 2006	HD, VIR, LTNP	CD25 FOXP3 immunohistochemistry		Plasma viral load is negatively correlated with tonsil CD25 + FOXP3 T cells numbers.	
Linet et al., 2007	HD, VIR (low CD4, high CD4)	CD25+ CD127low	Percentages of CD25+ CD127low CD4+ T cells correlate inversely with CD4 T-cell counts	Percentages of CD25+CD127lowCD4+ T cells positively correlate with plasma HIV RNA levels	Percentages of CD25+CD127lowCD4+ T cells positively correlate with percentages of HLA-DR+ CD4 T cells
Mezosi et al., 2007	HD, VIR, ART	FOXP3 FACS immunohistochemistry PCR	Inverse correlation between mRNA and protein FOXP3 expression and CD4 counts is observed in lymphoid tissue but not in PB	Positive correlation between FOXP3 mRNA and VL in PB but not in lymphoid tissue	
Chase et al., 2008	HD, VIR, ART, HIC	FOXP3 FACS and PCR CD25high CD62Lhigh	Positive correlation between FOXP3 mRNA and CD4 count		Inverse correlation between FOXP3 mRNA and percentage of HLA-DR+ cells among CD4
Karedi et al., 2008	PHI, VIR, ART	CD25high	Positive correlation between CD25high percentages and CD4 count	No correlation between CD25high percentages and VL	Percentages of CD25high CD4+ T cells inversely correlate with percentages of HLA-DR+ CD4 T cells
Kolte et al., 2008	HD, ART	CD25+CD45RA+	Positive correlation between CD25+CD45RA+ cells absolute numbers and CD4 count		Inverse correlation between FOXP3 mRNA and percentages of CD38+HLA-DR+ CD4 T cells
Ndlovu et al., 2008	HD, PHI, VIR	CD25+ CD127low	No correlation with percentage and counts in PHI. Positive correlation in VIR		No correlation between percentage and counts and CD38 expression (MF) on CD8 T cells in PHI. Negative correlation in VIR
Tenorio et al., 2008	HD, VIR, ART	CD25+ CD127low	Absolute number of total, naive, and memory T-reg cells directly correlates with CD4 count. Percentages of total and memory T-reg cells inversely correlate with CD4 count	Plasma HIV-1 viral load correlated inversely with the absolute number of memory and naive T-reg cells and percentage of naive T-reg cells	The absolute number of total, memory, and naive T-reg cells had a significant, inverse correlation with the percentages of CD38+HLA-DR+CD4+ and CD8+ T cells. The percentages of total, memory, and naive T-reg cells did not correlate significantly with the percent-activated CD4+ or CD8+ T cells.

Continues

Zhang et al., 2008	HD, HIV infected (asymptomatic and AIDS)	CD25+FOXP3+	Negative correlation between percentages and CD4 count	Positive correlation between percentages and VL	No correlation with CD4 and CD8 activation
Cao et al., 2009	HD, HIV infected (slow progressor, fast progressor)	CD25+ FOXP3+	Positive correlation between Treg numbers and CD4 count (in both SP and FP)		Positive correlation between Treg percentages and CD38+ HLA-DR+ CD4 T cells
Jiao et al., 2009	HD, VIR, ART (responder, non-responder, LTNP)	CD25+ FOXP3+ CD25high	Inverse correlation between CD25+ FOXP3+ cells percentages and CD4 counts Positive correlation between CD25+ FOXP3+ cells counts and CD4 counts		Positive correlation between percentage of apoptotic CD4+CD25high cells and percentage of CD38+ CD8 + cells. No correlation between percentage of apoptotic CD4+CD25high cells and percentage of HLA-DR+ CD8 + cells.
Tenorio et al., 2009	HD, VIR	CD25+ CD127low			No correlation.
Loke et al., 2010	HD, VIR, HIC	FOXP3		Viral load was directly related to the frequency of FoxP3 ⁺ Treg both in the resected biopsies and PB	
Piconi et al., 2010	ART (>500 CD4, >500 CD4)	CD25high FOXP3+ PD1 intracellular/extracellular	Inverse correlation of percentages of all studied subsets with CD4 count		
Prendergast et al., 2010	HD, VIR	CD25high FOXP3+			Inverse correlation between CD25high FOXP3+ absolute numbers and CD38 HLA-DR expression on CD8 T cells
Thorborn et al., 2010	HD, VIR	CD25+ FOXP3+	Positive correlation of Treg cell numbers with CD4 count		
Weiss et al., 2010	ART	CD25+ CD127low			Inverse correlation between percentage of CD25+ CD127low CD4 T cells and percentage of HLA-DR+ CD8 T cells
Brandt et al., 2011	HD, VIR, ART, HIC CD25+ FOXP3+	CD25+ CD127low FoxP3+	Inverse correlation between Treg percentages and CD3+CD4+ percentage among lymphocytes		Positive correlation between Treg percentages and percentage of CD38+ HLA-DR+ CD4 and CD8 T cells. Positive correlation between Treg percentages and percentage of Ki67+ CD4 and CD8 T cells.
He et al., 2011	HD, VIR, ART	CD25+ FOXP3+	Inverse correlation between CD25+ FOXP3+ percentages among CD4 and CD4 count		
Hunter et al., 2011	HD, VIR, ART, HIC	CD25+ CD127low			Positive correlation between CD25+ CD127low percentages and proportion of CD38+ HLA-DR+ CD8 T cells
Nikobva et al., 2011	HD, VIR, ART	CD25high Foxp3+ CD127low	CD39 expression on Treg correlates negatively with CD4+ T cell count	CD39 expression on Treg correlates positively with viral load	CD39 expression on Treg correlates positively with T cell activation
Schulze zur Wiesch et al., 2011	HD, VIR, ART, LTNP, HIC	CD25high Foxp3+ CD127low	Negative correlation Treg percentage vs CD4 count. Positive correlation Treg count vs CD4 count. CD39 expression on Treg correlates negatively with CD4+ T cell count	Positive correlation Treg percentage vs VL. Negative correlation Treg count vs VL. CD39 expression on Treg correlates positively with viral load	

Table 1.4 – Correlation of Treg levels to parameters of disease.

1.2 IL-7

1.2.1 IL7/IL7R Pathway - Molecular and cellular biology

1.2.1.1 IL-7

Interleukin 7 is a type I cytokine previously known also as “lymphopoietin 1” or “pre-B cell factor”.

Murine IL-7 was first discovered in 1988 as a soluble factor promoting the growth of murine B cell precursors (Namen et al. 1988). Subsequently, the human IL-7 gene was cloned (Goodwin et al. 1989). The murine IL-7 gene is 41 kb long, is located on chromosome 3 (locus 3 A1; 3 6.6 cM) and encodes for a protein of 154 amino acids with a 14,9 kDa molecular weight. The human IL-7 gene has a length of 72 kb, maps to chromosome 8 (locus 8q12-q13) and encodes for a 177 amino acids protein of 17,4 kDa. Comparison of the human nucleotide sequence with murine IL-7 shows high homology of the coding region (81%). A comparison of the human and murine IL-7 amino acid sequences again shows a strong degree of homology (60%). The major difference between the murine and the human proteins is that human IL-7 contains an insert of 19 amino acids (residues 96-114), not present in murine IL-7, which does not appear to be necessary for the biological activity of the cytokine (Goodwin et al. 1989; Lupton et al. 1990).

1.2.1.2.1 Sites of IL-7 Production

IL-7 production has been reported in thymic (Moore et al. 1993; Alves et al. 2009; Mazzucchelli et al. 2009; Shalapour et al., 2010) and bone marrow stromal cells (Funk et al. 1995; Mazzucchelli et al. 2009), which are considered the major source of this cytokine. IL-7 production has also been detected in spleen (Namen et al. 1988; Guimond et al. 2009), kidney (Namen et al. 1988; Sawa et al. 2009), liver (Golden-Mason et al. 2001; Sawa et al. 2009), intestine (Watanabe et al. 1995; Madrigal-Estebas et al. 1997; Shalapour et al., 2010) and skin (Heufler et al. 1993; Matsue et

al. 1993; Shalapour et al., 2010).

IL-7 synthesis in fetal thymus (Montgomery & Dallman 1997), fetal intestine (Murray et al. 1998) and embryonic brain (Michaelson et al. 1996) has been described.

In addition to stromal cells, several other populations of cells have been also shown to produce IL-7. These include endothelial cells, smooth muscle cells, fibroblasts (Kröncke et al. 1996), follicular dendritic cells (Kröncke et al. 1996) and cultured dendritic cells (Sorg et al. 1998; de Saint-Vis et al. 1998).

A point of major importance is that IL-7 production by normal lymphocytes have never been reported, rendering lymphocytes completely dependent from IL-7 production from other cell subsets. Another important point is that, despite some production of IL-7 from cells of the hematopoietic lineage (such as dendritic cells) has been reported, the major source of IL-7 is considered to be represented by tissue-resident non-hematopoietic cells (ex. stromal or epithelial cells).

1.2.1.2.2 Regulation of IL-7 production

Little is known about the mechanisms regulating IL-7 production; IL-7 secretion by stromal cells is believed to occur at a relatively constant rate and the increase in IL-7 levels described in lymphopenic settings (Fry et al., 2001; Guimond et al., 2009; see section 1.2.3.1) to result from a reduction in IL-7 consumption.

However several reports have challenged this view, proposing a more “dynamic” model of IL-7 secretion.

In vitro treatment with IFN- γ has been reported to increase IL-7 mRNA expression in a keratinocyte cell line (Ariizumi et al., 1995). Similarly, IFN- γ was shown to induce an increase in both IL-7 mRNA transcription and protein production in human colonic epithelial cell lines (Oshima et al., 2004).

Weitzmann and co-workers showed that interleukin-1 and TNF- α , two cytokines typically produced during inflammation, up-regulate production of IL-7 in human

stromal cells and osteoblasts (Weitzmann et al, 2000).

The first *in vivo* evidence of an inducible regulation of IL-7 production has been the demonstration that keratinocyte growth factor (KGF) treatment in mice is able to increase thymic IL-7 secretion (Min et al., 2002).

Sawa and collaborators elegantly showed that genetically determined activation of the IL-6/gp130/STAT3 pathway leads to an increased IL-7 production; similar results were obtained after exogenous IL-6 administration to wild type mice (Sawa et al., 2006). A more recent work demonstrated an inducible IL-7 production by the liver in response to type I IFNs inducing stimuli (Sawa et al.,2009).

Evidence for the existence of mechanisms of negative regulation of IL-7 synthesis derive from the *in vitro* observation that addition of TGF- β to stomal cell cultures inhibited IL-7 mRNA transcription and protein secretion (Tang et al., 1997). Whether this or other mechanisms negatively regulate IL-7 production *in vivo* remains to be further investigated.

In addition to the extrinsic control exerted by the aforementioned cytokines on IL-7 production, a recent work showed that IL-7 availability regulates IL-7 production by stromal cells by a negative feed-back mechanism (Guimond et al., 2009).

1.2.1.2 IL-7 Receptor

The IL-7 receptor (IL-7R) consists of two components, an α chain (IL-7R α or CD127), shared with thymic stromal lymphopoietin (TSLP), and a common gamma-chain (CD132), which is shared by the receptors for IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21. Signaling through the IL-7R requires both IL-7R α and the γ c component. Because CD132 is expressed ubiquitously on lymphocytes, IL-7 responsiveness is controlled largely by the presence or absence of IL-7R α .

1.2.1.2.1 IL7R α gene and protein

The murine IL-7R α gene is located on chromosome 15 and spans eight exons and seven introns with a size of 22.17 kb. In humans, the IL7R α gene is located on chromosome 5 spans eight exons and seven introns with a size of 19.79 kb.

IL7R α belongs to the type I cytokine receptor family. Cytokine receptors of this family are characterized by the presence in their extracellular domains of four conserved cysteine residues involved in intra-chain disulfide bonding and a membrane-proximal region WSXWS motif. In addition, their cytoplasmic domain contains a highly conserved proline-rich Box1 region.

IL7R α is a membrane-spanning glycoprotein of 439 AA including a 25 AA transmembrane domain and a 195AA cytoplasmic tail. While not presenting intrinsic tyrosine kinase activity, the IL-7R α intracellular domain contains four regions (A, S, T and the already mentioned BOX1) which serve as docking sites for the recruitment of several intracellular signaling molecules (reviewed in Jiang et al., 2005).

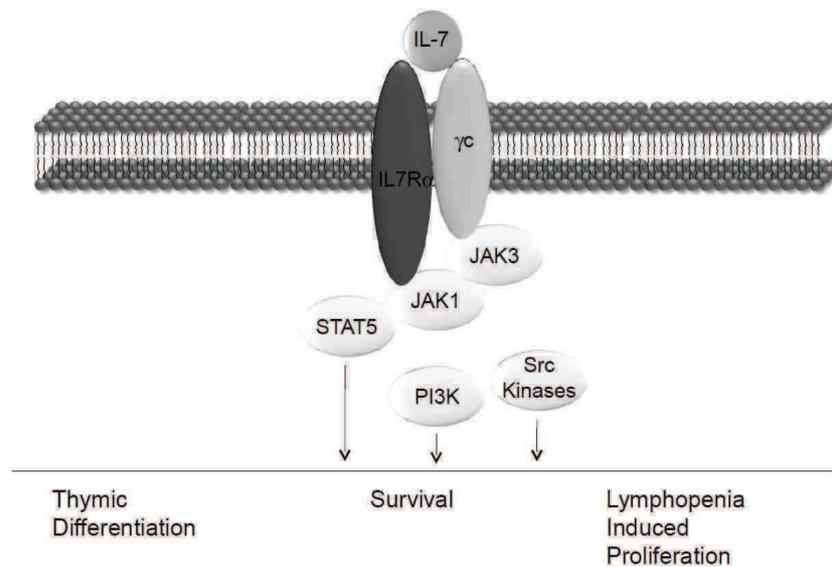


Figure 1.3 – IL-7/IL7R pathway. Schematic representation of the IL-7/IL7R pathway including molecules involved and main cellular effects.

1.2.1.2.2 Cell types expressing IL7R α

IL-7R α is expressed on hematopoietic cells of the lymphoid lineage from the stage of common lymphoid progenitors (CLP) (Kondo et al., 1997).

The expression of the receptor is preserved during the early phases of B cell development from the Pro-B cell stage to the Large Pre-B2 cell stage; thereafter IL-7R α expression is lost on B cells and, except for a controversial report (Hikida et al., 1998), there is no evidence for IL7R α expression on mature B cells.

Developing $\alpha\beta$ T cells express IL7R α throughout the different phases of the double negative (DN) stage; thereafter, IL7R α is down-regulated at the double positive (DP) stage and is re-expressed after the transition to CD4 or CD8 single positive (SP) stage. Both CD8 and CD4 mature T cells retain IL7R α expression in the periphery; the only exception seems to be represented by CD4+CD25+FOXP3+ regulatory T cells which have been reported to express low levels of IL-7R α (Gavin et al., 2002; Cozzo et al., 2003; Cox et al., 2005; Liu et al., 2006; Seddiki et al., 2006).

IL-7R α is also expressed on the cell surface of $\gamma\delta$ T cells and NKT cells, as well as by a small fraction of thymus-derived NK cells (Vosshenrich et al., 2006) and a subset of dendritic cells (Guimond et al., 2009). In addition, IL7Ra expression has been reported on Lymphoid Tissue inducers (LTi) (Yoshida et al., 1999), human marrow stromal cells (Iwata et al., 2002) and human endothelial cells (Dus et al., 2003).

Several types of cancer can express IL-7R α : B and T cell acute lymphoblastic leukemia, Hodgkin's lymphoma, cutaneous T cell lymphomas, colorectal cancer cells and renal cancer cells, as well as several malignant cell lines (including lung and breast cancer cell line, leukemia, melanoma) (Cosenza et al., 2002; Cattaruzza et al., 2009; Silva et al., 2011). Recently, gain-of-function mutations in IL-7R α have been identified in childhood acute lymphoblastic leukemias, conferring cytokine-independent growth of progenitor lymphoid cells (Shochat et al., 2011).

1.2.1.2.3 Regulation of IL-7R α expression

Two transcription initiation sites for IL-7R α have been reported, the first at approximately 942 base pairs (bp) of the translation start codon ATG (DeKoter et al., 2002), the second at position -90 (Xue et al., 2004).

In pro-B cells, PU.1 has been shown to bind a GGAA Ets-binding motif present in the promoter region within 197 base pairs upstream of the ATG translation start site (DeKoter et al., 2002). The transcription factor binding the PU.1 site differs between the B-cell and T-cell lineages. PU.1 is expressed in early lymphoid progenitors and its expression is retained throughout B cell development; however, expression of PU.1 is lost after commitment to T cell lineage development. GABP (GA-binding protein transcription factor) has been shown to bind the GGAA PU.1 site and to be required for IL-7R α expression by thymocytes and peripheral T cells (Xue et al., 2004).

Recently, two groups demonstrated a role for the transcription factor FOXO1 (Forkhead-Box-O 1) in IL-7R α expression (Ouyang et al., 2009; Kerdiles et al., 2009). These works showed that, in T cells, FOXO1 directly binds an evolutionarily conserved regulatory sequence of the IL-7R α gene located 3.5 kb upstream of the transcription initiation site and induces IL-7R α expression. FOXO1 deletion resulted in the blockade of IL-7-induced survival and substantially affected T cell homeostasis *in vivo*.

FOXP3, a transcription factor expressed in regulatory T cells (see section 1.3.1), has been reported to bind the IL-7R α promoter region (Liu et al., 2006). Associated with the inverse correlation between CD127 and FOXP3 expression (Liu et al., 2006; Seddiki et al., 2006), FOXP3 mediated repression of IL-7R α gene transcription has been suggested.

IL-7R α expression on mature T cells is strictly regulated and influenced by extrinsic stimuli, such as antigens and cytokines.

Several types of extrinsic stimuli able to modulate IL-7R α expression have been reported.

Glucocorticoids induce up-regulation of IL-7R α on both human (Frachimont et al., 2002) and murine (Lee et al., 2005) T cells; this process was shown to be dependent on the presence of a glucocorticoid response element (GRE) identified at 3.6 kb upstream of the translation initiation site of IL-7R α gene (Lee et al., 2005).

The inflammatory cytokine TNF α increases both IL7R α gene transcription and surface protein expression (Park et al., 2004).

Major insights about the regulation of IL7R α expression derive from the work of Park and colleagues (Park et al., 2004); the authors demonstrated that IL-7 itself down-regulates cell-surface IL-7R α expression by suppressing IL-7R α transcription in both CD4⁺ and CD8⁺ T cells. In CD8 T cells, this process involves the transcriptional repressor protein GF1; however GF1 does not seem to be necessary for cytokine-induced IL-7R α down-regulation in CD4 T cells and, in these cells, the molecular basis of the process remains to be elucidated.

In addition to IL-7, other cytokines of the γ c family (IL-2, IL-4, IL-15), as well as IL-6, are able to suppress IL-7R α expression on T cells surface (Xue et al., 2002; Park et al., 2004). IL-2 mediated suppression was shown to involve the activation of the phosphatidylinositol-3-kinase (PI3K) – AKT pathway (Xue et al., 2002).

TCR triggering in peripheral T cells induces transient down-regulation of IL-7R α (Schluns et al., 2000; Franchimont et al., 2002). An exception to this paradigm seems to be represented by memory CD8 T cells progenitors which have been reported to retain high levels of IL-7R α expression in a murine model of viral infection (Keach et al., 2003). However such a mechanism has not been confirmed in a peptide immunization model (Lacombe et al., 2005).

Globally, a common feature of the signals reported to suppress IL-7R α expression is their ability to promote T cell survival. IL-7R α down-regulation at the surface of cells that already received a survival signal could stop their IL-7 consumption. As IL-7 seems to be present at limited amounts *in vivo*, suppression of IL-7R α surface expression could represent an “altruistic” mechanism to optimize IL-7 availability (Park et al., 2004; Mazzuchelli et al., 2007).

1.2.1.3 IL-7 signaling

IL-7 signaling involves several pathways including the Janus kinase/Signal Transducer and Activator of Transcription (Jak/STAT) pathway, the phosphatidylinositol 3-kinase (PI3-kinase) pathway, and Src family tyrosine kinases.

1.2.1.3.1 Jak/STAT pathway

IL-7 binding to IL-7R α leads to heterodimerization of the α chain with the γ_c , which also possesses binding sites for IL-7. Jak3, a tyrosine kinase constitutively associated with the carboxy-terminal region of the γ_c , phosphorylates tyrosine residues in the cytoplasmic portion of IL-7R α , leading to recruitment of Jak1. This process creates docking sites for the transcription factors STAT1, 2, 3 and 5. STATs then dimerize, translocate to the nucleus, bind DNA and regulate the transcription of specific genes. Although IL-7 induces the phosphorylation of STAT1, 2, 3 and 5, STAT5 seems to play the pivotal role in transduction of IL-7 signaling. IL-7 induces phosphorylation of both STAT5 isoforms, STAT5A and B. Deficiency of STAT5A or STAT5B individually does not have severe consequences on T and B cell development; early studies of mice with combined STAT5A/B deficiency failed to reveal major impairment of lymphoid development (Teglund et al., 1998). These observations led to the conclusion that STAT5 has limited roles in IL-7 effects on immune cells development and function. However, the gene targeting strategy used in the original STAT5 knockout mice resulted in a partially functional STAT5 protein. More

recently, entire deletion of the Stat5a/b locus demonstrated an essential role of STAT5 in IL-7 signaling (Yao et al., 2005).

1.2.1.3.2 Phosphatidylinositol 3-kinase pathway

IL-7 has been clearly shown to activate the PI3 kinase pathway in human thymocytes (Dadi 1993). Following IL-7 stimulation, Jak3 was found to induce PI3 kinase activation by phosphorylating p85 (Sharfe 1995). Interestingly, distinct roles of the PI3-Kinase and STAT5 pathways in IL-7 effects on development of human thymocyte precursors have been proposed; by testing in a fetal thymic organ culture (FTOC) human thymic progenitors overexpressing IL-7 chimeric receptors that were no longer able to activate the PI-3K/PKB or the STAT5 pathway and Pallard et al. demonstrated that the activation of PI3-kinase was essential for the IL-7-mediated survival and proliferation of human T cell precursors (Pallard et al., 1999). Moreover, pharmacological inhibition of PI3-kinase completely blocked the survival effect of IL-7 on pro-T2 and -T3 cells, but not T1 cells (Khaled et al., 2002).

AKT, a key downstream target of PI3 kinase, has been shown to be activated after IL-7 treatment in an IL-7-dependent mouse thymocyte cell line (Li et al., 2004) and human thymocytes (Pallard et al., 1999). Activated AKT, in turn, phosphorylates the death protein Bad, inactivating it. However, the inactivation of Bad by IL-7 only partially depends on the PI3 kinase/AKT pathway (Li et al., 2004).

1.2.1.3.3 Src kinase pathway

Src family protein kinases include nine members, Src, Lck, Hck, Fyn, Blk, Lyn, Fgr, Yes and Yrk. Src family kinases are activated by IL-7 (Seckinger et al., 1994), but none have been shown to be uniquely required for IL-7 signaling.

1.2.2 Biological impact of IL7/IL7R pathway

1.2.2.1 IL-7 in lymphocytes development

Evidence of a unique, non-redundant role for IL-7 in murine B and T lymphocyte development comes from studies demonstrating the paucity of lymphocytes present in IL-7- and IL-7R α -deficient mice and following IL-7 or IL-7R α neutralization *in vivo*. Thymic cellularity is reduced 20-fold in IL-7 $^{-/-}$ mice (von Freeden-Jeffry et al., 1995) and 0.01–10% of normal in CD127 $^{-/-}$ mice (Peschon et al., 1994). Moreover, in mice IL-7 deprivation *in vivo* by long-term administration of a neutralizing anti-IL-7 antibody completely inhibits the development of B cell progenitors from the pro-B cell stage forward (Grabstein et al., 1993) and results in a > 99% reduction of thymic cellularity (Bhatia et al., 1995). Similar results were obtained using the A7R34 antibody, that recognizes CD127 and blocks the binding between IL-7 and IL-7R α (Sudo et al., 1993). In addition to its role on classical $\alpha\beta$ T cell development, IL-7 is also necessary for development of $\gamma\delta$ T cells as this cell subset does not develop in IL-7 KO (Moore et al., 1996) or CD127 KO (He et al., 1996; Maki et al., 1996) mice.

In humans, IL-7R α chain mutations have been reported and result in a particular T $^{-}$ B $^{+}$ NK $^{+}$ type of severe combined deficiency, confirming in humans that IL-7 is essential for T cell lymphopoiesis (Puel et al., 1998). However, because these patients had B cells, these results demonstrated that IL-7 is not absolutely required for B cell development in humans.

IL-7 exerts its activity on thymopoiesis by acting at different sites and through different mechanisms.

As mentioned, CD127 expression is regulated through the thymic developmental stages : CD127 is expressed at the double-negative stage, absent at the double-

positive stage, then re-expressed at the single-positive stage (Sudo et al., 1993). This IL-7R α expression pattern correlates with the responsivity to IL-7 of thymic cell subsets as measured by STAT5 phosphorylation (Van De Wiele et al., 2004). Forced transgenic expression of CD127 on DP cells causes increased death of DN cells (Munitic et al., 2004) suggesting that DP cells down-regulate CD127 in order to ensure continuous availability of IL-7 to DN and SP T cells.

IL-7 primarily acts as a survival factor for developing thymocytes through modulation of apoptosis-related molecules including Bcl-2 and Bax (von Freeden-Jeffry et al., 1997; Kim et al., 1998). This model of action is further supported by the demonstration that defective lymphocyte development in mice with impaired IL7/IL7R signaling can be at least in part rescued by either Bcl-2 overexpression (Maravskovsky et al., 1997) or Bax deficiency (Khaled et al., 2002).

Although protecting the cells from apoptosis restores $\alpha\beta$ T cells development in the absence of IL-7 signaling, gamma delta T cells are still lacking. Indeed, IL-7R-mediated signaling is necessary for the rearrangement of the TCR γ -chain locus (Maki et al., 1996; Candéias et al., 1997a). Involvement of IL-7 in rearrangement of the other TCR loci is less well established (Candéias et al., 1997b).

Finally, a recent study suggests that IL-7 signaling could be implicated in CD8 lineage choice during thymocytes transition to the single positive stage. Park et al. reported that IL-7 signaling in positively selected thymocytes induces expression of the transcription factor Runx3 and specifies CD8 lineage choice (Park et al., 2010).

1.2.2.2 IL-7 in T lymphocytes homeostasis

Survival - IL-7 is required for survival of CD4⁺ and CD8⁺ T Cells. First *in vitro* studies revealed a survival effect of IL-7 on T cells (Maraskovsky et al., 1996; Vella

et al., 1997; Hassan et al., 1998.). The first *in vivo* evidences of IL-7 implication in T cell homeostasis were obtained studying the decay of T lymphocyte after IL7/IL7R signaling blockade in thymectomized mice (Boursalian et al., 1999; Vivien et al., 2001). Adoptive transfer experiments confirmed the importance of IL-7 for naïve CD4 and CD8 T cells survival *in vivo* (Schluns et al., 2000; Tan et al., 2001).

IL-7 mainly promotes cell survival by preventing the mitochondrial pathway of apoptosis. In this respect, antiapoptotic Bcl-2 and Mcl-1 appear to play a dominant role as IL-7 signaling regulates Bcl-2 (von Freeden-Jeffry et al., 1997) and Mcl-1 expression (Opferman et al., 2003).

Bcl-2 and Mcl-1 are thought to mediate their function by directly regulating the activity of the death effectors Bax and Bak, which cause apoptosis by inducing the release of cytochrome c and other molecules from the mitochondria to initiate the activation of caspases. Indeed, using a IL-7 dependent thymocyte cell line it has been shown that withdrawal of IL-7 induces Bax translocation from cytosol to mitochondria finally resulting in cell death (Khaled et al., 1999). IL-7, inducing cytosolic retention of Bax, prevents this process.

Bad is another pro-apoptotic member of the Bcl-2 family and belongs to the group of “BH3 domain-only” proteins that bind to Bcl-2 and block its function. IL-7 induces Bad phosphorylation at three serine residues (Ser-112, -136, and -155) resulting in its sequestration and in prevention of apoptosis (Li et al., 2004).

The proapoptotic protein Bim, another BH3-only protein belonging to the Bcl-2 family, also seems to play a role in peripheral T-cell survival. Deletion of Bim partially protects peripheral T cells from IL-7 deprivation. (Li et al., 2010). IL-7 withdrawal does not alter the intracellular location of Bim, its association with Bcl-2 or its mRNA or protein expression. IL-7 seems to inhibit Bim activity at the post-translational level.

Regulation of metabolic activity - In addition to the aforementioned mechanisms, IL-7

signaling also induces T cell survival by regulating their metabolic activity. IL-7 mediated signals stimulate glucose uptake and cell-surface localization of Glut1 *in vitro* (Wofford et al., 2008). Moreover, IL-7 regulates glucose metabolism by modulating the intracellular glucose retention and the transcription of the glycolytic enzyme hexokinase II (Chehtane et al., 2010). Recently the essential role of IL-7 for the control of T cell glucose metabolism has been confirmed *in vivo* (Jacobs et al., 2010). After conditional deletion of IL-7R α in mature T cells, T cells failed to maintain a basal rate of glycolytic flux and undergo cell atrophy, which is characterized by decreased cell size and rate of growth. In contrast to *in vitro* studies loss of IL-7R α *in vivo* did not affect GLUT1 surface expression and glucose transport.

Lymphopenia induced proliferation - In addition to its role on T cell survival, Schluns et al. and Tan et al. also demonstrated that IL-7 is required for the proliferation of both CD8⁺ and CD4⁺ naive T cells in lymphopenic hosts, whereas other cytokines tested (IL-4, IL-15) were not required (Schluns et al., 2000; Tan et al., 2001). Importantly, similar results were obtained regardless of the method of lymphopenia induction (genetic or irradiation induced) eliminating the potential bias of irradiation or other exogenous environmental factors.

Investigating the molecular basis of IL-7-induced T cells proliferation, Li et al., showed that IL-7 down-regulate the cyclin-dependent kinase inhibitor p27Kip1 through a post-translational mechanism (Li et al., 2007). Moreover the authors showed that CD4 or CD8 T cells transferred into IL-7-deficient hosts underwent G1 arrest, whereas 27Kip1-deficient T cells underwent proliferation independently from the availability of IL-7.

Naive T cells also require contact with self-peptide major histocompatibility complexes (MHCs) to be driven into proliferation in condition of lymphopenia

(Goldrath et al., 1999; Kieper et al., 1999; Ernst et al., 1999). Clarifying the specific role of IL-7 in this phenomenon, Seddon et al. demonstrated that TCR signals and IL-7R signals are each able to stimulate T cell proliferation in lymphopenic hosts independently of one another, but can also synergize to facilitate proliferation (Seddon et al., 2002).

IL-7 in memory T cells homeostasis - If a role for IL-7 in naive T cell survival and homeostatic proliferation has been accepted since earliest studies, IL-7 requirement for memory T cells homeostasis has been more debated.

CD8 memory T cells seem to have no absolute requirement for IL-7/IL7R signaling for their maintenance in replete mice, and they require IL-15 for homeostatic proliferation in non-lymphopenic hosts (Ku et al., 2000). However, IL-7 does contribute to CD8 memory T cells proliferation after transfer into lymphopenic hosts (Goldrath et al., 2002; Tan et al., 2002) and its over-expression can overcome the dependency of memory phenotype CD8⁺ cells on IL-15 (Kieper et al., 2002).

A more complex picture resulted for the action of IL-7 on CD4 memory T cells. First studies demonstrated that polyclonal CD4 memory T cells injected into lymphopenic hosts proliferate independently of IL-7 availability (Tan et al., 2002). Subsequently, Seddon et al. showed that in the absence of TCR signaling, IL-7R signals promoted proliferation and survival of CD4⁺CD44^{hi} cells (Seddon et al., 2003). Moreover these authors showed that, in the absence of IL-7, wild type CD4⁺CD44^{hi} T cells proliferated normally but failed to accumulate, pointing to a role for IL-7 for *in vivo* survival of memory CD4 T cells. Further *in vitro* and *in vivo* experiment supported a role for IL-7 in memory CD4 T cells survival (Kondrack et al., 2003).

Accordingly with their low CD127 expression Treg seem to be the only T cell population whose peripheral homeostasis is IL-7 independent (Liu et al., 2010) (See section 1.3.4.2).

1.2.2.3 IL-7 and regulation of immune responses

1.2.2.3.1 IL-7 effects on effector T cells

Due to its well established role in lymphocyte development and peripheral T cell homeostasis, IL-7 is considered a “prototypical” homeostatic cytokine. As discussed above, during immune responses IL-7R α is down-regulated after activation and IL-7 is not thought to participate in the effector response, a phase considered dependent on other γ c cytokines, such as IL-2 and IL-15. However several lines of evidence indicate that IL-7 modulates effector T cells responses.

Soon after its discovery as a B-cell growth factor, it was shown *in vitro* that addition of IL-7 to ConA (Morrisey et al., 1989) or PMA (Chazen et al., 1989) stimulated murine T lymphocytes increased cell proliferation as revealed by a day 3 Thymidine incorporation assay. Similar results were obtained with anti-CD3 stimulation of murine (Costello et al., 1993) and human T cells (Armitage et al., 1990). Remarkably, IL-7 incubation during activation led to an increase in IL-2R α expression at T cells surface compatible with a synergic effect of IL-7 on IL-2 signaling (Morrisey et al., 1989; Armitage et al., 1990; Grabstein et al., 1990; Costello et al., 1993). However addition of anti-IL-2 blocking antibodies suppress IL-7 effect on T cell proliferation completely after PMA stimulation (Chazen et al., 1989), partially after α CD3 α CD28 stimulation (Costello et al., 1993) but has no effect during ConA, OKT3 or PHA stimulation (Morrisey et al., 1989; Armitage et al., 1990; Grabstein et al., 1990). Collectively these results indicate the existence of both IL-2 dependent and independent effects of IL-7 on effector T cell responses.

Unfortunately study set-up in these earliest reports did not allow distinguishing between true proliferative increases and effects on effector T cell survival. More recently, use of CFSE dilution method allowed better investigating the “proliferation

history” of cells activated in the presence or in the absence of IL-7. Using CFSE labeled TCR transgenic CD8 cells stimulated with their specific antigen in the presence or absence of IL-7, Saini et al. showed that the addition of IL-7 had no effect on either the proportion of cells triggered into division or the size of their proliferative burst after 72 hours of activation (Saini et al., 2008).

Interesting insights on the costimulatory action of IL-7 on T cell responses derive from experiments showing that T cells isolated from IL-7-treated mice have enhanced proliferative responses to *in vitro* stimulation (Komschlies et al., 1994; Geiselhart et al., 2001). Accordingly, *in vitro* responses of human T cells to PHA, anti-CD3 and PPD stimulation positively correlated to circulating levels of IL-7 *in vivo* (Churchman et al., 2008). As an explanation for this accelerated proliferative capacity, IL-7 was shown to induce T cells to move into cell cycle. Indeed, *in vivo* administration of 10µg of recombinant human IL7 to mice i.p. twice a day for 2 days led to an about 10-fold increase in CD4 and CD8 T cells that were in either S phase or G2/M (Geiselhart et al., 2001). Although this and subsequent studies show that IL-7 induces proliferation of T cells *in vivo*, i.e. in the presence of potential TCR engagement, whether IL-7 is able to induce cell cycle entry and proliferation of T cells independently of any other stimuli is still debated. Welch et al. showed that *in vitro* incubation of human peripheral CD4 and CD8 T cell with IL-7 alone induced cell proliferation (Welch et al., 1989). Such results were supported by some reports (Armitage et al., 1990; Londei et al., 1990) and challenged in others (Costello et al., 1993). Discrepancies in results could be due to differences in residual contaminating APC in the culture as well as in the composition of cell populations studied. Indeed less mature T cells appear to proliferate more vigorously to IL-7 than T cells from adults do. IL-7 more efficiently drives cell cycle entry and proliferation of naive CD4 cells from umbilical cord blood than CD4⁺ T cells isolated from adult peripheral blood (Fukui et al., 1997; Soares et al., 1998 ; Dardalhon et al., 2001). In accordance, naive CD4+

CD45RA⁺ cells expressing CD31, a molecule identifying recent thymic emigrant cells, represent the main population from adult peripheral blood that undergo IL-7–induced proliferation (Azevedo et al., 2009).

In addition to its controversial effect on T cell proliferation, IL-7 clearly affects effector T cell survival. *In vitro*, IL-7 has been shown to prevent cell death of *in vivo* activated T cells (Pellegrini et al., 2003; Pellegrini et al., 2009). Similarly, *in vivo* administration of IL-7 induces increases in effector CD4 and CD8 T cells numbers upon stimulation (Melchionda et al., 2005; Boyman et al., 2008; Pellegrini et al., 2009; Pellegrini et al., 2011) although one study failed to confirm these results in a model of LCMV infection (Nanjappa et al., 2008; see section 1.2.2.3.2). Interestingly Melchionda et al showed that injection of IL-7 as an adjuvant in a prime-boost immunization strategy while increasing effector cells directed against dominant antigens also dramatically enhanced effector CD8 responses directed to subdominant antigens (Melchionda et al., 2005).

In addition to affect quantitatively effector T cells responses, IL-7 appears to be able to promote CD4 and CD8 T-cell function. Pellegrini et al. showed that effector CD8⁺ T cells isolated from vaccinated IL-7–treated mice presented a tenfold increase in granzyme B and IFN γ production, an increased capacity to degranulate and, on a per cell basis, a higher cytotoxic activity (Pellegrini et al., 2009; Pellegrini et al., 2011). Concerning the CD4 T cell compartment, IL-7 treatment of immunized mice led to an increase in IL-2, IFN γ and IL-17 secreting CD4 T cells (Pellegrini et al., 2009; Pellegrini et al., 2011). The biological significance of such effects was confirmed *in vivo* by the increased efficacy in models of cancer (Pellegrini et al., 2009) and of chronic viral infection (Pellegrini et al., 2011).

Another mechanism through which IL-7 beneficially affects effector T cells responses is linked to its capacity to promote T cell refractoriness to inhibitory networks. First, IL-7 renders effector cells refractory to Treg cell–mediated inhibition (Ruprecht et al., 2005; Pellegrini et al., 2009). Second, IL-7 reduced the sensitivity to TGF β sig-

ning of effector CD8 T cells, a phenomenon that was associated with a reduction in FOXO3, Cbl-b and Smad2 levels (Pellegrini et al., 2009). Third, IL-7 repress Socs3 Inhibitory Pathway in effector cells (Pellegrini et al., 2011).

1.2.2.3.2 IL-7 effects on memory T cells development

If only a few studies have directly assessed the effect of IL-7 on effector T cells responses, the role of IL-7 in CD8 and CD4 memory T cells development has been an area of intense investigation.

A typical CD8 T cell response consists of three main stages: i) an effector cell expansion and differentiation phase, in which antigen specific T cell undergo intensive clonal expansion and differentiation into effector cells ; ii) a contraction phase, in which 90-95% of effector cells undergo apoptosis; iii) a final phase in which a population of stable memory cells is generated.

The issue of how memory CD8 T cells are generated still remains open. Two main CD8 T cell memory differentiation models have been proposed. According to the linear differentiation model, naive CD8⁺ T cells differentiate upon activation by the antigen into cytotoxic T lymphocytes (CTL), a fraction of which survive during the contraction phase to become memory CD8⁺ T cells (Opferman et al., 1999). Conversely, the asymmetrical differentiation model predicts that memory T cells differentiate through a lineage parallel to effectors (Chang et al., 2007).

As already discussed, naive CD8 T cells express high levels of IL-7R α but during activation the expression of the receptor is greatly reduced on effector T cells, to be finally re-upregulated on memory cells (Schluns et al., 2000). Using OT-1 transgenic T cells that were either normal or knock-out for IL-7R α , Schluns et al demonstrated that IL-7R α is dispensable for antigen-induced expansion but is essential for CD8⁺

memory T cell production (Schluns et al., 2000). These results support a model in which IL-7 availability does not affect T cells responses during the effector phases but acts at later stages to allow memory T cells generation by increasing cell survival during the contraction phase. Such a mechanism of action for IL-7 perfectly fits with a linear differentiation model of CD8 T cells memory differentiation.

Another potential mechanism of action for IL-7 has been proposed, which in contrast supports more an asymmetric model of memory differentiation. By carefully analyzing the kinetic of CD127 expression on antigen specific cells during murine LCMV infection, Kaech et al, observed that a small portion (5–15%) of early effector cells maintained a higher expression of IL-7R α , and that thereafter the proportion of IL-7R α hi LCMV specific CD8 T cells continued to increase until nearly all were IL-7R α hi at late time-points (Kaech et al., 2003). Importantly increased IL-7R α expression on effector cells correlates with increased survival signals and less apoptosis, suggesting that IL-7R α hi effectors could represent a population of memory T cells precursors. To experimentally address this hypothesis, using several adoptive transfer experiments, the authors showed that IL-7R α hi effectors, but not IL-7R α low cells, developed into long-lived memory cells and provided immunological protection upon re-challenge.

However, such an interesting model has been subsequently attacked by several evidences. First, following peptide immunization high levels of IL-7R α expression do not identify CD8+ memory T lymphocyte precursors (Lacombe et al., 2005). Subsequently, it has been shown that memory generation in IL-7 deficient environments was not altered (Klonowski et al., 2006). However a major drawback of such approach was that experiments were performed by adoptively transfer CD8 T cells into IL-7 $^{-/-}$ or IL-7R $\alpha^{-/-}$ mice, therefore in a highly lymphopenic environment, in which increased availability of other growth factors may also play a role. Indeed, using a conditional IL-7R transgenic model, Buentke et al. showed that IL-7R-deficient ef-

factors give rise *in vivo* to resting memory cells in lymphopenic environment, although IL-7R α expression is essential for survival of newly formed memory cells in replete hosts (Buentke et al. 2006). Moreover the authors showed that the ability of IL-7R α -deficient CD8 effectors to survive in empty but not replete hosts was dependent on IL-15 availability.

Finally, evidence reported by two groups using genetically engineered mice, in which IL-7R α was constitutively expressed at high level and was not reduced upon activation, revealed that expression of IL-7R α was not sufficient for the formation of memory CD8 T cells in models of viral and bacterial infection (Hand et al., 2007; Haring et al., 2008).

Usage of IL-7 as an adjuvant for strategies of vaccination or immunotherapy has been evaluated in preclinical studies. Evaluating the effects of IL-7 on long term memory responses induced by a prime-boost immunization strategy against the male antigen, the aforementioned study by Melchionda et al. demonstrated that IL-7 treated mice showed at late time points a significant increase in the frequency of memory cells producing IFN γ in response to the immunodominant CD8 epitope (Melchionda et al., 2005). More importantly this difference was associated with increased survival of IL-7 treated mice upon challenge with a male-antigen expressing tumor.

Trying to identify the phase of the response at which IL-7 can act to increase the memory T cell pool in both viral infection and DNA immunization models, Nanjappa et al. showed that IL-7 significantly increases memory T cell numbers when administered during the contraction phase, although the effects were undetectable when the cytokine was administered during the expansion phase (Nanjappa et al., 2008).

Collectively, published data lead to conclude that, although dispensable in particular situations such as lymphopenia, IL-7 availability affects the size of the memory T cells pool generated and maintained at the end of acute immune responses.

Conclusions drawn in CD8 T cells have been partially extended to the CD4 T cells compartment. Using an adoptive transfer model of *in vitro* or *in vivo* generated CD4 effector T cells two studies showed that in the absence of IL-7 signaling cell numbers recovered were diminished at late time points (Kondrack et al., 2003; Li et al., 2003). These experiments have led to a model, similar to that discussed for CD8 T cell, in which IL-7 regulates the transition of CD4 effectors to persistent memory cells.

1.2.3 IL-7 in disease

1.2.3.1 IL-7 and lymphopenia

Lymphopenia is a relatively frequent clinical condition which is characterized by a decrease in the number of lymphocytes. Lymphopenia is often encountered as a consequence of lymphodepleting therapies such as cancer chemotherapy or conditioning regimens for bone marrow transplantation. Moreover, a lymphopenia restricted to the CD4 T cell compartment is the hallmark of late stage of HIV infection.

IL-7/IL-7R axis alterations can be either the cause or the consequence of T cell lymphopenia.

As already mentioned genetic defects in IL-7R α or in proteins implicated in IL-7 signal transduction, such as JAK3 deficiency, results in SCID, which are characterized by almost complete absence T cell development and therefore extremely severe lymphopenia (Giliani et al., 2005).

Conversely levels of circulating IL-7 can be increased as a consequence of T cell lymphopenia associated with several conditions. The first study demonstrating a negative correlation between absolute lymphocytes count and IL-7 levels was performed in pediatric patients undergoing bone marrow transplantation (Bolotin et al.,

1999). Thereafter, those results were extended by demonstrating that low levels of CD3 T cells, and particularly of CD4 T cells, were associated with high levels of IL-7 in sera from patients with cancer-chemotherapy associated lymphopenia (Fry et al., 2001) as well as from HIV patients (Napolitano et al., 2001; Fry et al., 2001) and patients affected by idiopathic CD4 T cell lymphopenia (Fry et al., 2001; Malaspina et al., 2007). As discussed earlier, the inverse correlation between IL-7 levels and T cell numbers led to the formulation of a model in which IL-7 levels are determined by IL-7 consumption, and therefore by the numbers of target cells.

HIV infection - HIV infection presents some peculiarities among CD4 T cells lymphopenias in respect of IL-7/IL-7R axis biology. In addition to the aforementioned alterations in circulating IL-7 levels, several groups have reported a defect in binding and transduction of IL-7 signaling.

First, in patients with HIV infection, a reduced expression of CD127 on T cells is observed, especially within the CD8⁺ T cell compartment (Carini et al., 1994; Vingerhoets et al., 1998). Several non-exclusive mechanisms can be involved such as negative feedback of high IL-7 levels on CD127 expression (Rethi et al., 2005), hyper-activation state (Paiardini et al., 2005; Benito et al., 2008) or inhibitory effect of Tat protein on CD127 expression (Faller et al., 2006). CD127 expression on T cells is only partially restored after effective antiretroviral therapy (MacPherson et al., 2001). Interestingly Lécroux and colleagues showed that antiretroviral therapy initiation during primary HIV infection enhances CD127 expression on HIV-specific CD8⁺ T cells (Lécroux et al., 2009).

Cellular effects of IL-7 seem to be reduced as a results of impaired signal transduction. Early reports showed that the anti-apoptotic effects mediated by IL-7 were reduced in T cell from HIV-infected individuals (Carini et al., 1994;

Vingerhoets et al., 1998). This defect has been confirmed by other studies by measuring IL-7-driven T-cell proliferation or expression of the antiapoptotic molecule Bcl-2 (Colle et al., 2006). CD127 down-regulation can not entirely justify the defect as the positive linear relationship between CD127 expression and the magnitude of IL-7-induced responses found in healthy individuals is lost in HIV-infected patients, suggesting that, downstream of IL-7R, cellular defects may contribute to a decrease in IL-7 responsiveness (Colle et al., 2007). Studies of STAT5 phosphorylation upon IL-7 treatment have found a defect in IL-7 signal transduction in both CD4 and CD8 T cells from viremic HIV patients (Juffroy et al., 2010; Vranjkovic et al., 2011).

1.2.3.2 IL-7 and autoimmunity

A link between the IL-7/IL-7R pathway and autoimmunity is supported by genetic as well as experimental data.

Genetic studies have identified a polymorphism in IL-7R α chain as risk factors for Multiple Sclerosis (MS), a T cell mediated neurological disorder characterized by focal sclerotic lesions with myelin loss and infiltration of inflammatory cells in the central nervous system (Hafler et al., 2007; Gregory et al., 2007; Lundmark et al., 2007). These analyses identified a non-synonymous coding single-nucleotide polymorphism in IL-7R α (rs6897932) that affects alternative splicing, leading to the skipping of exon 6. This polymorphism leads to produce less membrane-bound IL7R α protein, leading to an increase of the soluble form of IL7R α (Gregory et al., 2007). Moreover IL-7R α and IL-7 mRNA expression is higher in the cerebrospinal fluid (CSF) of individuals with (MS) than in the CSF of individuals with other noninflammatory neurological disorders (Lundmark et al., 2007).

In a recent work, alterations in IL-7/IL-7R signaling components in MS patients have been associated to disrupted generation, composition and function of circulating Treg, leading to aberrant T-cell homeostasis (Haas et al., 2011). Moreover, the authors found, in

association to lower level of surface expression of IL-7R α by T cells, higher levels of circulating IL-7 in the sera from MS patients.

Elevated levels of IL-7 have been reported in several others immune-mediated diseases such as juvenile idiopathic arthritis (De Benedetti et al., 1995; Ruprecht et al., 2005), rheumatoid arthritis (van Roon et al., 2003), spondylarthritis (Rihl et al., 2008) and Sjögren's syndrome (Bikker et al., 2010). In contrast to lymphopenia, higher levels of IL-7 do not seem to be caused by reduced consumption as IL-7 accumulation seems to be unrelated to lymphoid depletion (Ponchel et al., 2005). Interestingly IL-7 production by the target organs of diseases, such as the synovium and the salivary glands, has been reported (Harada et al., 1999; Bikker et al., 2010). Moreover, inflammatory mediators such as IL-1 β and TNF α increase IL-7 synthesis by some target cells, such as synoviocytes (Harada et al., 1999).

We can therefore imagine a model in which inflammation induces IL-7 secretion by target cells which in turn lead to sustained pathological immune-responses.

Such a positive feedback loop could represent a potential target for IL-7 blocking therapeutic strategies. This working hypothesis has been experimentally evaluated in a pre-clinical model of collagen induced arthritis: IL-7R α blockade significantly reduced arthritis severity, T cell activity and inhibited local concentrations of proinflammatory mediators (Hartgring et al., 2010).

1.2.3.3 IL-7 therapy – Clinical Trials

A form of recombinant human, E. Coli produced, nonglycosylated IL-7 (CYT99007, Cytheris Inc., Rockville, MD) is currently under evaluation in several phase I and II clinical trials (Table 1.1). Most of the studied indications are conditions of severe lymphopenia, including HIV infection, idiopathic CD4 T cells lymphopenia, cancer-chemotherapy induced lymphopenia and post-allogenic bone marrow transplantation. In those conditions IL-7 is used with the aim to increase and accelerate immune-reconstitution. A second group of diseases, for which IL-7 evaluation is at a less

advanced stage, is represented by diseases not characterized by lymphopenia and in which IL-7 is tested for its immuno-modulatory and adjuvant properties. These indications include refractory solid tumors (alone or in combination with therapeutic vaccination); HBV and HCV infections.

Published phase I studies globally report a dose dependent expansion of both CD4 and CD8 T cell compartments (Rosenberg et al., 2006; Sportes et al., 2008; Sereti et al., 2009; Levy et al., 2009). The effects of rhIL-7 on T cell expansion have been attributed to a combination of increased cell cycling and diminished programmed cell death. Increases in circulating levels of IL-7 were paralleled by down-regulation of IL-7R α , thus self-limiting IL-7 function (Sportes et al., 2008; Sereti et al., 2009; Sportes et al., 2009) . However one study disagreed on this point (Levy et al., 2009). rhIL-7 therapy induces a preferential increase of naive and central memory CD4+ and CD8+ T cells (Sportes et al., 2008; Levy et al., 2009; Sportes et al., 2009).

An unexpected finding that has been observed during rhIL-7 trials involving HIV infected patients has been the appearance in some patients of transient increases in plasma HIV-RNA (“blips”), despite effective highly active antiretroviral therapy (HAART) (Levy et al., 2009; Sereti et al., 2009). This phenomenon has been attributed to reactivation of HIV replication from viral reservoirs.

Protocol	Eligible patient population	rhlL-7 dose and administration schedule	Date initiated	Status	ClinicalTrials.gov identifier	Reference
Cancer						
A Phase I study of subcutaneous rhlL-7 (CYT99007) in patients with refractory non-haematological malignancy	Refractory cancer	3-60 µg per kg every other day for 8 doses	April 2003	Not recruiting	NCT00062049	Sportes et al. 2008;
A study of subcutaneous rhlL-7 (CYT99007) in conjunction with peptide immunization in patients with metastatic melanoma	Melanoma	3-60 µg per kg every 3 days for 8 doses	September 2004	Not recruiting	NCT00091338	Rosenberg et al. 2006
A Phase I study of subcutaneous rhlL-7 (CYT107) in patients with refractory metastatic melanoma or renal cell carcinoma	Metastatic melanoma or renal cell carcinoma	30-120 µg per kg per week for 3 doses	May 2007	Recruiting	NCT00492440	-
A pilot study of tumour vaccination and rhlL-7, following standard multimodality therapy in patients with high-risk paediatric solid tumours	Paediatric solid tumours	20 µg per kg every other week for 4 doses	December 2009	Recruiting	NCT00923351	-
HIV infection						
A Phase I, randomized, placebo-controlled, double-blind study evaluating the safety of subcutaneous, single-dose rhlL-7 in HIV-1-infected patients who are receiving antiretroviral therapy	HIV infection	3-60 µg per kg for 1 dose	December 2004	Not recruiting	NCT00099671	Seret et al. 2009
A Phase I, randomized, placebo-controlled, double-blind study evaluating the safety of subcutaneous, single-dose rhlL-7 in HIV-1-infected patients who are receiving antiretroviral therapy	HIV infection	HIV infection 3-100 µg per kg for 1 dose	March 2005	Not recruiting	NCT00105417 (A5214)	-
A Phase IIIa randomized, placebo-controlled, single-blind, multicentre, dose-escalation study of subcutaneous, intermittent rhlL-7 (CYT107) in chronically HIV-infected patients with CD4+ T cell counts of 101-400 cells per mm ³ and receiving antiretroviral therapy	HIV infection	3-10 µg per kg every other day for 8 doses	May 2007	Not recruiting	NCT00477321 (INSPIRE)	Levy et al. 2009
An open-labelled, multicentre study of subcutaneous intermittent rhlL-7 (CYT107) in chronically HIV-infected patients with CD4+ T cell counts of 101-400 cells per mm ³ and plasma HIV RNA levels of less than 50 copies	HIV infection	20 µg per kg every week for 3 doses	January 2010	Not recruiting	NCT01190111 (Inspire-2)	-
A multicentre, open-labelled, controlled, randomized study of rhlL-7 (CYT107) treatment to restore and maintain CD4+ T cell counts above 500 cells per µl in HIV-infected patients with CD4+ T cell counts of 101-350 cells per µl after	HIV infection	20 µg per kg every week for 2 weeks; maximum of 4 cycles within 21 months and maximum of 3 cycles within 12	September 2010	Recruiting	NCT01241643 (Inspire-3)	-
Other						
rhlL-7 (CYT107) treatment of patients with idiopathic CD4+ T cell lymphocytopenia: expansion of CD4+ T cell populations	Idiopathic CD4+ T cell lymphocytopenia	3-20 µg per kg every week for 3 doses, from week 1 and then repeated from	February 2009	Recruiting	NCT00839436 (ICICLE)	-
A Phase I study of rhlL-7 (CYT107) in recipients of HLA-matched ex vivo T cell-depleted bone marrow or peripheral blood stem cell transplants	Haematopoietic stem cell transplantation	10-30 µg per kg every week for 3 doses	March 2008	Recruiting	NCT00684008	-
A Phase IIIa dose-escalation study of repeated administration of rhlL-7 (CYT107) add-on treatment in genotype 1 or 4 HCV-infected patients who are resistant to pegylated IFNα and ribavirin	HCV infection	3-20 µg per kg every week for 4 doses	July 2008	Recruiting	NCT01025297	-
A Phase IIIa dose-escalation study in Asia of repeated administration of rhlL-7 (CYT107) add-on treatment in genotype 1 HCV-infected patients who are resistant to pegylated IFNα and ribavirin	HCV infection	3-20 µg per kg every week for 4 doses	January 2009	Recruiting	NCT01024894	-
A Phase IIIa dose-escalation study in genotype 1 HCV-infected patients who are resistant to pegylated IFNα and ribavirin	HCV infection	3-30 µg per kg every week for 4 doses	January 2009	Recruiting	NCT01025596 (ECLIPSE-1)	-
A Phase III randomized, open labelled, controlled, dose-escalation study of repeated administration of rhlL-7 (CYT107) add-on antiretroviral treatment and vaccination in HBeAg-negative chronic HBV-infected patients	HBV infection	Not known	December 2009	Recruiting	NCT01027055	-

Table 1.5 - IL-7 therapy clinical trials.

2. AIM OF THE STUDY

As emphasized in the introduction part, CD25⁺ FOXP3⁺ regulatory T cells are now recognized as major players in maintenance of immune-tolerance. In this project we focused on an aspect we felt as under-investigated in Treg biology, namely Treg homeostasis.

2.1 – Role of IL-7 on CD25⁺ FOXP3⁺ regulatory T cells biology

When this project was conceived four years ago, two papers just completely changed Treg isolation strategies by introducing the notion that Treg lacked CD127 expression at their surface (Liu et al., 2006; Seddiki et al., 2006). As mentioned, this isolation approach presented some major limits as it excluded a CD127 expressing population whose physiological relevance was unknown. More importantly, lack of CD127 expression at Treg surface supported a model in which Treg homeostasis would be independent from IL-7. Given the major role played by IL-7 in T cell biology we decided to further investigate in a murine system the following issues :

- 1 Mechanisms regulating CD127 expression at Treg surface
- 2 Role of IL-7 in peripheral Treg homeostasis in lymphoreplete and lymphopenic contexts
- 3 Respective contribution of IL-2 and IL-7 in Treg homeostasis, with special emphasis on an eventual interplay

2.2 – Effects of HIV infection on regulatory T cells homeostasis

The role of Treg in HIV infection is still debated and conflicting results have been reported mainly due to the lack of specific Treg identification markers precluding their accurate quantification. In this work we decided to employ for the first time in HIV infection a novel consensus Treg identification strategy which allows distinction of naive and effector Treg. Applying this strategy to different groups of HIV infected patients we were able to investigate several points :

- effect of HIV infection on the homeostasis of Treg compartment
- quantification of Treg subsets in different groups of HIV-infected patients, namely viremic patients, including primary infected and chronically infected patients, and aviremic patients, including antiretroviral treated and HIV controllers patients.
- Correlation between Treg numbers and immune-activation status
- Correlation between Treg numbers and HIV specific CD8 T cells responses

3. - RESULTS

3.1 – ARTICLE 1

Increased CD127 expression on activated FOXP3+CD4+ regulatory T cells.

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Background : Regulatory T cells (Treg) are commonly identified by CD25 (IL-2R alpha) surface expression and/or intracellular expression of the FOXP3 transcription factor. In addition, Treg are also characterized by low CD127 (IL-7R alpha) expression when compared to conventional T cells and their biology in the periphery is considered essentially independent of IL-7. In this work we questioned whether low CD127 expression was an intrinsic hallmark of Treg or could be regulated by their activation status and/or environmental contexts.

Methods : We first studied CD127 expression on Treg surface upon *in vitro* activation. Using adoptive transfer and contact dermatitis models, we investigated *in vivo* CD127 expression on activated Treg both in lymphoid and non-lymphoid tissues. Finally, by analysing STAT5 phosphorylation and survival of Treg upon IL-7 treatment we assessed the functional relevance of CD127 expression at Treg surface.

Results : We demonstrated differential CD127 expression depending on Treg subsets considered. Notably, we observed high CD127 expression on inducible costimulatory molecule (ICOS)- and CD103-expressing Treg subsets. Since these two markers reflect activation status, we addressed whether Treg activation modulated CD127 expression. We demonstrated that in contrast to conventional T cells, Treg significantly upregulated CD127 expression during *in vitro* and *in vivo* activation using adoptive transfer and contact dermatitis models. High CD127 expression on Treg was also predominantly detected *ex vivo* in some specific sites, notably bone marrow and skin. Importantly, higher CD127 expression on Treg correlated with higher phosphorylation of STAT5 upon IL-7

exposure. High CD127 expression on Treg also provided survival advantage upon in vitro incubation with IL-7.

Conclusions : We demonstrated that low CD127 expression is not an intrinsic characteristic of Treg by showing that Treg exhibit high CD127 expression when activated in contrast to their low expression in non-immunized settings. We identified activated Treg as a potential target of endogenous or therapeutic IL-7.

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Increased CD127 expression on activated FOXP3⁺ CD4⁺ regulatory T cells

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Regulatory T cells (Treg) are commonly identified by CD25 (IL-2R α) surface expression and/or intracellular expression of the FOXP3 transcription factor. In addition, Treg are also characterized by low CD127 (IL-7R α) expression when compared to conventional T cells and their biology in the periphery is considered essentially independent of IL-7. We further investigated CD127 expression on Treg and we demonstrated differential CD127 expression depending on Treg subsets considered. Notably, we observed high CD127 expression on inducible costimulatory molecule (ICOS)- and CD103-expressing Treg subsets. Since these two markers reflect activation status, we addressed whether Treg activation modulated CD127 expression. We demonstrated that in contrast to conventional T cells, Treg significantly upregulated CD127 expression during *in vitro* and *in vivo* activation using adoptive transfer and contact dermatitis models. High CD127 expression on Treg was also predominantly detected *ex vivo* in some specific sites, notably bone marrow and skin. Importantly, higher CD127 expression on Treg correlated with higher phosphorylation of STAT5 upon IL-7 exposure. High CD127 expression on Treg also provided survival advantage upon *in vitro* incubation with IL-7. We thus demonstrated that low CD127 expression is not an intrinsic characteristic of Treg and we identified activated Treg as a potential target of endogenous or therapeutic IL-7.

Key words: CD127 (IL-7R α) · IL-7 · Treg



Supporting Information available online

Introduction

CD4⁺CD25⁺FOXP3⁺ Treg are a critical CD4⁺ T-cell subset involved in the control of immune homeostasis by preventing

autoimmune diseases and regulating chronic inflammation [1, 2]. Treg have been shown to exert suppressive activity by various mechanisms, notably by deprivation of IL-2 [3–5], inhibitory cytokine production (IL-10, TGF- β , IL-35), cytolytic function, metabolic disruption and modulation of APC function [6]. They are commonly identified by CD25 (IL-2R α) surface expression and/or intracellular expression of forkhead box P3 (FOXP3) transcription factor [7–9]. In addition to high CD25 expression, Treg are

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also characterized by low CD127 (IL-7R α) expression when compared to conventional T cells [10–12]. Although CD127 expression and consequently IL-7/IL-7R signaling is crucial to most peripheral T cells, Treg stand a remarkable exception and exhibit different cytokine requirement profile: IL-2 appears as the main survival factor, whereas IL-7 is thought to contribute moderately to Treg homeostasis [13–15]. High IL-2 requirement and low IL-7 influence on Treg are in accordance with the high level of CD25 and low level of CD127 expression on Treg. However, CD127 expression has been shown to be highly regulated on conventional T cells depending notably on IL-2 and IL-7 availability [16, 17] or TCR stimulation [18]. We thus questioned whether low CD127 expression was an intrinsic hallmark of Treg or could be regulated by their activation status and/or environmental contexts. First, we determined whether Treg exhibited some heterogeneity in CD127 expression *ex vivo*. When addressing Treg as subsets by introducing markers of Treg heterogeneity [19, 20], high CD127 expression correlated with the expression of markers such as CD103 and inducible costimulatory molecule (ICOS). Because these two markers have been described to identify activated Treg [21–25], these results suggested that in contrast to conventional T-cell activation, CD127 expression may be more prominent during Treg activation. To confirm this hypothesis, we developed different approaches. We detected higher CD127 expression on Treg upon *in vitro* activation. Using adoptive transfer and contact dermatitis models, we confirmed *in vivo* that CD127 is highly expressed on activated Treg both in lymphoid and non-lymphoid tissues. Collectively, these data demonstrated high CD127 expression correlated with Treg activation. Finally, since Treg activation also occurs in peripheral tissues, we performed tissue-specific analysis, revealing striking diversity in CD127 expression on Treg depending on the sites considered. To formally identify activated Treg as a preferential target of IL-7, we determined whether Treg transduced intracellular signaling mediated by IL-7. We demonstrated that high levels of CD127 expression on Treg correlated with increased IL-7 signal transduction. We finally confirmed the functional relevance of CD127 expression by demonstrating enhanced survival capacity of Treg expressing high level of CD127 (CD127^{hi}) upon *in vitro* incubation with IL-7.

Our results demonstrated that low CD127 expression is not an intrinsic characteristic of Treg and that differential CD127 expression on Treg depends on their localization and their activation status. They also revealed differential regulation of CD127 expression among conventional and regulatory CD4⁺ T cells upon activation, providing potential regulation of Treg biology depending on IL-7 availability.

Results

ICOS and/or CD103 identify CD127-expressing Treg *ex vivo*

Most CD4⁺FOXP3⁺ Treg have been shown to exhibit low expression of CD127 compared to conventional CD4⁺ and

CD8⁺ peripheral T cells both in humans and mice (11, 12). Regarding large phenotypic heterogeneity among Treg, we questioned whether CD127 expression could differ depending on the Treg subsets considered. To obtain a comprehensive characterization of Treg phenotypic subsets, we determined on FOXP3⁺CD4⁺ T cells the expression of several markers discriminating activation status (CD62L, CD44, CD69), or known to be preferentially expressed on Treg (GITR, CTLA-4) or representing co-stimulatory (CD28, ICOS, OX40, CD154, PD-1) and adhesion molecules (ICAM-1, CD103) (data not shown). Among the various molecules studied, we identified four markers for which the Treg population showed heterogeneity: in LN, CD62L was expressed by about half of Treg (56 \pm 6%); CD69 expression identified 34 \pm 1% of Treg; CD103-expressing Treg represented 21 \pm 1% of the total Treg population; and ICOS was expressed by 16 \pm 2% of Treg (percentages are mean \pm SD of a representative experiment as defined on a sample in Fig. 1A). Prior to the analysis of CD127 on each subset, we confirmed the suppressive capacity of these respective subsets. CFSE-labeled sorted naive CD4⁺ T cells (CD62L⁺CD25⁻CD4⁺ T cells) were stimulated *in vitro* with anti-CD3 with or without Treg and proliferation was assessed at days 3–4. As shown in Fig. 1B, all Treg subsets studied exerted a suppressive activity at least comparable with that of the whole CD4⁺CD25⁺ population in a 1:1 Treg:Teff ratio. We next analyzed CD127 cell surface expression on total Treg and Treg subset expressing or not CD62L, CD69, ICOS and CD103 (Fig. 1C). As previously published, total Treg exhibited low CD127 expression compared to conventional CD4⁺ T cells (CD127 MFI: 68 \pm 12 and 302 \pm 23, respectively). To note, level of CD127 expression on Treg was significantly different from isotype control staining, confirming low but existing CD127 expression on Treg. CD69- or CD62L-expressing Treg presented CD127 surface levels not significantly different from their negative counterparts. Importantly, CD103 or ICOS-expressing Treg exhibited significantly higher levels of CD127 compared to their negative counterparts (CD127 MFI: 111 \pm 3 on CD103⁺ compared to 62 \pm 14 on CD103⁻; 146 \pm 30 on ICOS⁺ compared to 67 \pm 12 on ICOS⁻). To determine whether ICOS- and CD103-expressing Treg subsets were independent or partially overlapping, we analyzed the co-expression of ICOS and CD103 on Treg and CD127 expression depending on ICOS and CD103 co-expression (Fig. 1D). CD103⁻ICOS⁻ Treg were predominant (73 \pm 1%) and exhibited low CD127 expression. The three subsets identified by CD103 and/or ICOS expression (*i.e.* CD103 or ICOS single positive, CD103/ICOS double positive) were similarly represented (9 \pm 1%, 7 \pm 1% and 9 \pm 1% of CD4⁺FOXP3⁺ cells isolated from LN, respectively) but exhibited very different CD127 profiles. Single positive cells for either ICOS or CD103 expressed CD127 levels comparable with those expressed by the whole Treg subset. Strikingly, levels of CD127 on CD103⁺ICOS⁺ cells were significantly higher when compared to total Treg (CD127 MFI: 195 \pm 12 on ICOS⁺CD103⁺ Treg fraction compared to 68 \pm 12 on total Treg). We thus demonstrated that in LN, CD103/ICOS expression identified a fraction of CD4⁺FOXP3⁺ T cells, which exhibited high CD127 expression.

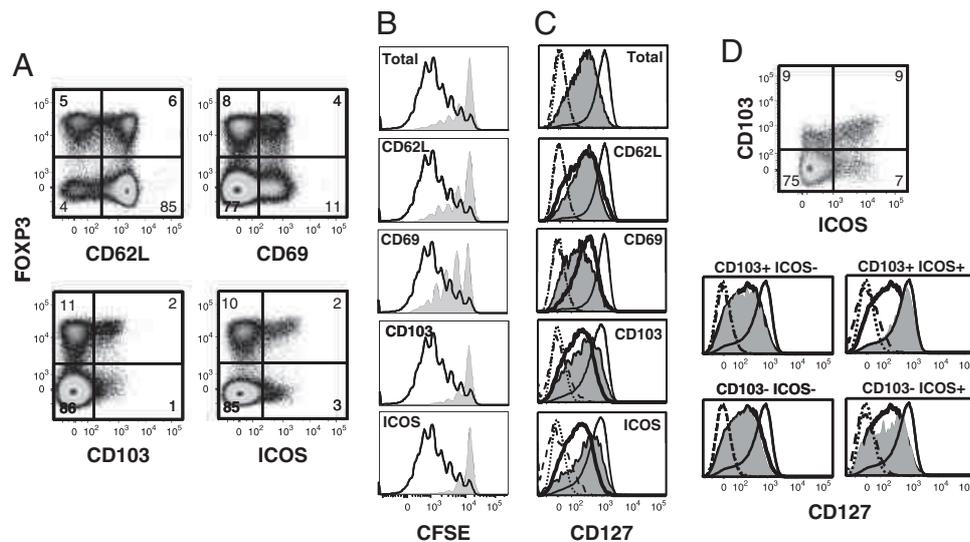


Figure 1. In lymph nodes, CD103 and ICOS expression identifies Treg presenting high levels of CD127. (A) Phenotypic heterogeneity of Treg. Dot plots show the expression of CD62L, CD69, CD103 and ICOS depending on FOXP3 expression on CD4⁺TCRβ⁺ LN cells. Percentages of cells from a representative sample are indicated in each quadrant. (B) Suppression assay of total, CD62L, CD69, CD103 or ICOS expressing CD4⁺CD25⁺ subpopulations. CFSE profiles of sorted naïve CD4⁺ T cells cultured 72–96 h in the presence of 0.5 μg/mL anti-CD3 with or without the indicated sorted CD4⁺CD25⁺ subsets (filled and open profile, respectively). Data are representative of three independent experiments. (C) CD127 expression on total and CD62L-, CD69-, CD103- or ICOS-expressing (grey histogram) or non-expressing (thick open line) FOXP3⁺ Treg compared to conventional FOXP3⁻CD4⁺ T cells (open histogram). Isotype control staining for both Treg subsets are represented (dotted and shaded lines). (D) CD103 and ICOS expression on LN CD4⁺FOXP3⁺ Treg. Histograms show CD127 expression on total Treg (thick open line) or indicated Treg subsets identified based on ICOS and/or CD103 expression (gray shaded profile) compared to conventional CD4⁺ T cells (open histogram). Isotype of total Treg and Treg subsets are presented (dotted and dashed lines, respectively). Gating strategy is shown in Supporting Information Fig. 1A. FACS data are representative of more than five independent experiments, using at least three C57BL/6 mice *per* experiment.

Treg upregulated CD127 during *in vitro* and *in vivo* activation

Since CD103 and ICOS have been described as Treg activation markers, we thus speculated that the CD127^{hi} Treg fraction could be more prominent during Treg activation. To directly address this assumption, we studied CD127 expression on Treg upon *in vitro* and *in vivo* activation. First, we determined CD127 expression simultaneously on Treg and conventional CD4⁺ T cells following *in vitro* anti-CD3 stimulation of total CD4⁺ T cells (Fig. 2A). At day 4, we confirmed increased ICOS expression during activation: ICOS-expressing Treg represented 92 ± 4% compared to 23 ± 2% *ex vivo* (percentages expressed are mean ± SD of a representative experiment, as defined on one sample in Fig. 2A). To note, ICOS expression was also increased on non-Treg CD4⁺ T cells but to a lesser extent (59 ± 9% upon activation compared to 6 ± 1% *ex vivo*). Four days after stimulation, CD127 expression was increased on FOXP3⁺ Treg, whereas CD127 downregulation was observed on some conventional FOXP3⁻CD4⁺ T cells (Fig. 2B). Interestingly, CD127 expression on activated Treg was similar to CD127 expression on activated conventional cells (CD127 MFI: 354 ± 123 on activated Treg compared to 248 ± 91 on activated conventional CD4⁺ T cells) (Fig. 2C).

To confirm these data *in vivo*, we used a classical adoptive transfer model: sorted CD4⁺CD25⁺ Treg were co-injected with CD45.1⁺ naïve CD4⁺ T cells into Rag-deficient hosts. Analysis

was performed 6 wk after transfer and Treg activation was confirmed by CD103 and ICOS expression (data not shown). Transferred conventional FOXP3⁻CD4⁺ T cells expressed lower levels of CD127 when compared to conventional CD4⁺ T cells isolated from WT mice (MFI: 196 ± 13 and 353 ± 45, respectively) (Fig. 3A and B). In contrast, activation of Treg induced higher CD127 expression compared to *ex vivo* isolated Treg (CD127 MFI: 154 ± 33 after transfer compared to 89 ± 10 *ex vivo*) (Fig. 3A and B). To determine whether higher CD127 expression relies on upregulation or expansion of pre-existing activated Treg, similar experiments were performed using “naïve-like” Treg (CD4⁺CD25⁺CD62L⁺CD103⁻CD69⁻ICOS⁻). CD45.2⁺ transferred “naïve-like” Treg expressed higher levels of CD127 than *ex vivo* Treg (CD127 MFI: 222 ± 22 after transfer compared to 89 ± 10 *ex vivo*) (Fig. 3C and D). High CD127 expression in “naïve-like” Treg supported upregulation rather than expansion of a pre-existing CD127^{hi} Treg fraction. We thus demonstrated that Treg, while expressing low levels of CD127 at the steady state, significantly upregulate this molecule upon *in vitro* and *in vivo* activation.

Activated Treg expressed CD127 in a model of acute skin inflammation

Although adoptive transfer is a useful model to study Treg activation and suppressive capacity *in vivo*, it requires transfer into empty hosts, which may introduce some bias concerning

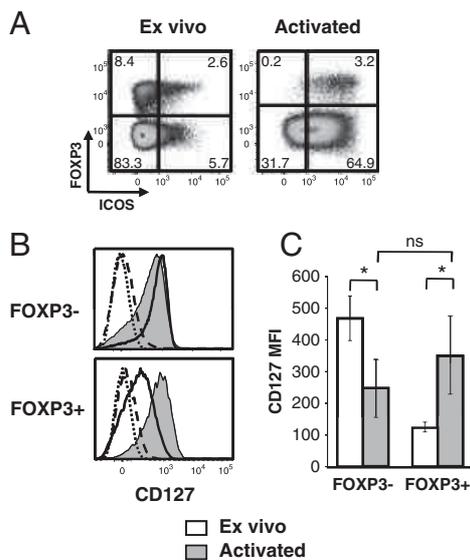


Figure 2. Treg upregulate CD127 upon *in vitro* activation. Total CD4⁺ cells isolated from LN and spleen of three to five C57BL/6 mice were activated *in vitro* with anti-CD3 for 4 days. (A) ICOS and FOXP3 expression on fresh (left panel) and stimulated CD4⁺ T cells (right panel). (B) CD127 expression on conventional FOXP3⁻ (upper panel) and regulatory FOXP3⁺ (lower panel) CD4⁺ cells upon *in vitro* activation. CD127 expression on control cells (open profile) or on activated cells (gray shaded profile) is shown. Isotype controls staining of both activated and *ex vivo* population are represented by the dotted and dashed lines, respectively. Cells were identified by a wide gate on FSC versus SSC plot to define lymphocytes followed by gating on CD4⁺ cells. (C) Mean CD127 MFI ± SEM of three independent experiments. **p*<0.05, two-tailed Mann-Whitney *U*-test.

CD127 expression analysis. It is well known that IL-7 availability directly interferes with CD127 expression at T-cell surfaces [16, 17] and that IL-7 cytokine levels are increased in lymphopenic states [26, 27]. We wanted to confirm our results in a non-lymphopenic model of Treg activation: a model of acute skin inflammation. Ear skin exposure to 2,4-dinitrofluorobenzene (DNFB) induces a strong inflammatory response accompanied by ear swelling and activation of resident and recruited T cells [28]. Conventional and Treg activation was studied using ICOS expression. Indeed, ICOS is the most consistent marker for Treg activation, whereas CD103 has been described to reflect tissue-specific Treg homing. Moreover, being a shared activation marker for conventional and regulatory CD4⁺ T cells, it also allows distinguishing activated conventional T cells. Analyses were performed 6 days after DNFB treatment. Inflammation was confirmed by significant increase in ear thickness (Fig. 4A) and increased CD4⁺ T-cell numbers in cervical LN draining the ear skin (Fig. 4B). In draining LN, a fraction of conventional CD4⁺ FOXP3⁻ cells underwent activation as revealed by ICOS upregulation. Simultaneously, an important fraction of CD4⁺ FOXP3⁺ Treg was activated and expressed ICOS (46 ± 6%). We next determined CD127 expression on activated regulatory and conventional CD4⁺ T cells as identified by ICOS expression. As expected, ICOS-expressing conventional CD4⁺ T cells downregulated CD127 at their surface. However, ICOS⁺ Treg

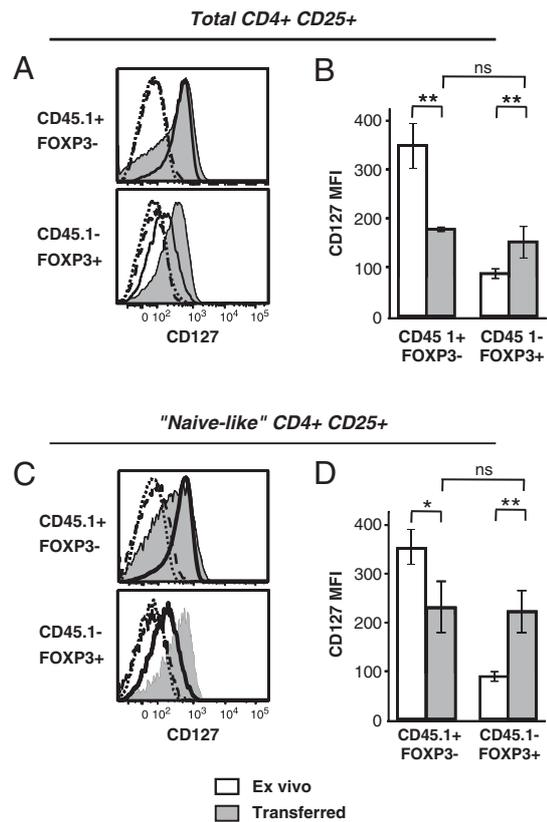


Figure 3. Treg upregulate CD127 upon *in vivo* activation. CD45.1⁺ naïve CD4⁺ T cells and CD45.2⁺ Treg or “naïve-like” Treg were sorted by FACS and co-injected into RAG-deficient mice. Phenotypic analysis was performed on mesenteric LN and spleen 6 wk after transfer. Cells were identified by gating of lymphocytes on FSC/SSC plot, followed by gating on CD4⁺ cells. CD45.1⁺FOXP3⁻ CD4⁺ cells and CD45.1⁻FOXP3⁺ Treg were further identified. (A, C) CD127 expression on conventional FOXP3⁻ (upper panel) and regulatory FOXP3⁺ (lower panel) CD4⁺ cells during *in vivo* activation when (A) total or (C) “naïve-like” Treg were co-injected. Isotype controls of activated and control fresh cells are represented by dotted and dashed lines. (B, D) CD127 MFI on CD4⁺ cells isolated from control WT mice (open profile) or transferred cells (gray shaded profile) when total (B) or “naïve-like” Treg (D) were co-injected. Data show mean ± SEM of three independent experiments using two to three mice per group in each experiment. **p*<0.05, ***p*<0.01, two-tailed Mann-Whitney *U*-test.

expressed higher levels of CD127 than their negative counterpart (Fig. 4C). To assess CD127 expression on CD4⁺ T-cell subsets at the site of inflammation, we analyzed lymphocytes infiltrating ear skin in both control and inflamed ear skin. In control mice, an important proportion of conventional and regulatory CD4⁺ T cells infiltrating ear skin expressed ICOS (Fig. 4D) suggesting that resident CD4⁺ T cells were in an activated state. During acute inflammation, a more diverse phenotype was observed both on conventional T cells and Treg, revealing notably an increasing proportion of ICOS⁻ conventional and Treg. The rationale for the appearance of phenotypically less activated T cells (as assessed by low ICOS expression) is unclear but may reflect recruitment from peripheral blood (PB) or downregulation of ICOS on pre-existing resident T cells during the acute phase of inflammation. Nevertheless, we assessed CD127 expression on skin-infiltrating CD4⁺

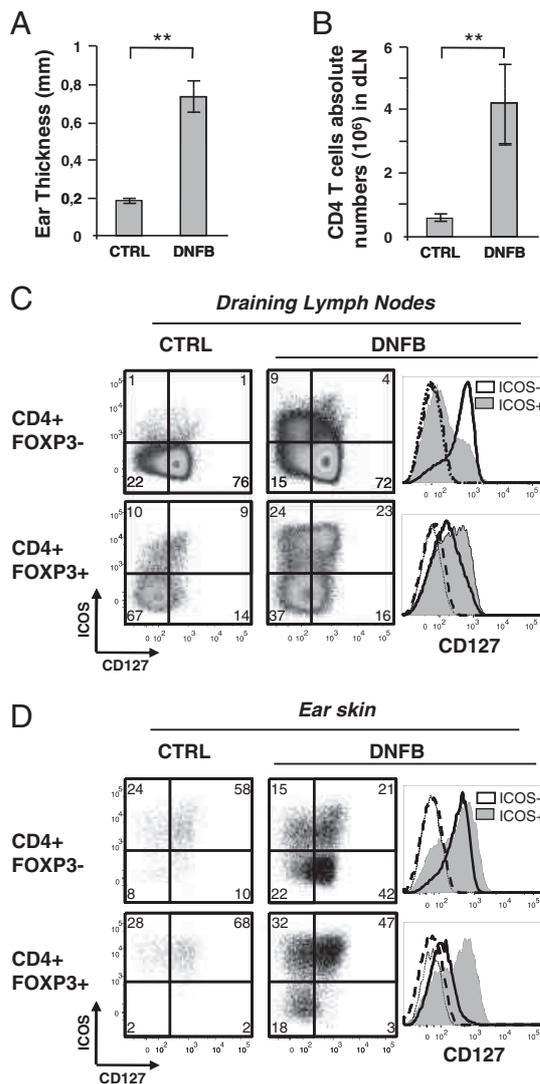


Figure 4. High CD127 expression on activated Treg in a model of acute skin inflammation. (A) Ear thickness and (B) CD4⁺ T-cell numbers in cervical LN draining ear skin 6 days after DNFB treatment. Data show mean ± SD of five mice from one representative of at least three independent experiments. ***p* < 0.01, two-tailed Mann-Whitney *U*-test. (C, D) ICOS and CD127 expression on CD4⁺FOXP3⁻ and CD4⁺FOXP3⁺ T cells from (C) draining LN and (D) ear skin. Histogram profiles show CD127 expression on ICOS⁻ (thick black line) or ICOS⁺ (gray shaded profile) cells from DNFB-treated mice. Isotype controls of ICOS⁻ and ICOS⁺ subsets are represented by the dotted and dashed lines, respectively. Gating strategy is shown in Supporting Information Fig. 1B. This figure is representative of at least three independent experiments using pool of 5–15 mice *per* experiment.

T-cell subsets expressing ICOS or not, similar to what we performed in draining LN. As shown in Fig. 4D, a fraction of ICOS⁺ conventional CD4⁺ T cells downregulated CD127 when compared to the ICOS⁻ population, whereas a fraction of activated ICOS⁺ Treg expressed higher levels of CD127 than their negative counterpart. The appearance of CD127^{hi} Treg among ICOS⁺ cells in acute inflamed skin may also rely on recruitment or migration of PB-derived T cells into inflamed sites but may also reflect different kinetics of expression of ICOS and

CD127 on activated Treg during the acute phase of inflammation. However, in both cases, high CD127 expression was predominantly observed on activated Treg in ear skin. Collectively, these *in vivo* results demonstrated that upon activation, conventional CD4⁺ T cells downregulate CD127, whereas Treg, which express low levels of CD127 at the steady state, strongly upregulated this molecule. These results also confirmed that CD127 expression on activated Treg can be equivalent to conventional T cells.

Different CD127 expression on Treg depending on their tissue localization

Considering the high CD127 expression profile from skin resident Treg in non-immunized mice, we next analyzed CD127 expression on Treg among various sites: LN, spleen (SP), PB, thymus, bone marrow (BM), peritoneal cavity, intestine, lung, liver and skin. CD127 expression was determined on tissue resident FOXP3⁺ and conventional FOXP3⁻CD4⁺TCR⁺ cells and isotype staining was performed as control (Fig. 5A). CD127 expression profile on Treg differed depending on the organs studied (Fig. 5B and C). Various proportions of Treg expressing high levels of CD127 were recovered, ranging from 10 ± 3% in the intestine to in the skin. PB (15 ± 8%), LN (17 ± 4%), thymus (19 ± 1%) and SP (28 ± 6%) exhibited intermediate proportion of CD127-expressing Treg. Strikingly, BM exhibited high percentage of CD127-expressing Treg (64 ± 3%) (Fig. 5B). Similar results were obtained analyzing MFI (Fig. 5C). Highest levels of CD127 expression on Treg were observed in BM and skin (CD127 MFI 312 ± 57 in BM and 383 ± 73 in skin compared to 85 ± 15 in LN). In these two organs, no concomitant upregulation was observed on conventional T cells when compared to LN counterpart, suggesting a Treg-restricted phenomenon. Our results demonstrated differential CD127 expression on Treg depending on their localization and they confirmed that low CD127 expression was not an intrinsic characteristic of Treg.

Higher levels of CD127 expression on Treg correlated with increased IL-7 signal transduction

To assess whether CD127 expression on Treg was associated with efficient IL-7 signal transduction, we examined STAT5 phosphorylation following IL-7 incubation *in vitro*. We used Foxp3-GFP reporter mice to identify Treg; similar results were obtained using CD25 as a marker for Treg identification. IL-2 treatment induced robust STAT5 phosphorylation in CD4⁺FOXP3⁺ but not in conventional CD4⁺FOXP3⁻ T cells (Fig. 6A). IL-7 treatment induced significant increase in phospho-STAT5 in Treg but to a lesser extent than observed in conventional CD4⁺FOXP3⁻ cells, as previously described [13, 29, 30]. Importantly, a dose-dependent effect was detectable among Treg but not among conventional T cells (Fig. 6A). We next studied STAT5 phosphorylation depending on CD127 expression on Treg. As shown in Fig. 1C, Treg exhibited heterogeneous CD127 expression. We thus determined pSTAT5 signaling on CD127^{hi} Treg

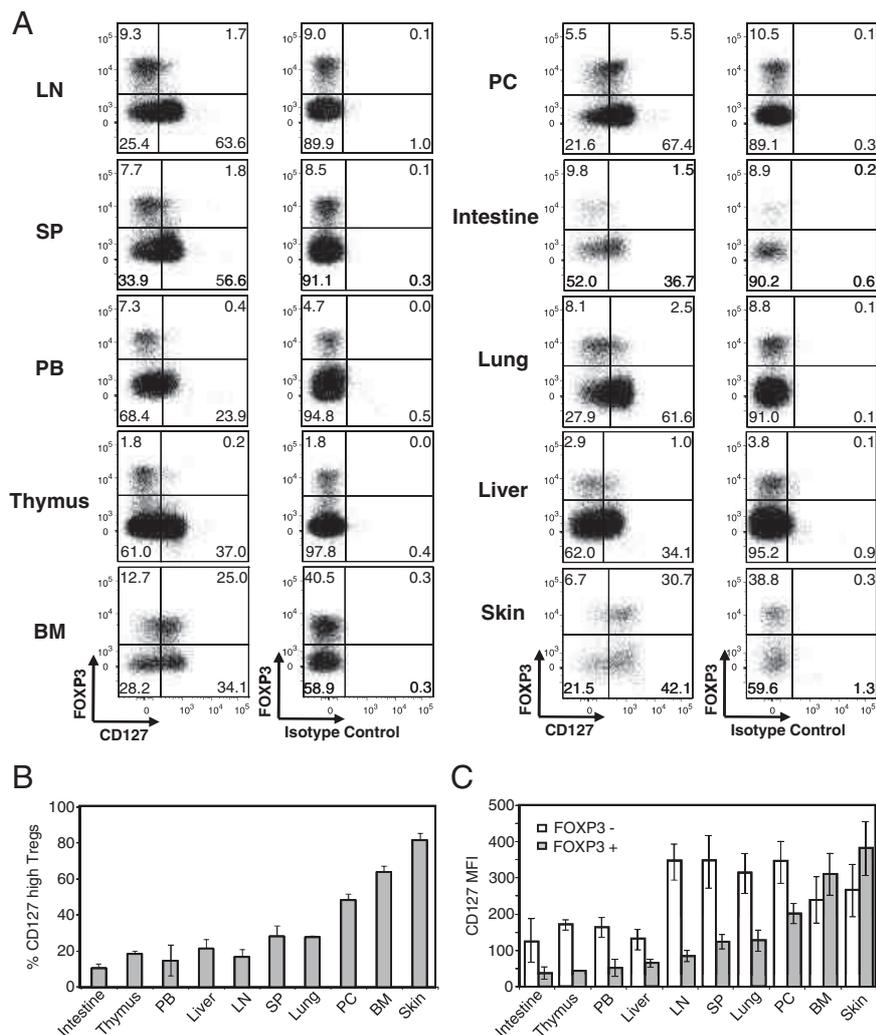


Figure 5. Different CD127 expression on Treg depending on the organs studied. (A) *Ex vivo* CD127 and FOXP3 expression on CD4⁺TCRβ⁺ cells isolated from LN, SP, peripheral blood (PB), thymus, BM, peritoneal cavity (PC), intestine, lung, liver and skin of C57BL/6 mice. Isotype controls are presented for all organs. FSC/SSC plot was used to gate lymphocytes in all organs except skin and intestine. In these organs, CD4 gating was directly performed in a CD4/FSC plot and followed by back-gating to the FSC/SSC plot to select more accurately lymphocytes and consequently TCRβ⁺CD4⁺ cells. Quadrants were defined based on isotype controls. Percentages of cells in each quadrant are indicated. (B) Percentages of CD127-expressing cells among FOXP3⁺CD4⁺ T cells in different organs are shown. (C) CD127 MFI on FOXP3⁻ (white bars) and FOXP3⁺ (grey bars) CD4⁺ T cells. Error bars represent SEM of three independent experiments. Data are representative (A) or show mean ± SEM (B, C) of at least three independent experiments, with 5–15 mice pooled in each experiment depending on the organs studied.

(top 10% of Treg gate) and Treg exhibiting low but significant CD127 expression (lowest 50% of Treg gate). Following treatment with low doses of IL-7 (1 ng/mL), an approximately eightfold increase in phospho-STAT5 MFI was detectable among CD127^{hi} Treg compared to a twofold increase in CD127^{lo} cells (Fig. 6B). A similar pattern of STAT5 phosphorylation in the two Treg sub-populations was observed after treatment with lower or higher doses of IL-7. These results indicate that CD127^{hi} Treg transduced IL-7 signals more efficiently than CD127^{lo} Treg.

Because Treg localized in the bone marrow (BM Treg) expressed high levels of CD127 at the steady state, we wanted to assess whether BM Treg could be more reactive to IL-7 than Treg from LN and SP. Interestingly, *in vitro* stimulation with IL-7 induced comparable amounts of phospho-STAT5 in conventional

and Treg from BM at all doses studied. Maximal levels of STAT5 phosphorylation were reached in BM Treg after exposure to very low doses (0.1 ng/mL) of IL-7 (Fig. 6C). Accordingly, we found that BM Treg expressed significantly higher levels of phospho-STAT5 than secondary lymphoid organs-derived Treg upon IL-7 stimulation at all IL-7 doses studied (Fig. 6D).

CD127 is also part of the receptor for thymic stromal-derived lymphopoietin (TSLP). We thus assessed whether differential levels of CD127 expression could influence the capacity of Treg to react to TSLP as well. TSLP induced a dose-dependent increase in STAT5 phosphorylation in both conventional and, to a lesser extent, Treg (Supporting Information Fig. 2A). No difference in STAT5 phosphorylation was detected in CD127^{hi} and CD127^{lo} Treg upon TSLP treatment at the doses of 1 and 10 ng/mL and

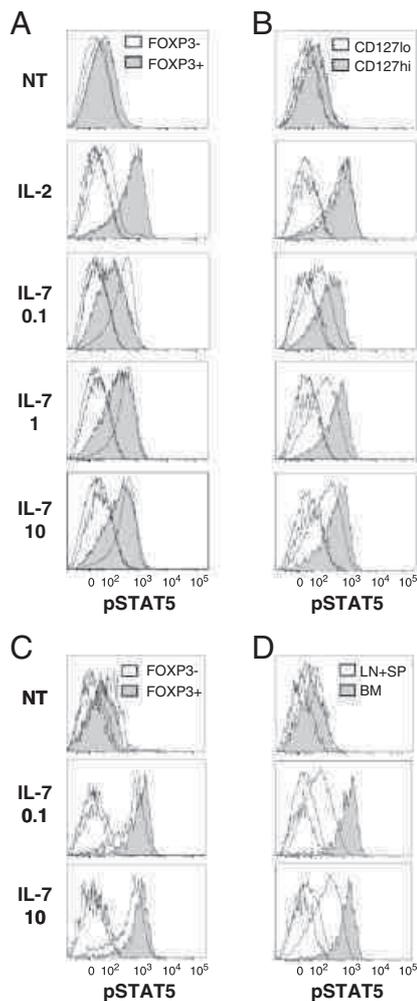


Figure 6. Different IL-7 signal transduction in Treg depending on CD127 expression. Cells from Foxp3-GFP mice were incubated with either medium alone, IL-2 (50 U/mL), or IL-7 (0.1, 1, 10 ng/mL) for 30 min. STAT5 phosphorylation was determined on FOXP3⁺ or FOXP3⁻ cells isolated from (A) pooled LN and splenic cells or (C) BM cells. (B) p-STAT5 staining depending on CD127 expression among FOXP3⁺ LN and spleen cells; CD127^{hi} Treg were identified by gating on top 10% CD127-expressing CD4⁺GFP⁺ cells (grey shaded profile); CD127^{lo} Treg were defined as 50% of CD4⁺GFP⁺ cells expressing the lowest CD127 levels (open profile). (D) Comparison of p-STAT5 staining among LN and spleen or BM isolated Treg. Gating strategy is shown in Supporting Information Fig. 1C. Results are representative of three independent experiments, using one Foxp3-GFP mouse for each experiment.

only a slight increase in pSTAT5 was detected in CD127^{hi} Treg compared to CD127^{lo} Treg upon exposure to high doses (100 ng/mL) of TSLP (Supporting Information Fig. 2B). Similar to what we performed for IL-7, we assessed whether BM Treg were more reactive to TSLP than Treg from LN and SP. Only a slight increase in pSTAT5 was detected in both conventional T cells and Treg from the BM upon high doses of TSLP treatment (Supporting Information Fig. 2C). Moreover, no significant difference between STAT5 phosphorylation in BM Treg and Treg from secondary lymphoid organs was observed (Supporting Information Fig. 2C). Collectively, our results demonstrate that Treg that

express more cell surface CD127 are more efficient at perceiving and transducing signals specifically mediated by IL-7.

IL-7 preferentially increases the *in vitro* survival of Treg expressing high levels of CD127

As we demonstrated that CD127^{hi} Treg transduced IL-7 signals more efficiently, we assessed whether IL-7 could exert functional effect preferentially on these cells. Conflicting data have been reported so far concerning the role of IL-7 on Treg survival. We thus re-address this question considering high and low CD127 expression on Treg. To this aim, we cultured FACS-sorted total, CD127^{lo} or CD127^{hi} CD4⁺CD25⁺ cells (Fig. 7A) in the presence or absence of IL-7. Upon overnight incubation, IL-7 significantly increased *in vitro* survival of total CD4⁺CD25⁺ cells ($p = 0.003$) (Fig. 7B). When considering CD127 expression, a minor but statistically significant increase in cell survival was observed on CD127^{lo} Treg upon IL-7 treatment ($p = 0.024$). Interestingly, a highly significant increase in survival was detected on IL-7 treated CD127^{hi} Treg compared both to non-treated cells ($p = 0.0002$) and to IL-7 treated CD127^{lo} cells ($p = 0.0003$). In conclusion, our results indicate that IL-7 preferentially promotes *in vitro* survival of Treg expressing higher levels of CD127.

Discussion

IL-7 and Treg are the two main regulators of homeostasis and immune responses. In contrast to their constitutive expression of IL-2R α (CD25), FOXP3⁺CD25⁺CD4⁺ Treg have been shown to exhibit low expression of CD127 compared to conventional CD4⁺ and CD8⁺ peripheral T cells [10–12]. Importantly, regulation of CD127 expression has proven crucial during thymocyte maturation [31] and it has been suggested to be a crucial step for effector or memory differentiation [32–34]. We thus further investigated CD127 expression on Treg. When dissecting Treg heterogeneity in lymphoid organs, CD127 expression on Treg was associated with the expression of CD103 and ICOS. These two markers have been shown to identify activated Treg [23, 35]. Using various context of activation (*in vitro*, model of adoptive transfer, skin inflammation model), we demonstrated that high CD127 expression on Treg was predominant during ongoing immune responses. These data differ from what has been described for conventional T cells which downregulate transiently IL-7R α expression during activation and suggest different regulation of CD127 expression on conventional and Treg. Whether TCR ligation and IL-2 signaling differently regulate CD127 expression or whether TCR ligation differs between conventional and Treg remain to be further investigated. Intrinsic difference in Treg intracellular machinery may also be responsible for such differential regulation. Interestingly, the opposite regulation of CD127 on Treg and effector cells during activation led to equivalent CD127 expression on these two subsets when activated. These data provide an additional mechanism for the

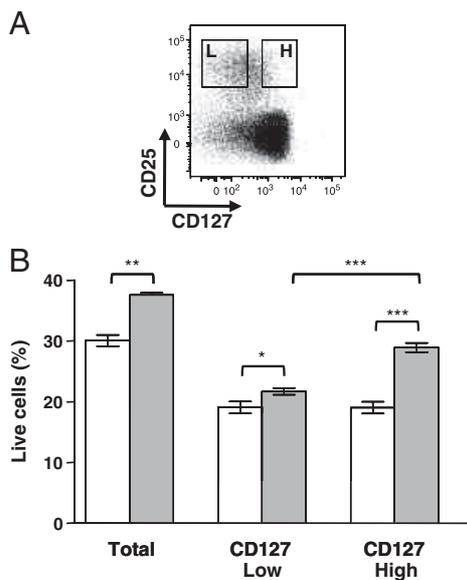


Figure 7. IL-7 preferentially promotes *in vitro* survival of Treg expressing higher levels of CD127. FACS-sorted total, CD127^{lo} or CD127^{hi} CD4⁺ CD25⁺ cells were cultured overnight in medium alone or in the presence of IL-7 (10 ng/mL). (A) Gating strategy to isolate the top 10% CD127-expressing CD25⁺ T cells (H gate) and CD127^{lo} Treg (L gate) by FACS. (B) Percentage of live cells after culture of indicated Treg populations in medium alone (open bars) or in the presence of IL-7 (filled bars). Live cells were defined as LIVE/DEAD negative cells among CD4 expressing lymphocytes. FACS sorting was performed on pooled mesenteric LN isolated from fifteen mice for each experiment. Data show mean \pm SD ($n = 3$) and are representative of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, unpaired two-tailed Student's *t*-test.

functional impact of IL-7 on Treg suppression, which has been shown to rescue effector cells from Treg-mediated apoptosis [4, 36]. Our data support the hypothesis that Treg and conventional T cells may compete for IL-7 during activation. They also support our previous results demonstrating that IL-7R expression on Treg was essential for Treg-mediated suppression of conventional T cells during lymphopenia-induced proliferation [37].

We also extended our analysis to peripheral tissues that are important localization for activated Treg. Depending on the organs studied, we observed strikingly different CD127 expression profile on Treg compared to Treg isolated from LN. PB and secondary lymphoid organs analysis revealed low CD127 expression on Treg *ex vivo*. Contrasting profiles were observed in mucosal sites: high CD127 expression on Treg was predominant in the skin but barely detectable in the intestine. Such heterogeneity among various mucosal sites suggests high CD127 expression is not a ubiquitous marker of activated Treg. Difference in CD127 expression depending on the tissue considered may rely on various mechanisms: differential antigenic load, differential kinetics of activation, differential activation/differentiation pathways and/or specific signals provided in specific sites, differential migration capacities of high CD127-expressing Treg, differential persistence depending on IL-7 (or TSLP) production on sites [38]. These latter hypotheses may also stand

for the high percentage of CD127-expressing Treg in the BM. The physiological relevance of such a specific profile in the BM is currently under investigation and may reflect local IL-7 production. Our data questioned Treg analyses excluding CD127-expressing cells [39, 40]. Such protocol, commonly used in clinical analyses, allows isolating a highly enriched Treg population but it may also exclude an activated population that could be especially relevant in chronic immune responses and pathological contexts. Although CD127^{hi} Treg are minor in PBMC, such a protocol may prove debatable depending on the tissue and the context considered.

Demonstrating differential CD127 expression among Treg subsets did not formally demonstrate any preferential reactivity to IL-7. To ascertain whether high CD127 expression enhanced IL-7-mediated signaling among Treg, we determined STAT5 phosphorylation upon *in vitro* IL-7 or TSLP incubation. Two strategies were considered: comparing high and low CD127-expressing Treg isolated from LN and SP or comparing total Treg isolated from secondary lymphoid organs and BM, which exhibit significant difference in CD127 expression. In both analyses, CD127^{hi} Treg exhibited higher phosphorylation of STAT5. Interestingly, IL-7-induced STAT5 phosphorylation in CD127^{hi} Treg was comparable with that detected in conventional FOXP3⁻CD4⁺ T cells. These results confirmed the functional relevance of differential CD127 expression on Treg in terms of reactivity to IL-7. A similar approach was performed to study the reactivity to TSLP, an IL-7-like cytokine expressed by epithelial cells, including keratinocytes, and important in allergic inflammation [41, 42]. We showed that TSLP induced STAT5 phosphorylation in Treg, but we failed to reveal any association between CD127 expression on Treg and TSLP signal transduction. Nevertheless, one may hypothesize that activated Treg could compete with effector T cells for TSLP and this may be one of the mechanisms by which Treg dampen inflammation at tissue sites. Collectively, these data demonstrated that activated Treg are an important target of IL-7/IL-7R α signaling. One obvious hypothesis is to consider IL-7 as an additional survival factor for activated Treg. So far, minor influence of IL-7/IL-7R α signaling on peripheral Treg has been reported in accordance with the low level of CD127 expression described on Treg [13–15]. However, Pandiyan *et al.* demonstrated that IL-7 addition enhances Treg survival during *in vitro* activation [43]. High CD127 expression on activated Treg reconciles these conflicting data since experimental protocols studying Treg sensitivity to IL-7 were performed either on resting [13] or activated Treg [43]. Indeed, we demonstrated enhanced *in vitro* survival of CD127^{hi} Treg compared to CD127^{lo} Treg upon 20 h IL-7 incubation. Finally, demonstrating high CD127 expression on activated Treg may be especially important during lymphopenic episodes [44] and in regard to the development of IL-7 based therapies in cancer and HIV infected patients notably [45–50].

In conclusion, we demonstrate that Treg exhibit high CD127 expression when activated in contrast to their low expression in non-immunized settings. Associated with the recent observation that DC express CD127 [27], it is remarkable to identify the three main partners involved in immune T-cell responses, *i.e.* conven-

tional T cells, DC and Treg, as potential targets of IL-7. Altogether, these data provide additional insights into the ubiquitous impact of IL-7 on various peripheral T-cell subsets. Importantly, our data substantiate a direct link between two main components of immune regulation that are Treg and IL-7 and identify activated Treg as a potential target of endogenous or therapeutic IL-7.

Materials and methods

Mice

Six- to eight-week-old C57BL/6 mice expressing CD45.1 or CD45.2 (Charles River, Janvier) and B6 RAG-2-deficient mice were used as donors and hosts, respectively. Foxp3-GFP mice were purchased from the Jackson Laboratory. All mice were kept under specific pathogen-free conditions and all experiments were performed according to institutional guidelines of the European Community.

Cell suspension

Single cell suspensions were prepared from LN, SP, liver, thymus and BM in HBSS containing 2% FBS (both from PAA Laboratories GmbH). For preparation of intestinal cell suspensions, colon pieces were incubated in PBS containing 5 mM EDTA for 15 min on a shaking incubator at 37°C prior to digestion. Supernatant was collected and remaining tissue was further digested. To digest tissues, intestine, lung and ear skin were finely minced and stirred in HBSS with 400 µg/mL Liberase (Roche) and 50 µg/mL Collagenase IV (Sigma) for 1 h at 37°C. Cells isolated from intestine, liver and lung were pelleted, resuspended in 37% Percoll (GE Healthcare) and spun at 2500 rpm for 25 min. Isolated lymphocytes were washed twice and used for subsequent flow cytometry analyses.

Flow cytometry

Extracellular staining was preceded by incubation with purified anti-CD16/32 Ab (FcγRII/III block, 2.4G2) (eBioscience) to block non-specific staining. Cells were stained with FITC-, PE-, PECy5-, PECy7-, APC- and APCAlexa750-labeled or biotinylated appropriate Ab including CD4 (GK1.5); TCR (H57-597); CD62L (MEL-14); CD69 (H1.2F3); CD25 (PC61.5); CD127 (A7R34); ICOS (7E.17G9); CD103 (2E7) or appropriate isotype Ab. Streptavidin-FITC, PECy5 or PECy7 were used to develop biotinylated Ab. All Ab were purchased from eBioscience. Intracellular FOXP3 staining was performed using eBioscience APC-conjugated FOXP3 staining buffer set (FJK-16s). Six-color flow cytometry was performed with a FACSCanto cytometer (BD Biosciences) and data files were analyzed using FlowJo software (Tree Star Inc.).

Cell purification

Cell sorting of naïve CD4⁺ T cells (CD4⁺CD62L⁺CD25⁻) and Treg (CD4⁺CD25⁺) was performed on a FACSVintage cell sorter (BD Biosciences). For suppressive assays and adoptive transfer experiments, Treg subsets were sorted based on CD62L, CD69, ICOS and CD103 expression following positive selection using anti-CD25 PE or biotinylated Ab incubation, and purification with anti-PE or anti-biotin beads, respectively (Miltenyi). For *in vitro* survival experiments, cells were isolated from mesenteric lymph nodes. CD127^{hi} Treg (top 10% of Treg gate) and Treg exhibiting low CD127 expression (approximately lowest 50% of Treg gate) were next FACS sorted among CD4⁺CD25⁺ cells.

In vitro cell cultures

Cells were cultured in RPMI 1640 medium containing penicillin, streptomycin, L-glutamine, HEPES buffer, non-essential amino acids, sodium pyruvate and β-mercaptoethanol and 10% heat inactivated FBS (all from PAA Laboratories GmbH). For activation experiments, total CD4⁺ T cells (1×10^5) were cultured in 96-well plates with splenocytes from RAG^{-/-} mice (1×10^5) and 0.5 µg/mL anti-CD3.

Suppression assays

Suppression assays were performed using allotypic marker CD45.1/CD45.2 to distinguish conventional from CD25⁺ sorted population. CFSE (Sigma) labeled CD45.1⁺CD62L⁺CD25⁻CD4⁺ cells (3×10^4) were cultured for 72–96 h in 96-well plates with splenocytes from RAG^{-/-} mice (3×10^4), 0.5 µg/mL anti-CD3 (eBiosciences), in the presence or absence of the indicated subset of CD4⁺CD25⁺CD45.2⁺ cells. CFSE labeling was performed using standard methods.

Adoptive transfer experiments

FACS sorted CD45.1 naïve CD4⁺ T cells (1×10^5) and CD45.2 Treg (0.5×10^5) were co-injected into RAG2-deficient mice. Six weeks after transfer, SP and mesenteric LN were harvested.

Contact dermatitis model

Acute skin inflammation experiments were conducted as described [28]. Briefly, 0.5% DNFB (Sigma) diluted in acetone/olive oil vehicle (4:1 v/v) was applied onto ears of C57BL/6 mice. Control mice were treated with vehicle alone. Six days later, ear thickness was measured with a digital caliper, cervical draining LN and ears were isolated.

STAT5 phosphorylation experiments

Cells isolated from Foxp3-GFP mice were incubated for 30 min at 37°C with or without IL-2, IL-7 (Immuno Tools) or TSLP (R&D) and immediately fixed in 2% paraformaldehyde. Cells were made permeable by incubation in 90% methanol and then were stained with primary rabbit Ab to phosphorylated STAT5 or isotype control (Cell Signaling) revealed with an anti-rabbit Alexa-647 conjugated secondary Ab.

In vitro survival experiments

5×10^4 FACS-sorted total CD4⁺CD25⁺, CD4⁺CD25⁺CD127^{lo} or CD4⁺CD25⁺CD127^{hi} were cultured overnight (18–20 h) in medium alone or in the presence of IL-7 (10 ng/mL). Cell survival was determined using the fluorescence-based LIVE/DEAD assay (Invitrogen).

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Abbreviations: ICOS: inducible costimulatory molecule · DNFB: 2,4-dinitrofluorobenzene · SP: spleen · PB: peripheral blood · TSLP: thymic stromal-derived lymphopoietin

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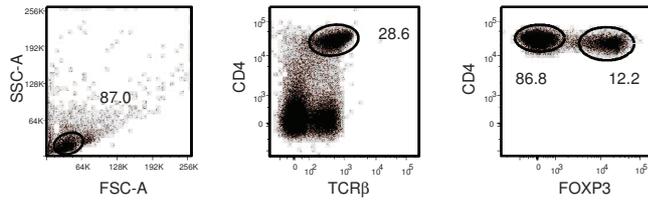
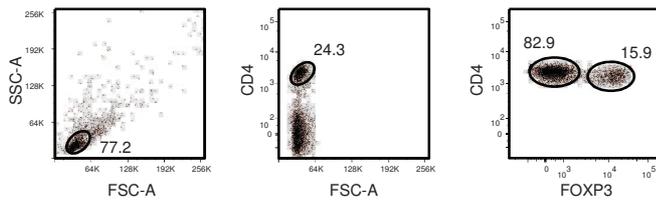
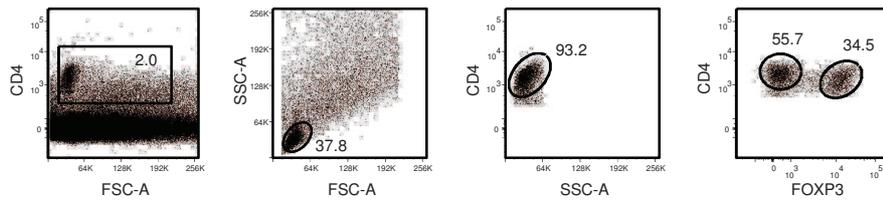
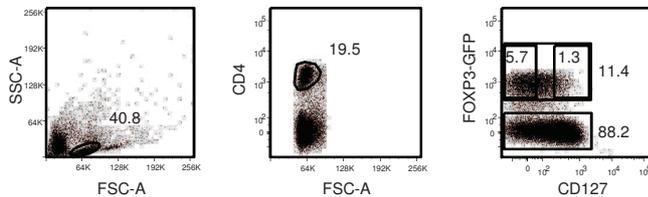
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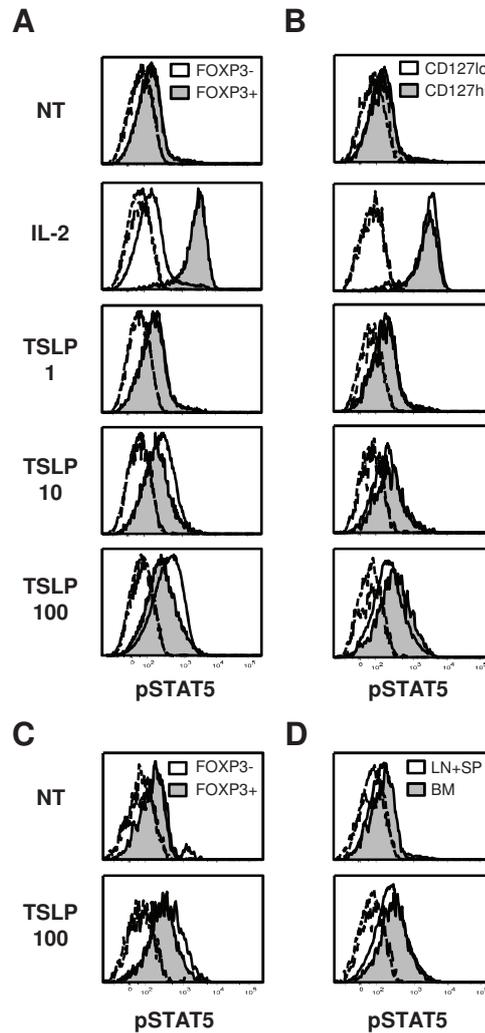
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Increased CD127 expression on activated FOXP3⁺CD4⁺ regulatory T cells

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A**B****- Draining Lymph node****- Ear Skin****C**

Supplemental Figure 1. Gating strategies for FACS analyses. (A) Gating strategy for Treg immunophenotyping in WT mice. The gating strategy to identify Treg included successively forward (FSC) and side scatter (SSC) gating, selection of cells positive for TCR β and CD4, and identification of FOXP3 expressing cells. (B) Gating strategy for Treg identification in DNFB model. For lymph nodes, Treg identification was performed using FCS/SSC plot followed by CD4 selection and identification of FOXP3 expressing cells. For ear skin analysis, a wide CD4 versus FSC gate followed by back-gating to the FSC/SSC plot was used to better define CD4⁺ lymphocytes. (C) Gating strategy for STAT5 phosphorylation studies. A FSC versus SSC plot was used to define lymphocytes followed by gating on CD4⁺ cells. Treg were identified as FOXP3-GFP⁺ cells. CD127^{hi} Treg were identified by gating on top 10% CD127-expressing CD4⁺GFP⁺ cells; CD127^{lo} Treg were defined as 50% of CD4⁺GFP⁺ cells expressing the lowest CD127 levels.



Supplemental Figure 2. No difference in TSLP signal transduction in Treg depending on CD127 expression. Cells from Foxp3-GFP mice were incubated with either medium alone, IL-2 (50 U/ml) or TSLP at indicated doses (1, 10 and 100 ng/ml) for 30 min. STAT5 phosphorylation was determined on Foxp3⁺ or Foxp3⁻ CD4⁺ cells isolated from (A) pooled LN and splenic cells or (C) BM cells. (B) p-STAT5 staining depending on CD127 expression among Foxp3⁺ LN and SP cells, CD127^{hi} Treg were identified by gating on top 10% CD127 expressing CD4⁺ GFP⁺ cells (grey shaded profile); CD127^{lo} Treg were defined as 50% of CD4⁺ GFP⁺ cells expressing the lowest CD127 levels (open profile). (D) Comparison of p-STAT5 staining among LN and SP or BM isolated Treg. Results are representative of three independent experiments using one GFP Foxp3 mouse for each experiment.

3.2 – ARTICLE 2

Interleukin-7 governs CD4+ CD25+ FOXP3+ regulatory T cell numbers

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Background : Mechanisms governing peripheral CD4+FOXP3+ regulatory T cells (Treg) survival and homeostasis still remain ill defined. Some subset specific factors, such as TGF β , interleukin-2 (IL-2) and B7 costimulatory molecules, have been identified as essentials for the maintenance of the peripheral Treg compartment. Conversely, Treg dependency upon classical T cell homeostatic factors such as IL-7 is still debated. In this work, we formally investigated in murine models the role of IL-7 in Treg homeostasis in vivo.

Methods : Using mice in which IL-7 signaling was genetically impaired (IL7R α ^{-/-} and IL7^{-/-} mice) or increased (IL-7Tg mice) as well as IL-7 treatment in normal mice, we investigated the role of IL-7 availability in controlling the size of the Treg pool. Moreover we used the adoptive transfer model to study the contribution of IL-7 in Treg expansion in lymphopenic settings.

Results : We demonstrated that IL-7 availability regulated the size of the peripheral Treg cell pool. Moreover, we showed that IL-7 administration increased Treg cell numbers by inducing a thymic-independent Treg peripheral expansion. Finally we demonstrated that IL-7 participates to the peripheral expansion of Treg after adoptive transfer into lymphopenic hosts.

Conclusions : Our results definitively identify IL-7 as a central factor contributing to Treg peripheral homeostasis, thus reassembling Treg to other T cell subsets in respect of their need for IL-7 for their peripheral maintenance.

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Interleukin-7 governs CD4+CD25+ FOXP3+ regulatory T cells peripheral homeostasis

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ABSTRACT

Mechanisms governing peripheral CD4⁺FOXP3⁺ regulatory T cells (Treg) survival and homeostasis still remain ill defined. Some subset specific factors, such as TGF β , interleukin-2 (IL-2) and B7 costimulatory molecules have been identified as essentials for the maintenance of the peripheral Treg compartment. Conversely, Treg dependency upon classical T cell homeostatic factors such as IL-7 is still debated. In this work, we formally investigated in murine models the role of IL-7 in Treg homeostasis *in vivo*. We demonstrated that IL-7 availability regulated the size of the peripheral Treg cell pool. Moreover, we showed that IL-7 administration increased Treg cell numbers by inducing a thymic-independent Treg peripheral expansion. Finally we demonstrated that IL-7 participates to the peripheral expansion of Treg after adoptive transfer into lymphopenic hosts. Our results definitively identify IL-7 as a central factor contributing to Treg peripheral homeostasis, thus reassembling Treg to other T cell subsets in respect of their need for IL-7 for their peripheral maintenance

INTRODUCTION

CD4⁺ CD25⁺ FOXP3⁺ regulatory T cells are a population of CD4⁺ T cells crucial for the regulation of immune responses and in preventing autoimmunity and chronic inflammation [1]. Mice and humans genetically deficient in Treg cells as a result of mutations in FOXP3, a critical transcription factor for Treg development and function, typically present severe lymphoproliferation and immune-pathology [2], [3], [4], [5]. Treg exert their major role in the maintenance of immune-tolerance through several mechanisms including secretion of inhibitory molecules, suppression of antigen-presenting cells function, cytolysis and effector cells metabolic disruption [6].

Despite the importance of the Treg compartment for the maintenance of immune tolerance and the intensive investigation focusing on Treg cell biology during the last few years, little is known about the factors controlling peripheral Treg survival and homeostasis. While Treg seem to rely on some subset specific factors, such as TGF β [7], [8], [9], IL-2 [10], [11], [12], [13] and B7 costimulatory molecules [14], [15], their dependency upon factors classically involved in naïve or memory T cells homeostasis, such as IL-7 signaling, still remains controversial.

IL-7 is a member of the gamma chain cytokines family which plays a critical role in T cell development and peripheral homeostasis [16]. All major CD4 T cell subsets, including naïve, memory and Th17 CD4 T cells, strictly rely on IL-7 for their peripheral homeostasis [17], [18], [19], [20]. The only important exception seems to be represented by CD4⁺ FOXP3⁺ Treg which have been reported to express low levels of the IL-7 receptor alpha chain (CD127) [21], [22], [23] and whose peripheral biology is believed to be essentially IL-7 independent [20], [24].

We demonstrated recently in a murine system that low CD127 expression is not a universal characteristic of Tregs and that CD127 expression on Tregs depends on their tissue localization and activation status [25]. Moreover, we and others have previously shown that *in vitro* IL-7 treatment in-

duces significant STAT5 phosphorylation in Tregs [26], [24], [27], [25] and that IL-7 is able to increase *in vitro* Treg survival [25], [28].

In this study, we investigated the role of IL-7 on Treg homeostasis *in vivo*. We demonstrated that IL-7 availability regulated the size of the peripheral Treg cell pool. Moreover, we showed that IL-7 administration increased Treg cell numbers by inducing a thymic-independent peripheral expansion. Finally we demonstrated that IL-7 participates to the peripheral expansion of Treg after adoptive transfer into lymphopenic hosts.

Our results definitively identify IL-7 as a central factor contributing to Treg peripheral homeostasis. Such a conclusion has major implications for the development of IL-7 based strategies to ameliorate immune-reconstitution in lymphopenic settings while preventing immune pathology.

RESULTS

IL-7 availability affects the size of the conventional and regulatory T cell pools to a similar extent

In order to assess *in vivo* the influence of IL-7 on Treg homeostasis, we first examined mice in which IL-7 signaling was genetically disrupted (IL-7R α ^{-/-} or IL-7^{-/-}) or increased (IL7Tg).

In agreement with published reports, IL-7R α ^{-/-} or IL-7^{-/-} mice display similar percentages of thymic FOXP3⁺ cells among single positive CD4 cells when compared to normal B6 mice (Fig. 1A). Similar proportions were observed in thymi from IL-7Tg mice. Study of absolute thymic T cell numbers revealed that the expected reduction in FOXP3⁻ CD4⁺ single positive thymocytes in IL-7R α ^{-/-} or IL-7^{-/-} mice was associated to a proportional reduction in the FOXP3⁺ CD4⁺ single positive compartment (Fig. 1B). Accordingly IL-7 over-expressing mice presented higher numbers of both FOXP3⁻ and FOXP3⁺ CD4⁺ single positive thymocytes. Collectively these data suggest that IL-7 signaling similarly affects conventional T cells (Tconv) and Treg cell thymic development. We then focused on splenocytes in order to evaluate IL-7 contribution in controlling Treg cell peripheral cell numbers. As already described, higher percentages of FOXP3⁺ cells were found among splenic CD4 T cells from IL-7R α ^{-/-} and IL-7^{-/-} mice when compared to control B6 mice (Fig 1A). Such a difference is probably linked to impaired peripheral survival of conventional naïve CD4 T cells in the absence of IL-7 signaling. Interestingly, IL-7Tg mice presented a perfectly preserved proportion of splenic Treg cells among CD4 T cells.

Analyzing T cells numbers, we observed as expected that conventional CD4⁺ FOXP3⁻ T cell numbers were significantly reduced in IL-7R α ^{-/-} and IL-7^{-/-} mice (13.8-fold and 5.4-fold reduction respectively) while they were present in higher numbers in IL-7Tg mice (Fig. 1B). Accordingly, IL-7R α ^{-/-} and IL-7^{-/-} mice presented significantly reduced but yet substantial numbers of peripheral Treg cells compared with their wild type counterparts, while IL7Tg mice presented a 4.7-fold increase in Treg cell number (Fig. 1B). Interestingly, the extent of Treg number variation in

spleen from KO or Tg mice strictly reflected the variation we observed in the conventional FOXP3-CD4 T cell compartment.

Collectively, these results suggest that IL-7 similarly affects conventional and regulatory T cell numbers by acting on thymic development and/or peripheral homeostasis.

IL-7/ α IL-7 complex treatment induces Regulatory T cells peripheral expansion

To better assess the peripheral role of IL-7, we determined the effects of exogenous IL-7 treatment on Treg cells. C57Bl/6 mice were injected with IL-7/ α IL-7 complexes or PBS at d0, d2 and d4. Such a treatment protocol has been reported to induce strong peripheral expansion of conventional CD4 and CD8 T cells in non-lymphopenic hosts [29]. Flow cytometry analysis at day 7 from the first injection revealed a slight reduction in the percentage of FOXP3⁺ among CD4⁺ in the lymph-nodes (NT 11.1% \pm 1.0% vs IL-7 9.2% \pm 1.1%, $p=0.044$) but not in the spleen (NT 10.8% \pm 1.4% vs IL-7 11.4% \pm 0.6%, $p=0.586$) (Fig. 2A). When considering absolute numbers, IL-7 treatment increased both CD4⁺ FOXP3⁻ and FOXP3⁺ cell numbers (\sim 2 fold increase in both LN and SP) (Fig. 2B).

Increased peripheral T cell numbers could have resulted from effects on peripheral proliferation, cell survival and/or thymic production.

To assess whether IL-7 treatment was affecting Treg proliferation, we examined Ki67 staining which identifies proliferating cells. IL-7 treatment had only minimal effects on cell cycle entry of Treg, while conventional CD4 T cells exhibited substantial increases in proliferation (Fig. 2C). These results suggest IL-7 treatment is likely to increase Treg cell numbers in the periphery by preferentially affecting survival rather than proliferative capacity.

To assess IL-7 effects on Treg cellularity while formally excluding any potential contribution of IL-7 effects on thymopoiesis, we adoptively transferred CFSE stained CD4 T cells from CD45.1 mice into CD45.2 B6 mice prior to IL-7 complexes treatment. Use of CD45.1 and FOXP3 staining allowed us to distinguish progeny of adoptively transferred cells from host cells (Fig. 3A). A two

fold expansion was observed in FOXP3⁺ CD4⁺ transferred CD45.1 Treg cells isolated from IL-7 complexes treated mice when compared to PBS injected mice (Fig. 3B). Even in this experimental setting, such a Treg cell expansion reflected a similar effect on the conventional T cell compartment (Fig. 3B). In order to evaluate the contribution of cell proliferation in the observed expansion, we analysed the CFSE dilution profile of adoptively transferred cells (Fig. 3C). IL-7 complexes treatment induced an increase in the CFSE_{low} proliferating fraction in both conventional and Treg cell compartments. In accordance to data obtained with the Ki67 staining, IL-7 induced increase in proliferation was more important in T_{conv} than in Treg cell compartments.

Collectively, these results indicate that IL-7 therapy affects both conventional and regulatory T cell numbers to a similar extent by acting on their proliferation and their peripheral survival.

IL-7 directly induces Treg peripheral expansion in lymphopenic hosts

Increase in Treg cellularity upon IL-7 treatment may also reflect increasing proliferation of conventional/effector cells, which presumably correlated with high IL-2 production. To directly address the impact of IL-7 on Treg biology, we used adoptive transfer into lymphopenic hosts, in which Treg expansion in the absence of potential IL-2 producer T cells could be observed. We transferred purified CFSE labelled CD4⁺ CD25⁺ cells into IL-7 competent or deficient empty hosts (Rag^{-/-} or Rag^{-/-} IL-7^{-/-} mice) (Fig. 4). Treg recovered from spleen of both groups were identified by FOXP3 intracellular staining at day 1 and 6 (Fig. 4A). At day 1, Treg cell numbers recovered from spleen were slightly but statistically different between IL-7 deficient and IL-7 competent hosts. Such higher splenic homing in Rag^{-/-} IL-7^{-/-} recipients presumably reflect the absence of LN structures in the absence of IL-7. At d6, Treg recovery in IL-7 competent hosts was increased compared to day 1, whereas Treg numbers recovered in IL-7 deficient hosts were reduced compared to d1 values (Fig. 4B). As previously described, T cell numbers are resultant of peripheral proliferation and cell survival in the absence of thymic export. However the increasing numbers recovered from d1 to d6 in IL-7 competent recipients necessarily identified an impact of IL-7 on T

cell proliferation. We thus analysed the CFSE dilution profile of injected Tregs in both hosts (Fig. 4C). Higher proportion of dividing FOXP3⁺ Tregs was evident in IL-7 competent hosts compared to IL-7 deficient hosts. Notably, FOXP3⁺ Treg proliferation was still detectable in IL-7 deficient hosts suggesting that other factors were involved in Treg proliferation in lymphopenic settings. Collectively, these data demonstrate that Treg expansion and accumulation in lymphopenic settings is IL-7 dependent.

DISCUSSION

All major CD4 T cell subsets, including naïve, memory and Th17 CD4 T cells, strictly rely on IL-7 for their peripheral homeostasis. The only important exception seems to be represented by CD4⁺ FOXP3⁺ Treg cells, which are believed to essentially rely on other factors which are more specific for this cells subset such as IL-2, TGF β and costimulatory molecules. This study aims to further investigate the effects of IL-7 on the homeostasis of CD25⁺ FOXP3⁺ regulatory T cells.

We demonstrated that IL-7 availability controls the size of the Treg pool. IL-7 treatment in normal mice induced an increase in conventional CD4 T cell numbers that was strictly paralleled by an increase in Treg cell numbers. Accordingly, mice in which IL-7 signaling was impaired presented a reduction in Treg cell numbers similar to those observed in conventional T cell numbers, while IL-7Tg mice, in which IL-7 production is constantly increased, showed an expansion of the Treg compartment. Our data perfectly corroborate results obtained in humans during clinical trials investigating the effects of IL-7 therapy on immune-reconstitution in lymphopenic settings. Indeed, three published reports analyzed the proportion of Treg cells in peripheral blood from patients treated with IL-7 [30], [31], [32] and detected either slight reduction or no difference in the percentage of Treg cells among CD4 T cells after IL-7 treatment. As total CD4 T cell count increased in IL-7 treated patients, we can imagine that, even in human studies, Treg changes in absolute numbers paralleled those detected in conventional CD4 T cells. Collectively, both human and mice studies reveal that IL-7 levels equally affect the size of conventional and Treg subsets thus maintaining the physiologic balance between these two compartments.

Several reports investigated the role of IL-7 on thymic Treg development [33], [24], [34] and tried to extend the analysis to the peripheral effects of IL-7. Using adoptive transfer of total CD4 T cells into irradiated Rag^{-/-} or Rag^{-/-}IL7^{-/-} mice, Mazzuchelli et al, failed to detect any differences in Treg numbers recovered concluding that IL-7 availability does not affect Treg

expansion during lymphopenia [24]. Unfortunately, total lymph-node cells were transferred, impeding any conclusions on the direct effect of IL-7 on Treg cells in this setting. Using adoptive transfer of purified CD4⁺CD25⁺ into Rag^{-/-} or Rag^{-/-}IL7^{-/-} mice we showed here that Treg cells transferred into Rag-deficient mice lacking IL-7 proliferated and accumulated less than Treg transferred into IL-7 competent Rag-deficient mice. While demonstrating that IL-7 plays a major role in Treg expansion during lymphopenia, the residual proliferation still present in the absence of IL-7 indicates that other factors participate to the phenomenon. Treg are believed to be continuously activated by tissue self-antigens [35]. Reduction of cell competition for MHC-self antigen complexes in lymphopenic environments could increase proliferation. Another potential explanation is that accumulated bacterial products in lymphopenic hosts could drive Treg expansion via, for example, Toll-Like Receptors triggering [36].

Although several gamma chain cytokines, including IL-2, IL-4, IL-7, IL-15, IL-21, have been reported to increase Treg survival *in vitro* [28], only IL-2 has been clearly established as a major factor controlling Treg cell homeostasis *in vivo* [10], [11], [12], [13], [37], [38]. In this study we provide evidence that IL-7 controls Treg cell numbers *in vivo*. We showed here that in the absence of IL-7 signals, a polyclonal population of Treg proliferated poorly upon adoptive transfer into lymphopenic hosts. Importantly such lymphopenia-driven expansion was shown to be IL-2 independent [13], allowing us to conclude on the direct effect of IL-7. However, one may argue that this effect was mainly related to the absence of IL-2 signaling [34]. Whether IL-7 still influences Treg homeostasis when significant amount of IL-2 are present remains to be further investigated. Zhang et al. showed that a lymphopenic environment enhances IL-2 induced Treg expansion [37]. As lymphopenia is typically characterized by increased levels of IL-7, we may speculate that IL-2 and IL-7 could synergize to induce Treg expansion and/or survival.

In summary, our data indicate that IL-7 plays a key role in Treg cell homeostasis. Our findings have

several important implications. They establish a role for IL-7 in Treg homeostasis, thus reassembling Treg to other T cell subsets in respect of their need for IL-7 for their peripheral maintenance. Moreover, they indicate that IL-7 availability similarly affects both the conventional and the regulatory T cell pool, maintaining their proportions relatively unchanged. This balanced effect of IL-7 can be important to prevent immune-pathology during both spontaneous and therapeutic IL-7 induced immune-reconstitution.

MATERIAL AND METHODS

Mice

6 to 8 weeks old C57Bl/6 were purchased from Charles River (Janvier). Mice deficient for Rag gene (Rag^{-/-}) or for Rag and IL-7 genes (Rag^{-/-}IL7^{-/-}) were used as hosts for adoptive transfer experiments. IL-7R α ^{-/-} mice were purchased from the Jackson Laboratory. IL-7^{-/-} and IL-7 transgenic (IL-7Tg) mice were kindly provided by Dr. Sophie Ezine. All mice were kept under specific pathogen free conditions and all experiments were performed according to institutional guidelines of the European Community.

Cell purification

Single cell suspensions were prepared from lymph nodes (pooled inguinal, brachial, axillary and mesenteric) and spleens in HBSS containing 2% FCS (PAA Laboratories GmbH). For total CD4 T cells transfer, CD4 T cells were isolated using BD magnetic beads. For Treg adoptive transfer experiments, CD4⁺ CD25⁺ cells were isolated by magnetic beads accordingly to manufacturer instructions (Miltenyi). Cell purity for CD25 and FOXP3 was >90%.

Adoptive transfer experiments

Purified CD4⁺ CD25⁺ cells (1×10^6) were injected into Rag-deficient mice (Rag^{-/-}) or Rag^{-/-} mice that were also deficient for IL-7 (Rag^{-/-} IL-7^{-/-}). One and six days after transfer, spleens were harvested and analyzed.

CFSE labelling

When required, cell division of transferred T cells was assessed by CFSE labelling (Sigma) using standard methods. Cells were resuspended in PBS in a concentration of 10^7 /ml and incubated with CFSE at final concentration of 5 μ M for 10 min at 37°C, followed by two washes in ice cold HBSS

containing 10% FCS.

Cytokines

Recombinant murine IL-7(rmIL-7) was purchased from Immunotools. Anti-IL-7 mAb M25 was purchased from BioXcell respectively. Before injection, cytokines/antibody complexes were generated as previously described [29]. Briefly, 1 µg of cytokine was co-incubated with 5 µg of the specific monoclonal antibodies for 30 min at +37°C.

FACS analysis

Extracellular staining was preceded by incubation with purified anti-CD16/32 antibodies (FcγRII/III block, 2.4G2) (eBioscience) to block nonspecific staining. Cells were stained with FITC-, PE-, PECy5-, PECy7- APC- APCAlexa750- or APC-H7-labeled or biotinylated appropriate antibodies including : CD4 (GK1.5); TCRβ (H57-597); CD25 (PC61.5) or appropriate isotype Abs. Streptavidin-FITC, PECy5 or PECy7 were used to develop biotinylated Abs. All Abs were purchased from eBioscience except APC-H7-labeled antibodies (BD Bioscience). Intracellular FOXP3 staining was performed using eBioscience PE- or APC- conjugated FOXP3 staining buffer set (FJK-16s). Six-color flow cytometry was performed with a FACSCanto cytometer (BD Biosciences) and data files were analyzed using FlowJo software (Tree star Inc).

Statistical analyses

Statistical analyses were performed using unpaired Student T test with Graph Pad Software.

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LEGENDS

Figure 1. IL-7 signalling is involved in both thymic development and peripheral maintenance

of FOXP3⁺ Treg. (A) Dot plots gated on CD4⁺ CD8⁻ TCRβ⁺ thymocytes and splenic CD4⁺ TCRβ⁺ cells from normal B6, IL7R^{-/-}, IL7^{-/-} and IL7Tg animals. Samples were counter-stained for FOXP3. Numbers indicate the relative percentages. (B) Absolute numbers of CD4⁺ FOXP3⁺ T cells from normal B6, IL7R^{-/-}, IL7^{-/-} and IL7Tg animals. Experiments were repeated independently twice with four to six mice per group; the data shown are representative of one experiment. The degree of statistical significance (p values), relative to normal B6 numbers, is indicated.

Figure 2. IL-7 complexes treatment induced both conventional and regulatory T cell

expansion. C57Bl/6 mice were i.p. injected with PBS or IL-7/anti-IL-7 complexes (1 μg rmIL-7 plus 5 μg M25) three times at 2 day intervals. LN and SP cells were analysed 7 days after the first injection. (A) Representative dot plots of CD25 vs FOXP3 expression on CD4⁺TCRβ⁺ cells recovered from PBS or IL-7 complexes treated mice. (B) Total number of CD4⁺TCRβ⁺FOXP3⁻ conventional T cells (left panels) and CD4⁺ TCRβ⁺FOXP3⁺ regulatory T cells (right panels) from lymph nodes and spleen. (C) Percentage of cells expressing the proliferation marker Ki67 in CD4⁺TCRβ⁺FOXP3⁻ conventional T cells and CD4⁺ TCRβ⁺FOXP3⁺ regulatory T cells from LN (left panel) and spleen (right panel). Percentage of Ki67 positive cells were determined based on isotype control. Results were pooled from 2 separate experiments, using three to four mice per group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$ (unpaired Student's *t* test).

Figure 3. IL-7 induced expansion of both conventional and regulatory T cells is thymic

independent. Total CD4 T cells from CD45.1 mice were transferred into CD45.2 C57Bl/6 hosts. Mice were then treated with IL-7/anti-IL-7 complexes as in Fig. 2. (A) Representative dot plots of

CD45.1 vs FOXP3 expression on CD4⁺TCRβ⁺ cells recovered at day 7 from lymph nodes and spleens of PBS or IL-7 complexes treated mice. (B) Total numbers of adoptively transferred CD45.1 FOXP3⁻ conventional T cells (left panels) and CD45.1 FOXP3⁺ regulatory T cells (right panels) from lymph nodes and spleen. (C) Representative profiles of CFSE dilution of CD45.1 Tconv and Treg isolated from PBS or IL-7 complexes treated mice. Experiments were repeated independently three times with two to four mice per group. Data shown are representative of one experiment.

Figure 4. IL-7 promoted expansion of CD4⁺CD25⁺ Treg cells in lymphopenic hosts. 1.10⁶ CFSE-labeled, purified CD4⁺CD25⁺ T cells were transferred into Rag^{-/-} and Rag^{-/-}IL-7^{-/-} mice. (A) Representative dot plots of FOXP3 vs TCRβ expression on splenic lymphocytes at day 6 after transfer. (B) Total splenic numbers of CD4⁺TCRβ⁺FOXP3⁺ cells recovered per mouse at day 1 and 6. (C) Representative profiles of CFSE dilution of CD4⁺TCRβ⁺FOXP3⁺ cells at day 6 after transfer, and graph of CFSE^{low} Tregs percentage recovered from three experiments using two to three mice per group.

FIGURE 1

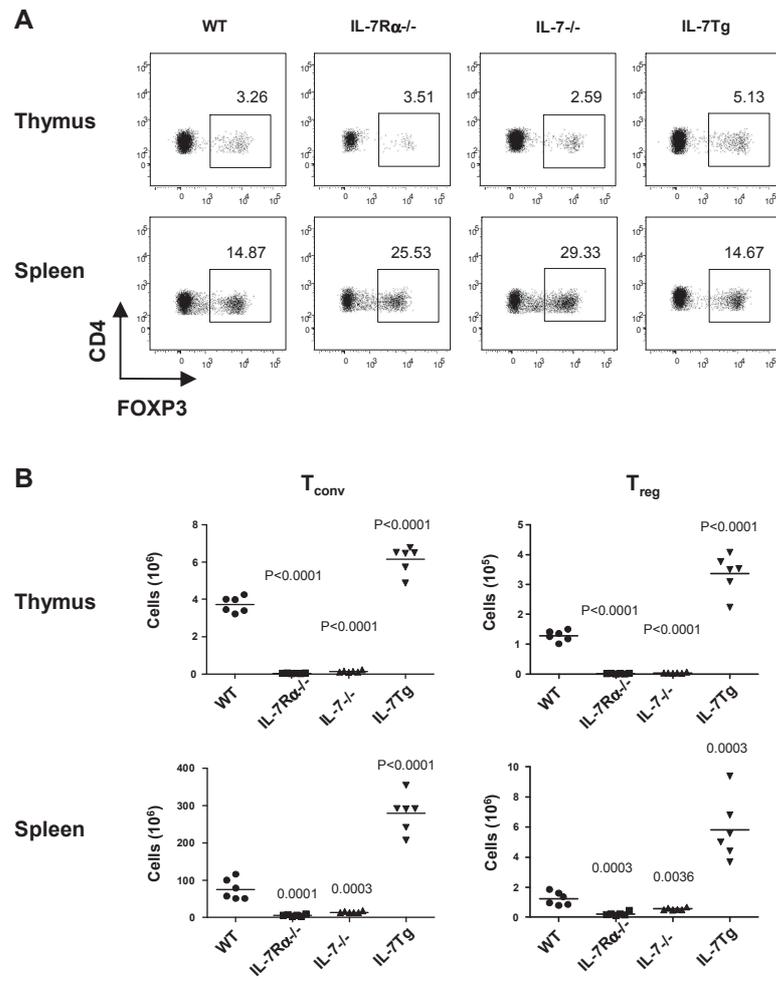


FIGURE 2

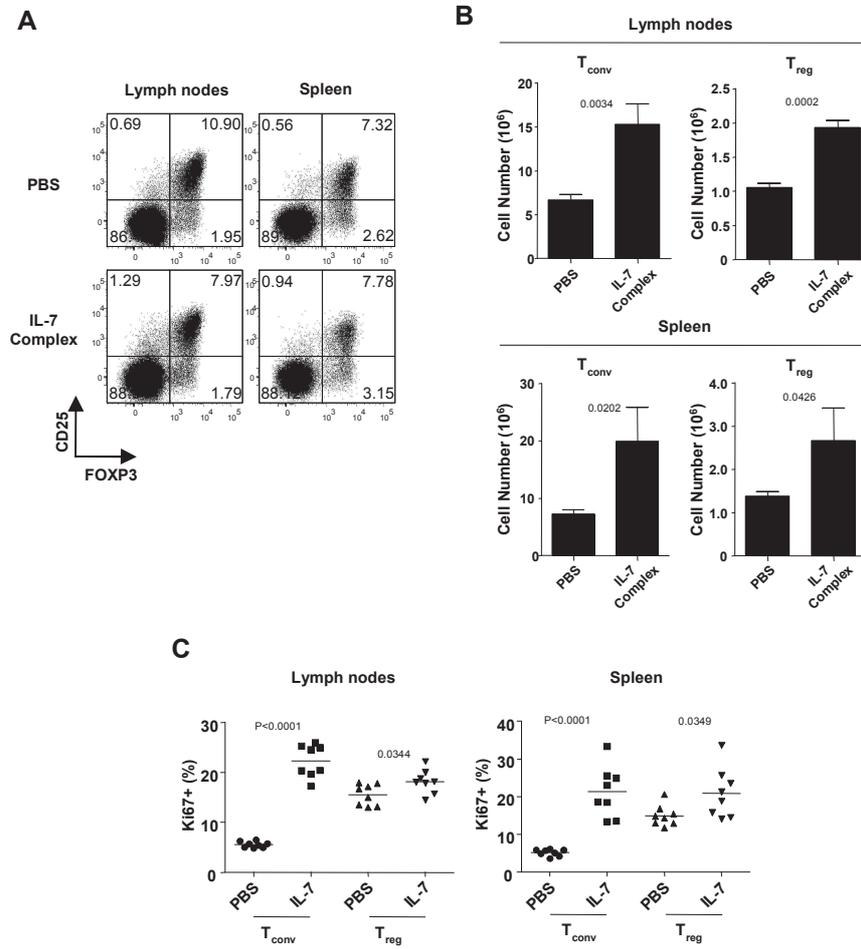


FIGURE 3

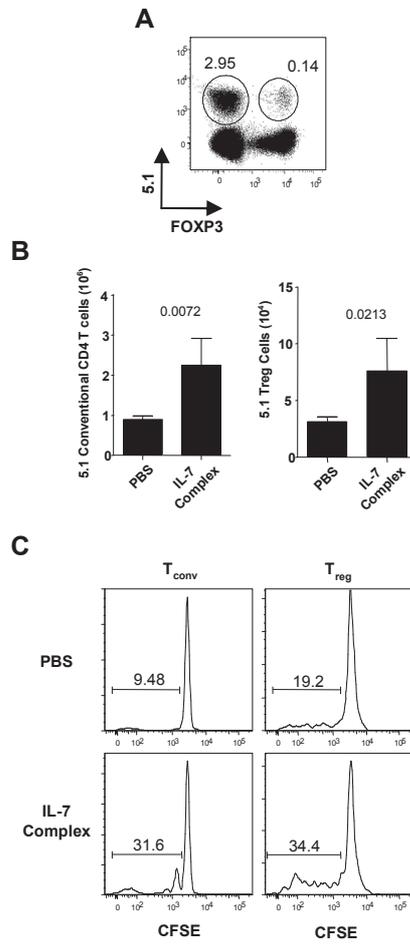
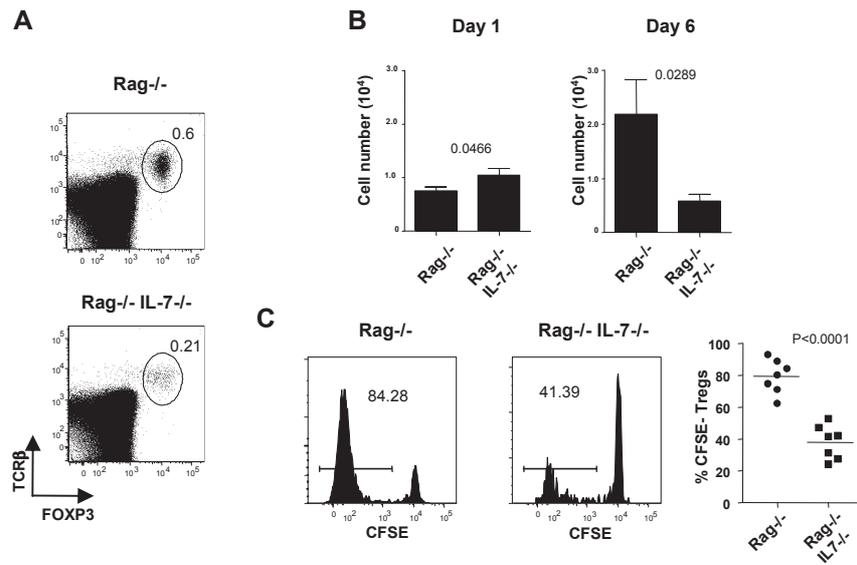


FIGURE 4



3.3 - PRELIMINARY RESULTS

3.3.1 - IL-7 modulates CD25 expression on regulatory T cells *in vitro* and *in vivo*

Our previous results indicated that IL-7 directly contributed to Treg homeostasis. However IL-2 is considered the major player governing Treg cell numbers. We therefore speculated that IL-7 could act in synergy with IL-2 to guarantee optimal Treg homeostasis. As CD25 expression at the cell surface is essential for IL-2 consumption by Tregs, we assessed the possibility that CD25 expression on Tregs cells can be dynamically regulated by IL-7 availability. Indeed, early reports showed that IL-7 induces CD25 expression on the surface of activated conventional T cells (see introduction). To this aim, we FACS sorted CD4⁺ GFPFOXP3⁺ lymph nodes and spleen cells from FOXP3GFP mice and cultured them overnight with medium alone or in the presence of IL-7 or IL-2 as control. As expected, CD25 expression decreased on CD4⁺ FOXP3⁺ T cells incubated in the absence of cytokine (Fig. 3.1 A, B) when compared to levels expressed *ex vivo*. As expected, the addition of IL-2 to overnight cultures sustained high levels of CD25 expression on Treg cells (Fig. 3.1 A,B). Interestingly, the addition of IL-7 to cultures prevented the decrease in CD25 expression on Treg cells in a dose dependent manner (Fig. 3.1 A, B and data not shown). We performed similar analysis at the mRNA level to determine whether IL-7 affected CD25 surface expression through a transcriptional mechanism. As expected, CD25 mRNA decreases upon *in vitro* 24h incubation in the absence of cytokines while addition of IL-2 to culture medium maintained CD25 transcription (Fig. 3.1 C). As demonstrated at the protein level, Treg incubated in the presence of IL-7 maintained CD25 transcription (Fig. 3.1 C). These *in vitro* results demonstrate that IL-7 is able to sustain CD25 transcription and surface expression on Treg cells in the absence of IL-2 signaling.

To measure the effect of IL-7 on CD25 expression *in vivo*, we examined CD25 expression on Tregs from mice in which IL-7 signaling was disrupted (IL-7R^{-/-} or IL-7^{-/-}) or increased (IL-7Tg). Percentage of CD25 expressing cells and median MFI were determined using isotype control staining to define positive expression and to calculate

Δ MFI respectively (Fig. 3.2). The percentage of CD25 expressing Treg was lower on splenic FOXP3⁺ CD4⁺ cells from IL-7RKO or IL-7KO mice than on wild type cells (WT 72.8% \pm 3.4%, IL-7RKO 38.3% \pm 6.6%, IL-7KO 54.1% \pm 8.8%).

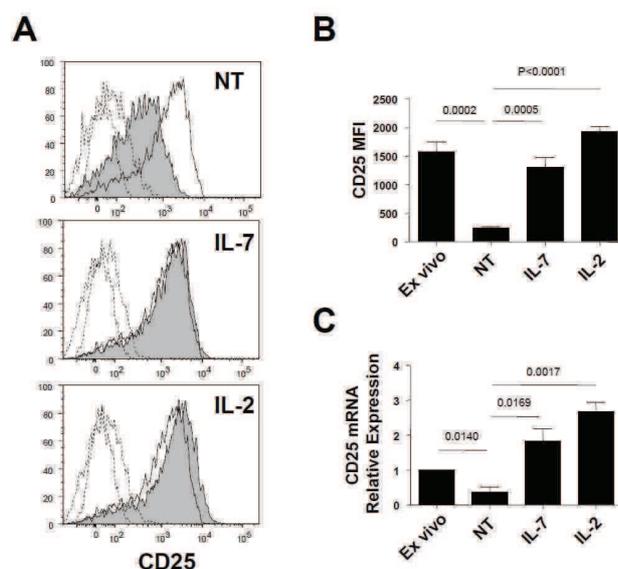


Figure 3.1 - IL-7 signaling dynamically regulated CD25 expression on Treg cells in vitro. FACS sorted CD4⁺ FOXP3GFP⁺ and CD4⁺FOXP3GFP⁻ cells were untreated or treated overnight with IL-2 (2 ng/ml) or IL-7 (10 ng/ml). (A) Representative FACS profiles and MFI of CD25 expression on FOXP3GFP⁺ after culture are shown. Mean \pm SEM from three independent experiments are shown. (B) CD25 mean fluorescence intensity and (C) CD25 mRNA relative expression by PCR. Results are representative of three independent experiments performed in triplicates.

Conversely, CD25 expression was modestly but significantly increased on splenic Treg cells isolated from IL7Tg mice when compared to wild type mice (Fig. 3.2 A-B). Studies of median of CD25 expression on Treg from the different mouse strains showed equivalent profile: reduced CD25 MFI on Treg isolated from IL-7R KO and IL-7 KO mice, whereas a significant increase was detected on IL-7 Tg Treg (Fig. 3.2 B). These results indicate that IL-7 quantitatively affects CD25 expression on peripheral CD4⁺ FOXP3⁺ T cells *in vivo*. In addition, we determined whether IL-7 has similar effects on wild type Treg *in vivo*. CD4

T cells were isolated from B6 lymph nodes and spleen, CFSE stained and adoptively

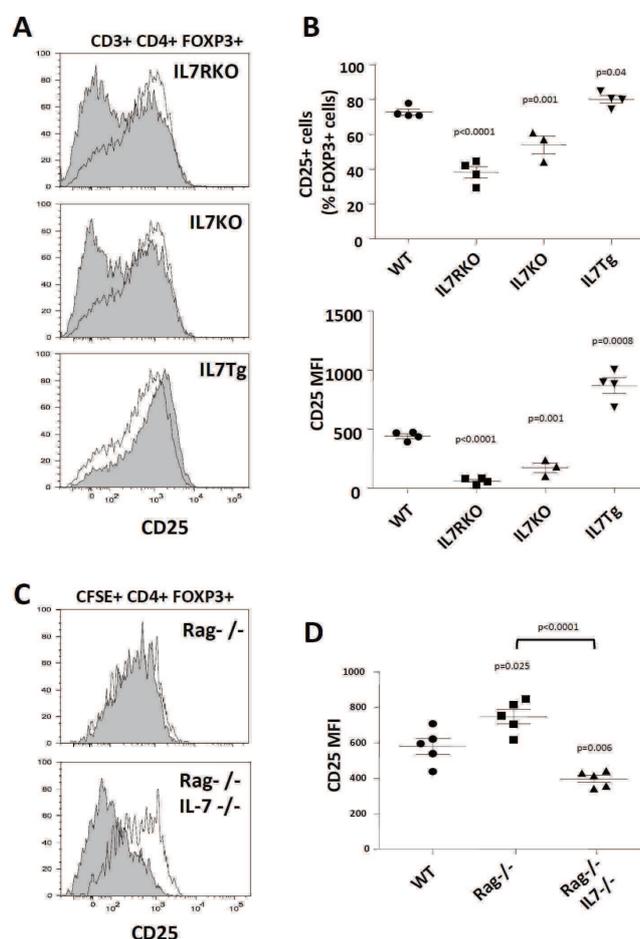


Figure 3.1 - IL-7 signaling dynamically regulated CD25 expression on Treg cells in vivo. Analysis of WT, IL7RKO, IL7KO and IL7Tg mice. Representative FACS profiles (A), percentage and MFI (B) of CD25 expression on splenic CD4+TCR β +FOXP3+ Treg cells are shown. Data are representative of at least three independent experiments with four to six mice per group. (C, D) Total CFSE stained CD4 T cells were adoptively transferred into either normal B6 mice (WT), IL-7 competent or IL-7 deficient Rag $^{-/-}$ mice. Spleen were harvested after 24 hours and CFSE+CD4+TCR β +FOXP3+ analysed for CD25 expression. (F) Representative FACS profiles of CD25 expression on Treg recovered from WT mice (open line) or the indicated Rag deficient mice (gray shaded profile), (G) CD25 expression MFI from one representative experiment are shown. Results are representative of three independent experiments with two to five mice per group.

transferred into either wild type, RAG $^{-/-}$ or IL-7-deficient RAG $^{-/-}$ IL-7 $^{-/-}$ host mice. Injected CD4 T cells were examined in host spleens 18h after transfer. At this time point, no

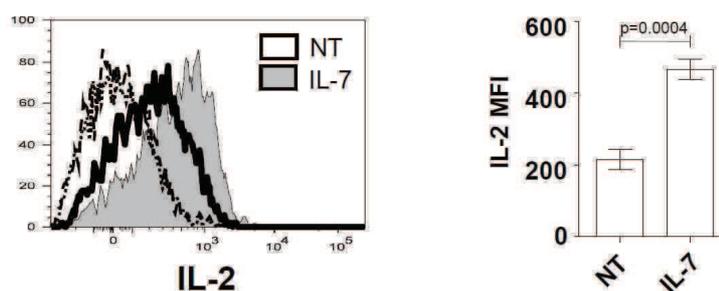
proliferation of injected cells was observed as previously described (Park 2004, Guimond 2009) (data not shown). No CD25 up-regulation was detected on CD4⁺ FOXP3⁻ cells in all studied hosts (data not shown). In contrast, Treg cells transferred into RAG^{-/-} IL-7^{-/-} hosts significantly down-regulated CD25 surface expression when compared to Treg transferred into either WT or IL-7 replete Rag^{-/-} hosts (Fig. 3.2 C-D). Collectively, these *in vivo* experiments support our *in vitro* results and further document that endogenous IL-7 is necessary for maintenance of CD25 expression on Treg cells *in vivo*.

3.3.2 - IL-7 signaling modulates Treg IL-2 binding capacity and IL-2 signal transduction

CD25 is necessary for the constitution of IL-2 receptor with the highest affinity and plays a major role in Treg biology. To assess whether CD25 modulation by IL-7 also affected IL-2 binding capacity by Treg, we first determined the binding capacity for IL-2 of FOXP3-GFP⁺ Tregs previously incubated overnight with IL-7 or not by performing short (30 min) IL-2 fluorokine incubation. As shown in Fig. 3.3A, prior IL-7 incubation provided enhanced binding capacity for IL-2: a two fold increase in IL-2 fluorokine was detected. Similar experiments were performed *ex vivo* by analyzing binding of IL-2 fluorokine by splenic Tregs isolated from WT, IL-7R KO, IL-7 KO and IL-7 Tg mice. Tregs issued from IL-7R KO and IL-7 KO exhibited significantly reduced IL-2 binding capacities compared to WT Tregs and conversely IL-7 Tg mice exhibited significantly higher binding capacity (Fig. 3.3). Collectively these results demonstrated that IL-7 signaling affects IL-2 binding capacity by Tregs. We next evaluated whether the decrease in CD25 expression and IL-2 binding capacity observed in Treg in the absence of IL-7 signaling led to a decreased IL-2 signaling transduction. To this aim we assessed STAT5 phosphorylation following short term (10 min) IL-2 incubation on CD4⁺FOXP3⁺ splenocytes from WT or IL-7RKO mice. As shown in Fig. 3.4A, following exposure to IL-2, STAT5 was promptly phosphorylated in WT Treg in a dose dependent manner. Quantitative analysis of pSTAT5 revealed that IL-7RKO Treg cells presented lower pSTAT5 than

WT Treg cells at low doses (Fig 3.4B), suggesting that these Treg cells are less responsive to IL-2. These finding indicates that IL-7 signaling may optimize Treg responsiveness to IL-2.

A



B

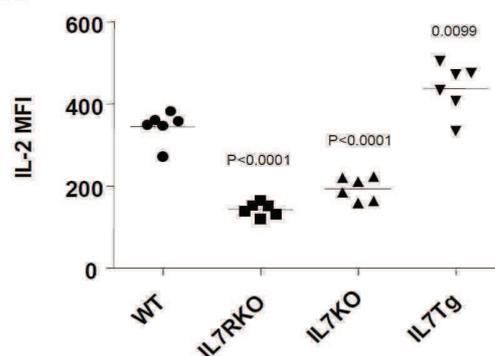


Figure 3.3 - IL-7 availability affected IL-2 binding capacity by Treg. (A) Total CD4⁺ cells isolated from FOXP3GFP mice were cultured in the absence (NT) or in the presence (IL-7) of IL-7 at 10 ng/ml. After 24 hours cells were harvested and incubated for 30 min with biotinylated IL-2. Binded biotinylated IL-2 was revealed with fluorochrome-conjugated streptavidine. Shown are representative profiles of IL-2 fluorescence on CD4⁺GFP⁺ cells cultured with (gray shaded profiles) or without (open line) IL-7. Median Fluorescence Intensities of triplicates \pm SD are shown. Data are representative of three independent experiments performed in triplicate. (B) Splenocytes from WT, IL7RKO, IL7KO and IL7Tg mice were analyzed directly *ex vivo* for IL-2 binding capacity as in (A). Shown are IL-2 Median Fluorescence Intensities (MFI) on CD4⁺TCR β +FOXP3⁺ cells. Results are representative of two independent experiments with four to six mice per group.

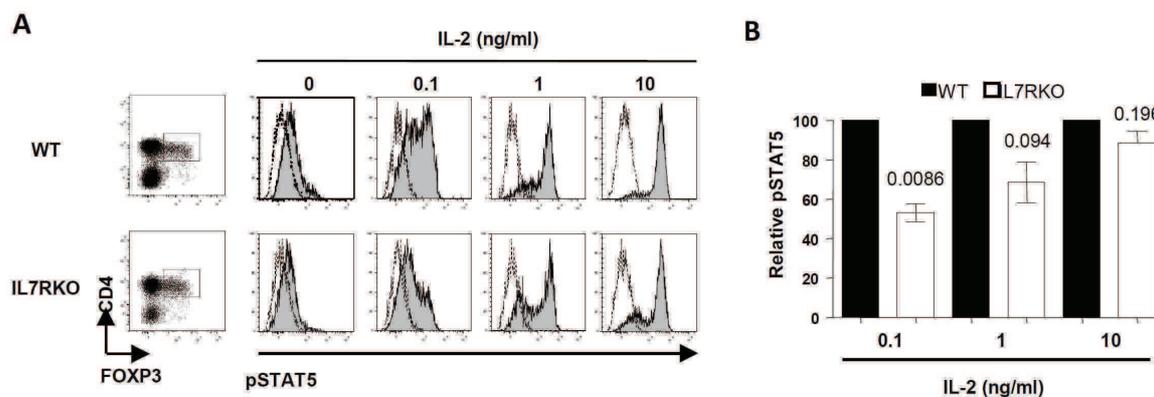


Figure 3.4. Attenuated IL-2 signaling in IL7R-deficient Treg. WT or IL7RKO splenocytes were treated with increasing doses of rmIL-2 for 10 min. Stat5 phosphorylation was then determined by flow cytometry in CD4+TCR β +FOXP3+ cells. (A) Representative dot plots gated on TCR β + lymphocytes showing gating for CD4+FOXP3+ cells and FACS profiles of phosphor-STAT5 at different IL-2 doses. (B) Relative phospho-STAT5 Median Fluorescence Intensities (MFI) in IL7RKO Treg. At any given IL-2 doses, MFI of pSTAT5 staining in IL7RKO Treg was normalised to pSTAT5 staining in WT Treg. Results are the mean \pm SEM from three independent experiments.

3.3.3 - IL-7 affects IL-2 induced Treg expansion *in vivo*

Although not necessary for Treg proliferation during lymphopenia (Setoguchi et al., 2005), IL-2 administration in this setting has been shown to strongly increase Treg expansion (Zhang et al., 2005). Zhang et al. have suggested that IL-2 and lymphopenia could somehow synergize to induce Treg expansion. As lymphopenia is typically characterized by increased levels of IL-7 and having shown that IL-7 availability increases Treg reactivity IL-2 we speculated that IL-7 could affect IL-2 induced Treg expansion. We transferred purified Treg cells into Rag deficient mice competent or not for IL-7 that were additionally treated with IL-2/ α IL-2 complexes at d1, 2 and 3. At day 6, we determined CD4⁺ FOXP3⁺ Treg proliferation profiles and cell numbers. As expected, Treg proliferation benefited from IL-2 injection in Rag deficient hosts (0.5% non dividing cells upon IL-2 compared to 15.7% in non treated hosts (data not shown)). IL-2 induced strong

Treg proliferation independently in cells transferred in both IL-7 competent or IL-7 deficient *Rag*^{-/-} mice (Fig. 3.5 A) suggesting IL-7 availability did not affect significantly IL-2 dependent Treg proliferation. We next determined T cell recovery at day 1 and day 6 in both hosts. Treg cell recovery at d1 was established before first IL-2 injection and no statistically significant difference was detected between IL-7 deficient and competent hosts (data not shown). Interestingly, at day 6, Treg cell recoveries did not directly correlate to their proliferation profile. Tregs number recovered in IL-7 deficient hosts was approximately four-fold reduced compared to IL-7 competent mice. These results indicate that IL-7 modulates IL-2 induced Treg expansion in lymphopenic hosts by probably affecting Treg accumulation rather than Treg proliferation.

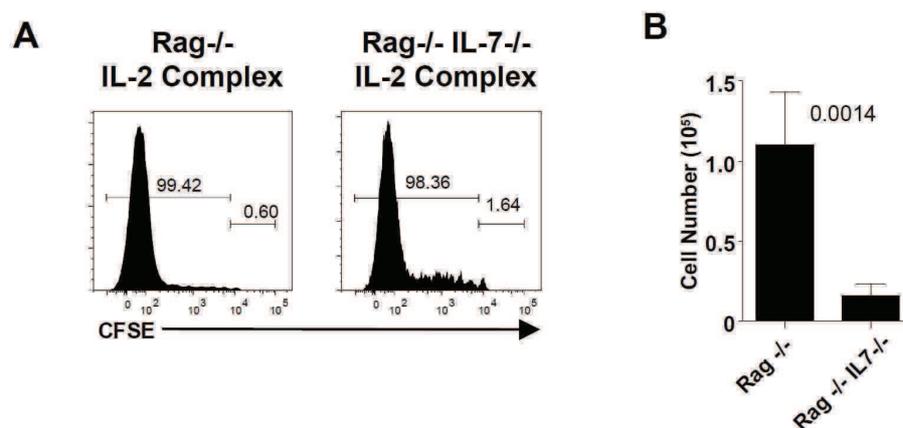


Figure 3.5 - IL-7 signaling was required for IL-2 induced expansion of CD4⁺CD25⁺ Treg cells in lymphopenic hosts. CFSE-labeled, purified CD4⁺CD25⁺ T cells were transferred into *Rag*^{-/-} and *Rag*^{-/-}*IL-7*^{-/-} mice and IL-2/aIL-2 (1 μg/5 μg) complexes were administered at day 1, 2 and 3 after transfer. Spleens from transferred hosts were analysed at day 6. (A) Representative profiles of CFSE dilution of CD4⁺TCRβ⁺FOXP3⁺ cells. (B) Absolute numbers of splenic CD4⁺TCRβ⁺FOXP3⁺ cells recovered per mouse at day 7. Results are represented as the mean ± SD of 4 mice from an experiment representative of three. (C) Percentage of CD4⁺TCRβ⁺FOXP3⁺ cell numbers recovered in *Rag*^{-/-}*IL-7*^{-/-} mice when compared to numbers recovered in IL-7 competent *Rag*^{-/-} mice. Mean ± SEM from three independent experiments are shown. Experiments were performed three times with two to four mice per group.

3.4 – ARTICLE 3

Early and long lasting alteration of Effector CD45RA-Foxp3high Regulatory T cells homeostasis during HIV infection

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Background : Regulatory T cells (Treg) quantification in HIV infection remains ill defined due to the lack of reliable specific markers to identify human Treg and the diversity of clinical stages of HIV infection.

Methods : Employing a recently described Treg identification strategy based on CD45RA and FOXP3 expression, we performed here an extensive quantification of total, naive (CD45RA+ Foxp3low) and effector (CD45RA- Foxp3hi) Treg in different contexts of HIV infection: primary HIV infection, long term viremic patients, HAART treated aviremic patients and HIV controllers.

Results : We showed that, whereas total Treg percentages were mildly affected by HIV infection, Treg absolute numbers were significantly reduced in all groups studied. We demonstrated that whereas naive Treg numbers were essentially preserved, effector Treg were consistently affected during HIV infection. Moreover, we showed that effector Treg numbers but not total or naive Treg numbers negatively correlated with the magnitude of HIV specific CD8 T cell responses, providing *ex vivo* evidence of Treg involvement in HIV immunity.

Conclusion : Our data demonstrate that naive and effector Treg were differently affected during HIV infection. Effector Treg were consistently altered in all groups considered whereas naive Treg numbers were essentially affected during PHI. Moreover, we identified the predominant role of effector Treg on HIV specific CD8 T cell response but not on chronic immune activation.

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Early and long lasting alteration of Effector CD45RA-Foxp3high Regulatory T cells homeostasis during HIV infection

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ABSTRACT

Regulatory T cells (Treg) quantification in HIV infection remains ill defined due to the lack of reliable specific markers to identify human Treg and the diversity of clinical stages of HIV infection. Employing a recently described Treg identification strategy based on CD45RA and FOXP3 expression, we performed here an extensive quantification of total, naïve (CD45RA+ Foxp3low) and effector (CD45RA- Foxp3hi) Treg in different contexts of HIV infection: primary HIV infection, long term viremic patients, HAART treated aviremic patients and HIV controllers. We showed that, whereas total Treg percentages were mildly affected by HIV infection, Treg absolute numbers were significantly reduced in all groups studied. We demonstrated that whereas naïve Treg numbers were essentially preserved, effector Treg were consistently affected during HIV infection. Finally, we demonstrated that effector but not total or naïve Treg numbers negatively correlated with the magnitude of HIV specific CD8 T cell responses.

INTRODUCTION

HIV infection is characterized by massive CD4 depletion, inefficient HIV specific immune responses and chronic immune hyper activation. All these phenomena contribute to disease progression to AIDS. Multiple mechanisms leading to dysfunctions of both HIV specific and non specific immune responses are involved. In that respect, alterations of regulatory T cells (Treg) numbers and/or functions have been considered. Treg have been shown to exert suppressive activity during infectious immune responses¹. However, the overall influence of Treg during HIV infection remains ill defined: Treg induced suppression may prove deleterious to the development of efficient HIV specific immune responses or beneficial by preventing inappropriate immune activation². Suppressive activity of Treg on HIV specific responses has been repeatedly described³⁻⁵. However, very few consensual data are available concerning Treg functions and numbers during HIV infection. A strong suppressive activity of Treg has been initially associated to decreased viral load³ but most studies point to a pathogenic role of Treg on HIV infection^{4,5}. Recently, CD8 T cells restricted by protective HLA alleles have been characterized by their capacity to evade Treg suppression, thus associating delayed disease progression and low Treg suppression⁶. It has also been demonstrated that Treg proliferate during HIV infection⁷ and are directly targeted by HIV infection⁸⁻¹⁰. However, Treg quantification in HIV infection remains controversial in part due to the lack of reliable specific marker to identify human Treg. CD25 and to a lesser extent Foxp3 expression are transiently expressed by activated T cells, a bias especially deleterious in the context of chronic immune activation. These uncertainties in Treg characterization led to conflicting data on the quantification of regulatory T cells¹¹. Combination of CD127 low expression and CD25 or Foxp3 expression is currently used to enhance Treg characterization presumably by excluding effector non Treg cells^{12,13}. However, the purity of regulatory T cells reached using such strategy remains under debate¹⁴. This strategy also excludes a fraction of Treg which may include activated Treg as demonstrated in mice¹⁵. Importantly, heterogeneity in Treg quantification appears to differ depending on clinical stage of HIV infection. Whereas Treg percentages are shown to be consistently increased in HIV infected patients with low CD4 count¹⁶⁻²², various results emerged depending on the viral, immunological and clinical status of HIV infected patients. During primary

HIV infection, decreased Treg percentages have been described^{23,24} although results differ depending on the staining strategy²⁴. Treg percentages in HIV controllers were alternately described as unchanged²⁵⁻²⁶ or reduced²⁷.

The aim of this work was to readdress Treg quantification during HIV infection by integrating the diversity of HIV infections and novel Treg identification strategy. We distinguished different groups of HIV infected patients based on viral levels, time of infection and/or clinical profile. Primary HIV infected viremic patients (PHI), untreated viremic patients (VIR), ART treated aviremic patients (ART), and HIV controllers (HIC) were considered separately. Treg quantification was performed using a novel consensus combination proposed by Miyara et al.²⁸. CD45RA/RO and Foxp3 combination provides a unique tool to distinguish effector non regulatory T cells from Treg. More importantly, such combination also allows evaluating functional Treg activity by defining “naïve” (CD45RA⁺Foxp3^{low}) and “effector” (CD45RA⁺Foxp3^{hi}) regulatory T cells distribution. We quantified Treg using both classical (CD127^{low}CD25⁺Foxp3⁺) and novel (CD45RA and Foxp3 combination) identification strategies. We demonstrated that Treg counts were consistently decreased in all HIV infected group studied whatever the combination used. Such consistency was not observed when considering Treg percentages. Importantly, naïve and effector Treg subsets were differently affected depending on the stage of HIV infection. PHI patients exhibited significant decrease of both naïve and effector Treg. In contrast, during chronic stages, decreased Treg count essentially relies on effector Treg decline, whereas naïve Treg were mildly affected.

METHODS

Study Participants.

Peripheral blood samples from 30 HIV-uninfected blood donors were obtained from the Etablissement Français du Sang (Hospital Saint Louis, Paris, France). With their informed consent, we collected samples from 19 patients with PHI enrolled in the French ANRS multicenter PRIMO cohort (Agence Nationale de Recherche sur le SIDA, CO06). Primary infection was defined by HIV RNA positivity and by a negative or emerging antibody response. We also studied 16 untreated chronically-infected patients which had been infected for a median of 48 months and are referred to as viremic patients. We included in the study 18 ART treated aviremic individuals maintaining plasma HIV RNA levels <75 copies/ml on ART for more than one year. The remaining 22 patients were enrolled in the French HIV-Controllers cohort (ANRS CO18) [inclusion criteria: HIV infection >10 years, 90% of plasma HIV RNA values <400 copies/ml with no ART]. Clinical and biologic characteristics of study participants are shown in Table 1.

Laboratory studies

Cell preparation

Peripheral blood mononuclear cells (PBMCs) were isolated from anticoagulated blood by Ficoll density gradient centrifugation. Cells were either immediately used for subsequent analysis or cryopreserved in liquid nitrogen. Human leukocyte antigen typing was done with the complement-dependent microlymphocytotoxic technique (One Lambda, Montpellier, France).

Flow cytometry and regulatory T cells identification

Absolute numbers of CD4⁺ T cells for healthy donors were determined in fresh whole blood by the use of TruCount tubes and CD3 FITC/CD4 PE/CD45 PerCP TriTest (BD) according to the manufacturer's instructions.

Conjugated antibodies for surface markers were purchased from BD Biosciences (San Jose, CA): CD3 (APC-H7), CD4 (PE-Cy7), CD45RA (FITC), CD25 (PE-Cy5) and eBioscience: CD127 (PE).

Intracellular detection of FoxP3 with anti-hFoxP3 (APC, clone 236A/E7 [e-Bioscience]) was performed on fixed and permeabilized cells following manufacturer's instructions (e-Bioscience). Six-color flow cytometry was performed with a FACSCanto cytometer (BD Biosciences) and data files were analyzed using FlowJo software (Tree Star Inc.).

Peptide-human leukocyte antigen class I multimers

HIV-specific CD8⁺ T cells were identified by using soluble PE-labeled or APC-labeled peptide-human leukocyte antigen (HLA) class I multimers (Proimmune, Oxford, United Kingdom; Beckman Coulter, Villepinte, France) derived from the HIV Gag, Nef, Pol and Env proteins. The following epitopes were used: the HLA-A*0201-restricted peptide ligands SLYNTVATL (Gag 77–85) and ILKEPVHGV (Pol 476–484), the A*0301-restricted peptide ligands RLRPGGKKK (Gag 20–28) and QVPLRPMTYK (Nef 73–82), the A*1101-restricted ligand AVDLSHFLK (Nef 84–92), the A*2402-restricted peptide ligand RYPLTFGWY (Nef 134–143), the B*0702-restricted peptide ligand IPRRIRQGL (Env 848–856), the B*0801-restricted peptide ligands GEIYKRWII (Gag 259–267) and FLKEKGGL (Nef 90–97), the B*2705-restricted peptide ligand KRWIILGLNK (Gag 263–272), and the B*5701-restricted peptide ligands KAFSPEVIPMF (Gag 162–172), TSTLQEQIGW (Gag 240–249), and QASQDVKNW (Gag 308–316).

Activation profile on conventional T cells

Freshly collected whole-blood samples were assessed for the frequency of CD4⁺ and CD8⁺ T cells expressing CD38 and HLA-DR (BD Bioscience). T cell activation was assessed as the percentage of CD4⁺ expressing HLA-DR and CD8⁺ T cells expressing HLA-DR or co expressing HLA-DR and CD38.

ELISPOT assay

IFN γ -enzyme-linked immunosorbent spot assay was used to quantify ex vivo HIV-specific CD8⁺ T cell responses to peptides corresponding to optimal HIV-cytotoxic T lymphocyte (CTL) epitopes derived from the HIV-1 Env, Gag, Pol, and Nef proteins (National Institutes of Health HIV Molecular Immunology Database: http://www.hiv.lanl.gov/content/immunology/tables/optimal_ctl_summary.html). For each subject, optimal peptides were tested depending on the results of HLA typing. IFN- γ spot-forming cells (SFCs) were counted with a KS-ELISPOT system (Carl Zeiss Vision) and expressed as SFCs/10⁶ PBMC. The number of specific SFCs was calculated after subtracting the negative control value (unstimulated cells).

Statistical Methods

Statistical analysis was performed using GraphPad prism software. Groups were compared using the nonparametric Kruskal Wallis test followed by Dunn's multiple comparison tests. Spearman's rank test was used to determine correlations. P values above 0.05 were considered not statistically significant. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

RESULTS

Decline in Treg numbers in all HIV infected patients studied

Because Foxp3 expressing CD4 T cells comprise both regulatory T cells and effector non regulatory T cells, Foxp3 detection is commonly associated to CD25 expression and/or low CD127 expression to exclude effector cells. Alternately, Miyara and Sakaguchi have developed a novel strategy allowing naïve and effector Treg identification using differential expression of CD45RA and Foxp3 staining, which has been validated in autoimmune context²⁹ (Fig. 1A). To fairly evaluate the relative advantages of both identification strategies in the context of HIV infection, we first analyzed “total” Treg defined by addition of naïve (CD45RA+Foxp3low) and effector (CD45RA-Foxp3hi) Treg compared to identification by Foxp3+CD25+CD127low expression on CD4 T cells (Fig. 1B and 1C, respectively). Regardless the identification strategy used, significant reduction in Foxp3 expressing CD4 T cells numbers was detected in all HIV infected groups compared to healthy donors. Importantly CD45RA+/- Foxp3low/hi detection allowed discarding an important and variable number of effector non Treg cells (CD45RA-Foxp3low). Quantification of Treg by CD45RA and Foxp3 combination appears more stringent, but does not drastically modify the conclusion usually drawn on total Treg numbers during HIV: decline in Treg cell numbers was consistently detected in all HIV patients studied, in accordance with studies demonstrating HIV infection affected both Treg and conventional CD4 T cells. Collectively our data demonstrate that disruption of the Treg pool is long lasting and occurs in all groups of HIV infected patients studied.

Tregs percentages are reduced in PHI and HIC patients

Reduction of Treg numbers may reflect the impact of HIV on all CD4 T cells. To determine whether Tregs were differentially affected among CD4 T cells, we determined Treg percentages using each identification strategy. Percentages of Treg detected among CD4 T cells differed depending on the combination used: lower percentages were consistently recovered using CD45RA/Foxp3 combination compared to Foxp3+CD25+CD127low identification. However, both detection strategies revealed significant reduction in Treg percentages during PHI and among HIC patients compared to healthy

donors (Fig. 2), the lowest percentage being observed during PHI. No significant difference was observed in viremic patients regardless the combination used. Observations differed in ART treated patients who exhibited significant reduction in CD127^{low}CD25⁺Foxp3⁺ Treg percentage but no significant differences when using CD45RA/Foxp3 detection strategy. These results confirm the discrepancy between Treg percentages among CD4 T cells and Treg cell numbers: whereas Treg numbers are consistently reduced in all HIV infected patients studied, Treg percentages are significantly altered in PHI and HIC, but not in VIR and ART patients using the more stringent definition.

Effector Treg are preferentially affected during HIV infection

The novel consensus combination defined by Miyara and Sakaguchi²⁸ allowed identification of naïve (CD45RA⁺Foxp3^{low}) and effector (CD45RA⁻Foxp3^{hi}) Treg fractions, thus integrating phenotypic and functional Treg heterogeneity to analysis. Although a slight reduction of naïve Treg numbers was detected in all groups, statistical reduction of naïve Treg numbers was uniquely detected in PHI group (Fig. 3A). Importantly, no statistical differences were detected in naïve Treg percentages in all groups of HIV infected patients compared to healthy donors. In contrast, effector Treg absolute numbers were statistically reduced in all groups studied (Fig. 3B). However percentages of effector Treg among CD4 T cells were solely statistically reduced in PHI and HIC patients. Collectively, analyses of naïve and effector Treg numbers suggested different outcome for naïve and effector Treg during HIV infection. Naïve Treg counts were affected during the early phase of infection (PHI) but not in the other groups considered (VIR, ART and HIC). In contrast, effector Treg numbers were consistently affected all along the course of HIV infection.

Moreover, alteration in effector Treg numbers affected all groups of patients whereas alterations in percentages were less consistently detected. This suggests that quantification of Treg numbers is more sensible than quantification of percentages.

Correlation of effector Treg counts to CD4 count was lost in all HIV infected groups studied except in HIC patients

To evaluate the potential impact of Tregs on HIV related disease progression, we next performed correlations between total, naïve and effector Tregs counts and CD4 count. When classical CD127^{low}CD25⁺Foxp3⁺ total Tregs identification was used, highly significant correlation was obtained between Tregs and CD4 count in healthy donors ($p < 0.0001$) as well as in aviremic HIV patients ($p = 0.0005$ ART and $p = 0.0003$ HIC) (Fig. 4A). Conversely this correlation was lost in viremic patients both during primary infection ($p = 0.1727$ PHI) and at the chronic stage ($p = 0.0628$). When Treg subsets were considered (Fig. 4B and 4C), naïve or effector Treg counts were highly correlated to CD4 T cell count in healthy donors. In all groups of patients studied, correlation between naïve Treg count and CD4 count was observed ($p = 0.0306$ PHI, 0.0152 VIR, 0.0037 ART and 0.0078 in HIC) (Fig. 4B). In contrast, a significant correlation between effector Treg numbers and CD4 count was detected in HIC patient ($p = 0.0026$) whereas it was lost in viremic ($p = 0.5715$), in PHI ($p = 0.9483$) and ART ($p = 0.1941$) patients. Collectively, these data suggest that the relationship between naïve Treg and CD4 count is mildly affected during all HIV stages studied whereas effector Treg correlation to CD4 count is altered with the exception of HIC patients.

Naïve Treg but not effector Treg counts inversely correlate with viral load

We next compared in viremic patients (including PHI and VIR) correlation obtained between CD127^{low}CD25⁺Foxp3⁺ Treg, naïve Treg and effector Treg counts and viral load. Using classical CD127^{low}CD25⁺Foxp3⁺ Treg identification, no significant correlation was obtained between Tregs and viral load (Fig. 5A). However, significant inverse correlation was detected between viral load and naïve Treg, but not effector Treg numbers (Fig. 5B and 5C). Because effector but not naïve Treg were presumably the main suppressive component on HIV infection, these data suggest that changes in Treg numbers during HIV infection are consequential rather than causal to high viral load.

Inverse correlation between HIV specific CD8 T cell responses and effector Treg number

We next investigated the direct impact of Treg on chronic immune activation evaluating HIV specific and non specific responses. No correlation was observed between naïve or effector Treg numbers and global T cell activation as assessed by determining percentages of HLA-DR+ CD38+ among CD4 and CD8 T cells in all groups of HIV infected patients nor in healthy donors (data not shown). However, *in vitro* experiments have clearly demonstrated the suppressive activity of Treg on HIV specific CD8 T cell responses³⁻⁵. We thus determined whether correlation exists between effector Treg numbers and HIV specific CD8 T cells responses. IFN- γ ELISPOT analyses following stimulation with HIV specific peptides were performed on HIV infected patients (n=27, including 7 PHI, 5 VIR, 5 ART and 10 HIC) (Fig. 6A). No correlation between Treg count and HIV specific CD8 T cells were reached when considering “total” or “naïve” Treg. In contrast, effector Treg count negatively correlated with SFC quantification of HIV specific CD8 T cells (p=0.0459). Regarding the viral status of HIV infected patients, negative correlation was significantly detected in viremic (p=0.0082) but not aviremic patients (p=0.6482) (data not shown). To confirm the specific impact of effector Treg on HIV specific CD8 T cell responses, we evaluated the *ex vivo* percentages of HIV specific CD8 T cell expressing CD38 and HLA-DR, two reliable markers of CD8 T cell activation in HIV infected patients (n= 29: 8 PHI, 12 VIR, 5 ART, 4 HIC) (Fig. 6B). Similarly to SFC analysis, an inverse correlation was statistically detected between effector Treg numbers and HIV specific HLA-DR+ CD38+ activated CD8 T cells (p=0.0028). Inverse correlation was also detected when considering viremic (p=0.0240) but not aviremic patients (p=0.3738) (data not shown). These data are in accordance with numerous *in vitro* suppression assays demonstrating the suppressive capacity of Treg on HIV specific responses³⁻⁵. Importantly, these data demonstrate that effector Treg counts, but not naïve or total Treg directly impact on HIV specific responses and thus identify the predominant role of effector Treg on HIV specific responses.

DISCUSSION

An important limitation to human Treg studies is represented by the lack of specific identification markers precluding their accurate quantification. In a recent publication, Miyara et al²⁸ developed a novel identification strategy using combination of CD45RA and Foxp3 expression. Such combination allowed discriminating between naive and effector Treg, thus providing a more accurate quantification of Treg.

Using such novel combination, we readdressed Treg quantification in the context of HIV infection analysing primary HIV infected, viremic, HAART treated, spontaneous controllers patients and healthy donors. Special attention was given to PHI and HIC cohorts. Although Treg identified by CD45RA/Foxp3 combination appears more stringent than CD127^{low}CD25⁺Foxp3⁺ staining (Fig. 1), Treg numbers were still consistently reduced in all groups of HIV infected patients studied when compared to healthy donors, as previously described^{17,18,30}. Decrease in Treg percentages during PHI has been previously described²³ and may reflect Treg susceptibility to HIV infection^{8,10} or recruitment to inflamed sites³¹⁻³³. When considering HIC patients, decrease in Treg percentages and numbers was observed compared to healthy donors as previously demonstrated²⁷. Data obtained when using CD45RA/foxp3 combination rather confirmed those observed with previous Treg identification strategy.

We next evaluated whether naive/effector Treg distinction provided novel insight on Treg biology during HIV infection. We showed that naive and effector Treg count were differently affected during HIV infection. Initial decay of both naive and effector Treg was observed during PHI. Interestingly, in all other groups of HIV infected patients, effector Treg decay was consistently observed whereas naive Treg counts were mildly affected. The mechanisms involved in such specific effector Treg defects are unclear. Persistent effector Treg decay suggests low restoration and/or low persistence of effector Treg compartment. Defect of effector Treg does not appear to rely on the absence of naive Treg precursors since naive Treg numbers are essentially preserved/restored. Limited activation or conversely over activation leading to terminal differentiation and death may participate to such defects. Alternately, decay in effector Tregs that are especially sensitive to apoptosis may rely on highly defective survival.

Effector Treg decay is especially striking in the context of HIV because high level of immune activation theoretically favours Treg survival. Treg survival has been shown to highly depend on IL-2 availability and IL-2 producing cells^{34,35} but HIV infection is characterized by altered IL-2 production³⁶. Effector Treg decay during chronic phases may thus reflect altered IL-2 production.

Dissecting naive and effector Treg allowed approaching functional suppressive capacity of Treg: naive Treg exhibit low *in vivo* suppressive activity, but upon *in vitro* stimulation, they exhibit high proliferation and survival capacity leading to suppressive functions. Conversely, effector Treg are directly suppressive, but exhibit low proliferative and survival capacity upon *in vitro* stimulation³⁷. We thus attempted to evaluate the impact of effector Treg on HIV related disease progression. We favoured determination of Tregs absolute counts because Treg percentages among CD4 T cells depend on both regulatory and conventional CD4 T cells alterations. Secondly, as Tregs suppression affects CD4 T cells, but also CD8 T cells, B cells and innate cells³⁸, determining Treg percentages among CD4 T cells may preclude the observation of broader Treg effects. When considering correlation between Tregs and CD4 count, effector Treg count positively correlated with CD4 T cell numbers in HIC patients but not in PHI, VIR or ART. Because positive correlation is also observed in healthy subjects, these data suggest that Treg homeostasis was less altered in HIC patients compared to the other chronically infected patients. When considering correlation between Treg and viral load, we showed an inverse correlation between naive (but not effector) Treg and viral load, suggesting a limited impact of effector Tregs on viral control. Correlation of naive Treg with viral load is likely to represent the impact of virus on Treg compartment as early as the primary HIV infection rather than the impact of Treg on viral load. Collectively, these correlations rather identified Treg decay as a consequence of HIV infection. Secondly, we analyzed whether reduced effector Treg numbers directly affect HIV specific and non specific immune responses. Data addressing correlation between Treg and immune activation have been so far extremely heterogeneous and contradictory presumably due to variability in identification strategies and clinical status^{5,24,27,39-42}. In the present study, no significant correlation between effector Treg numbers (nor percentages) and non specific chronic activation was detected (data not shown) supporting the hypothesis of a low impact of Treg on chronic immune

activation. However, addressing chronic immune activation based on phenotypic characterization may not prove accurate enough. Indeed, Treg suppression does not necessarily abrogate T cell activation, but rather modulate the expansion/differentiation stage^{38,43}. Finally, we determined whether correlation exist between effector Treg and HIV specific CD8 T cell responses. Focusing on HIV specific CD8 T cell responses, we observed that effector Treg (but not naïve or total) Treg numbers correlated with the magnitude of HIV specific CD8 T cell responses in viremic patients as assessed by ELISPOT assay following *in vitro* peptide stimulation and as confirmed by phenotypic analysis. These results are in accordance with numerous *in vitro* suppression assays demonstrating the suppressive capacity of Treg on HIV specific responses³⁻⁵ and further identify the predominant role of effector Treg on HIV specific responses.

In conclusion, our data demonstrate that naive and effector Treg were differently affected during HIV infection. Effector Treg were consistently altered in all groups considered whereas naive Treg numbers were essentially affected during PHI. Moreover, we identified the predominant role of effector Treg on HIV specific CD8 T cell response but not on chronic immune activation.

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CONFLICT OF INTERESTS

The authors declare no competitive financial interests.

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TABLE

Table 1 Characteristics of HIV infected patients and healthy controls

105 subjects were included in this study and included in 5 groups: healthy donors (HD), HIV infected individuals studied during primary HIV infection (PHI), HAART naïve viremic patients (VIR), aviremic patients (VL < 50 copies/mL) HAART treated for more than a year (ART) and HIV controllers defined on spontaneous viral control for more than 10 years (VL < 50 copies/mL) (HIC). Median value and 25th and 75th percentile are presented (in brackets) for age, viral load and CD4 count are presented for each group.

LEGENDS

Figure 1: Regulatory T cell counts

(A) Representative dot plots of CD45RA and Foxp3 expression on CD4 T cells recovered from HD, PHI and HIC are shown. (B, C) Graph representing total Treg counts assessed either by summing naïve (CD45RA+ Foxp3low) and effector (CD45RA- Foxp3hi) Treg (B) or classical CD127low CD25+ Foxp3+ staining (C) in PBMC from healthy donors (HD open circles) and HIV infected patients [PHI (filled red squares), VIR (open red squares), ART (open green triangles) and HIC (filled green triangles)]. Statistical significance was indicated as * when p value < 0.05, ** when p value <0.0, *** when p value <0.001 when detected.

Figure 2: Regulatory T cell percentages

Graph representing total Treg percentages among CD4 T cells assessed either by CD45RA/Foxp3 staining (A) or classical CD127low CD25+ Foxp3+ staining (B) in PBMC from healthy donors (HD open circles) and HIV infected patients (PHI (filled red squares), VIR (open red squares), ART (open green triangles) and HIC (filled green triangles)). Statistical significance was indicated as * when p value < 0.05, ** when p value <0.01 when detected and *** when p value <0.001.

Figure 3: Persistent decline in effector Treg counts in HIV infected patients

Naïve and effector regulatory T cell count were defined as CD45RA+ Foxp3low CD4 T cells and CD45RA- Foxp3hi CD4 T cells respectively as described in Fig. 1A. (A, B) Graph representing naïve (A) and effector (B) Treg count (left) and percentages (right) in all groups previously described: healthy donors (HD open circles) and HIV infected patients (PHI filled red squares, VIR open red squares, ART open green triangles and HIC filled green triangles). Statistical significance was indicated as * when p value < 0.05, ** when p value <0.01 when detected and *** when p value <0.001.

Figure 4: Effector Treg numbers correlated with CD4 count

Correlation between total CD127^{low} CD25⁺ Foxp3⁺ (A), naïve (B) and effector (C) Treg counts and CD4 T cell counts were in all groups previously described: healthy donors (HD open circles) and HIV infected patients (PHI filled red squares, VIR open red squares, ART open green triangles and HIC filled green triangles). Statistical significance was indicated as * when p value < 0.05, ** when p value < 0.01 when detected. Correlations were evaluated using a Spearman rank correlation coefficient test. Correlation curve and spearman r correlation are indicated when correlations were significant.

Figure 5: Naïve but not effector Treg counts correlated with viral load

Graphs showing correlations between “total” (as defined by CD127^{low} CD25⁺ Foxp3⁺ CD4 T cells) (squares) (A), naïve (circles) (B) and effector (circles) regulatory T cell counts (C) and viral load in viremic patients. Correlation curve are indicated when significant. Statistical significance was indicated as * when p value < 0.05.

Figure 6: Inverse correlation between HIV specific CD8 T cell responses and effector Treg count

Correlations between “total” (as defined by CD127^{low} CD25⁺ Foxp3⁺ CD4 T cells) (squares), naïve (circles) and effector (circles) regulatory T cell counts and HIV specific CD8 T cell responses. (A) Correlations with ELISPOT SFC count upon HIV specific peptide stimulation of 27 HIV infected patients. (B) Correlation with percentages of activated CD8 T cells, defined by HLA-DR and CD38 co-expression on HIV specific CD8 T cell subsets detected in HIV infected patients (n= 29). Correlation curve and statistical significance are indicated as * when p value < 0.05.

Table 1

Table 1

	HD	PHI	VIR	ART	HIC
Number of subjects	30	19	16	18	22
Age years	37.5 (32-69)	32 (29-51)	35 (32-56)	36 (31.5-55)	47.5 (45-62)
Viral Load Log ₁₀ copies/ml	/	4.9 (4.7-6.3)	4.4 (4-5.5)	<1	<1
CD4 count Cells/ μ l	1120 (763-2601)	527 (421-1316)	442 (358-897)	817 (757-1655)	788 (656-1328)

Figure 1

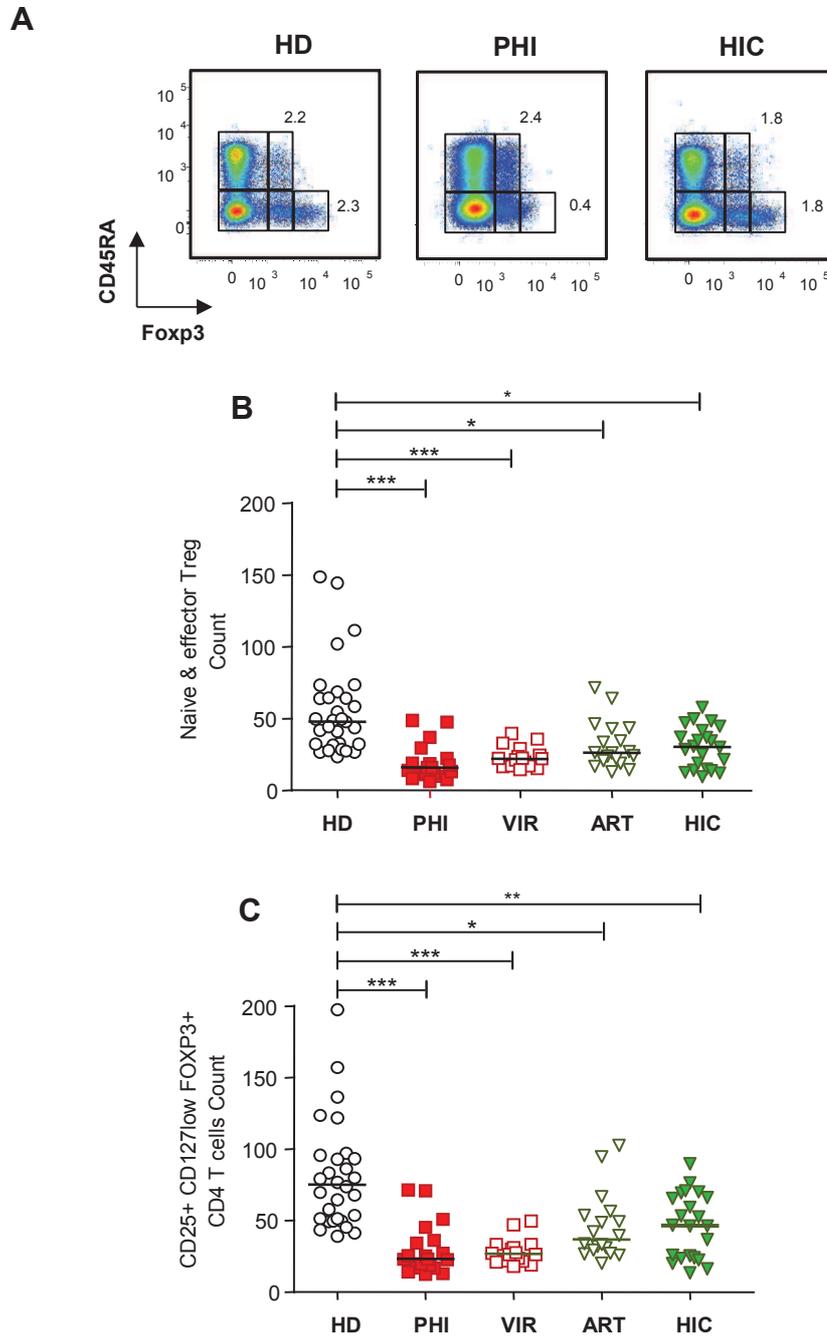


Figure 2

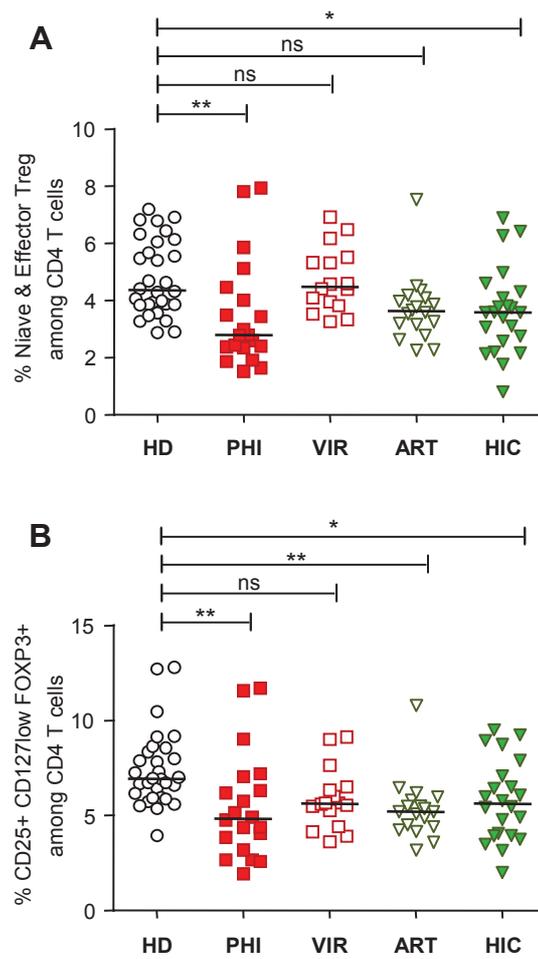


Figure 4

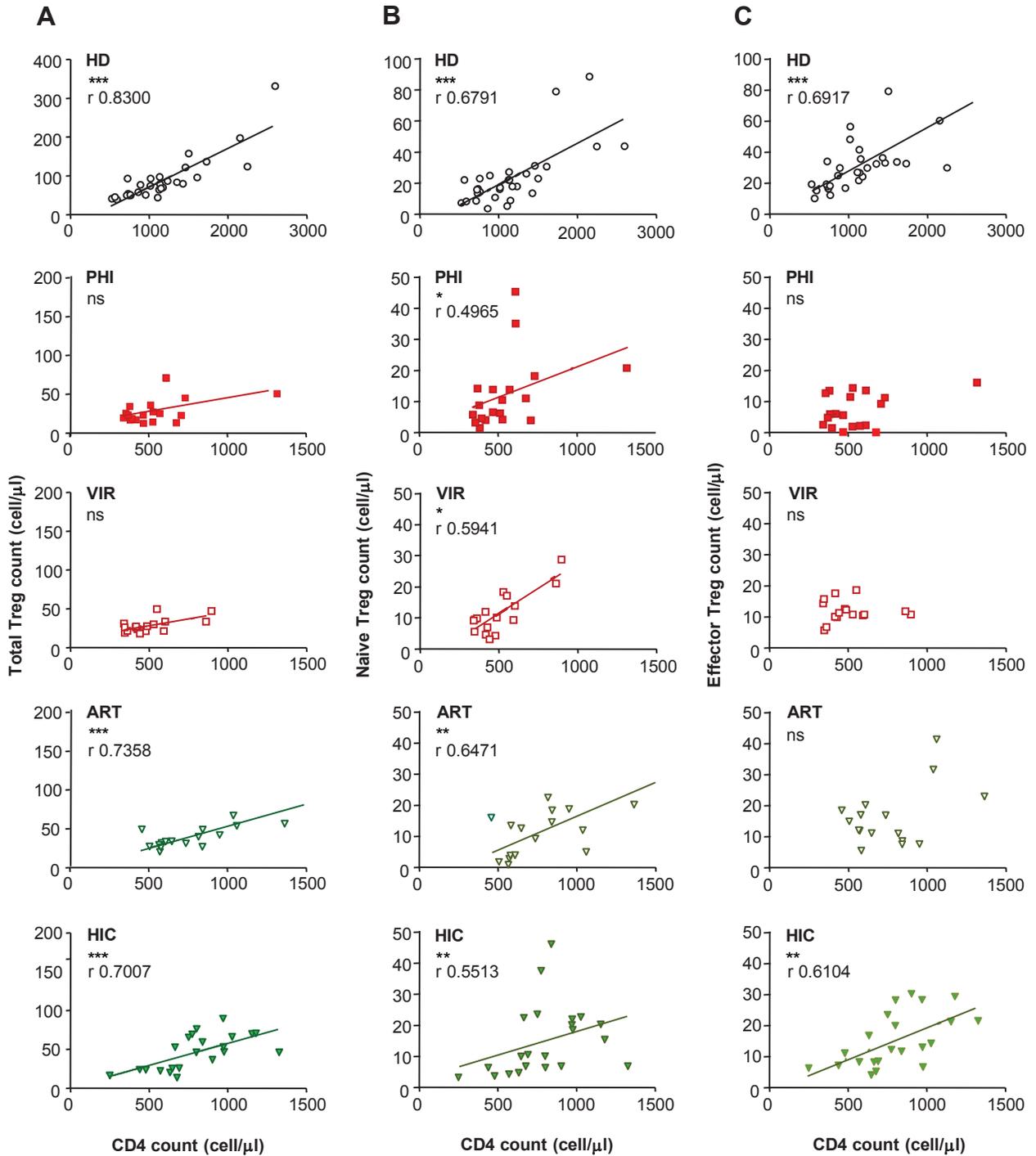


Figure 5

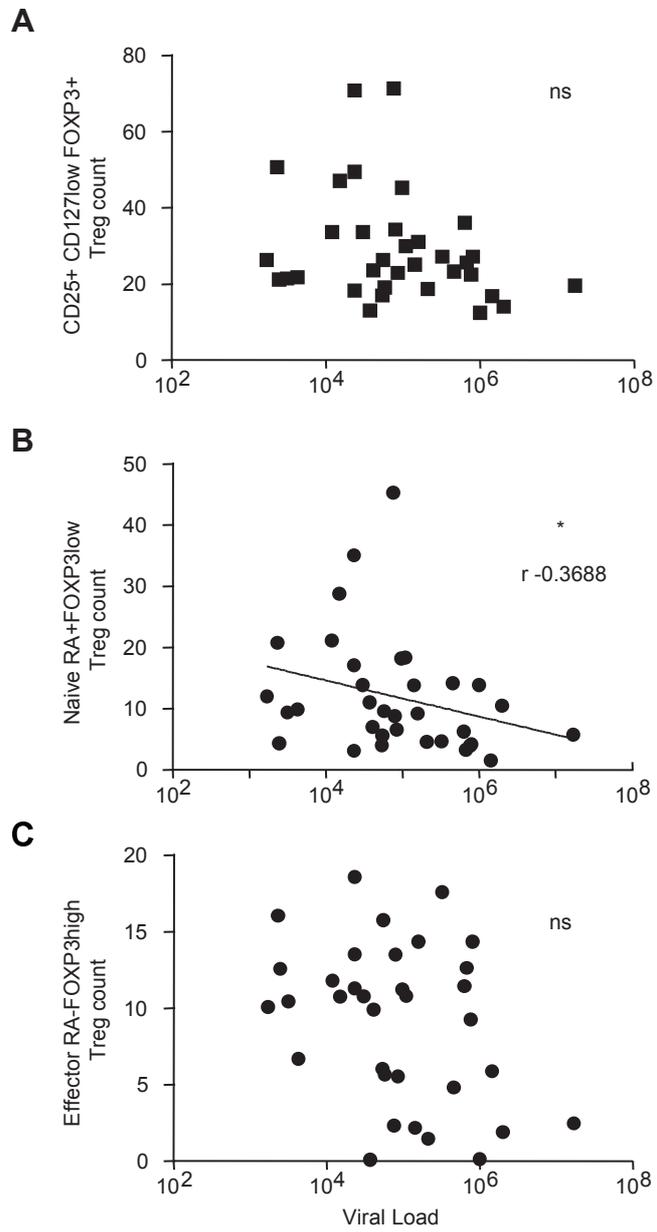
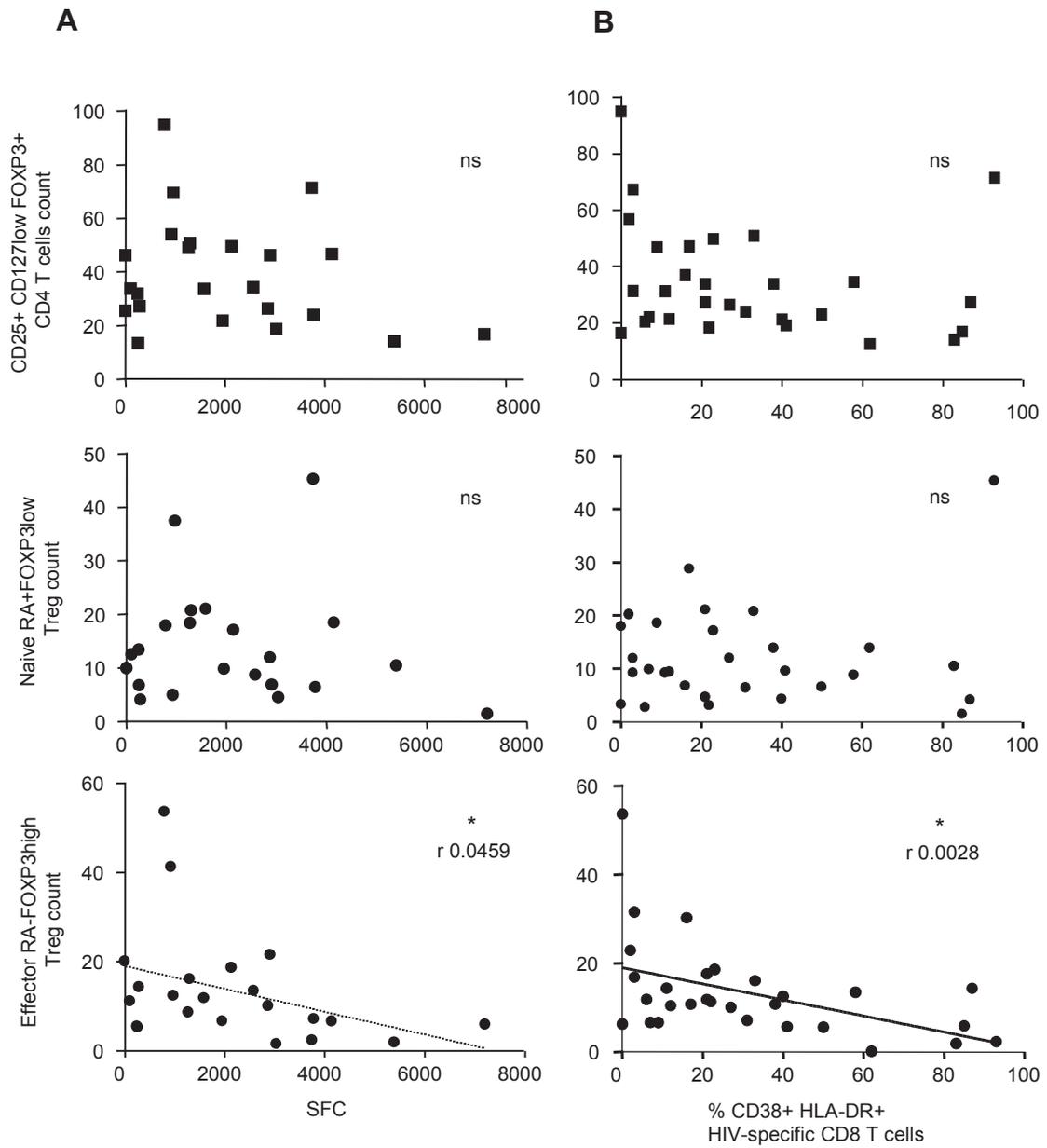


Figure 6



4. DISCUSSION AND PERSPECTIVES

Treg represent a crucial cell subset for the maintenance of the immune-tolerance and numerous efforts have been undertaken in recent years to understand their biology in order to modulate their function in therapeutic approaches. Despite this, mechanisms regulating Treg homeostasis remain ill-defined. In this thesis work we investigated Treg homeostasis using two different though complementary approaches. In the murine system we investigated the fundamental aspects governing Treg homeostasis focusing on the role played by IL-7. In a second part we studied in the human system the modifications induced by HIV infection on Treg cell numbers and we assessed the potential implications of Treg homeostasis alterations in HIV patho-physiology.

4.1 - CD127 on Treg : regulation of expression

As discussed in the introduction (section 1.1.2.3), CD127 expression at T cells surface is a finely regulated process which is modulated by several factors, including TCR triggered activation, signaling mediated by gamma chain cytokines such as IL-2 and IL-7, proinflammatory cytokines such as TNF α and therapeutics such as glucocorticoids. In the first part of this thesis work we tried to identify whether CD127 regulation also occurred at Treg cell surface and to better define mechanisms regulating CD127 expression at Treg surface.

Activation of peripheral CD4 and CD8 T cells induces transient down-regulation of CD127 cell surface expression (Schluns et al., 2000; Franchimont et al., 2002). In contrast we demonstrated here that *in vitro* and *in vivo* activation of Treg cells induces CD127 up-regulation at cell surface. Our data suggest that TCR signaling could differentially regulate CD127 expression on conventional and regulatory T cells. Intrinsic difference in Treg cells intracellular machinery may be responsible for such a differential regulation. Indeed, lower levels of CD127 expression at Treg surface are believed to be the result of direct suppression of CD127 gene

transcription. It has been shown that Foxp3 target CD127 promoter thus suppressing its transcription (Liu et al., 2006). We can imagine that other transcription factors could interfere with CD127 transcription during cell activation. In this respect, a good candidate could be represented by retinoic acid receptor-related orphan receptor gamma t (ROR γ t), a transcription factor required for the generation of T helper 17 cells expressing the proinflammatory cytokine IL-17 (Ivanov et al., 2006) as these two factors have been shown to physically interfere (Zhou et al., 2008; Ichiyama et al., 2008). It has been shown that a population co-expressing both FOXP3 and ROR γ t does exist (Lochner et al., 2008). We performed phenotypic characterization of ROR γ t population and observed that these cells express higher levels of the activation markers CD103 and ICOS and, accordingly, higher levels of CD127 (data not shown). These data suggested that ROR γ t could be expressed in Treg during activation and whether this expression directly affects CD127 expression levels at Treg surface (or interfere with Foxp3 mediated expression) remains to further investigated.

Whether other factors could differentially affect CD127 expression on conventional and regulatory T cells is currently under investigation (Figure 4.1).

IL-2 stimulation of conventional CD4 effector T cells has been shown to potently diminish CD127 mRNA transcription and protein expression (Xue et al., 2002). Whether IL-2 signaling similarly affects CD127 expression at regulatory T cells surface is currently under investigation. Preliminary results we obtained employing *in vivo* IL-2 administration into normal mice reveal that IL-2 signaling positively affects CD127 expression at Treg surface (data not shown). However *in vivo* IL-2 administration does not allow any conclusion on a direct effect independently from cell activation, as this treatment induces strong Treg proliferation. Further investigations are needed.

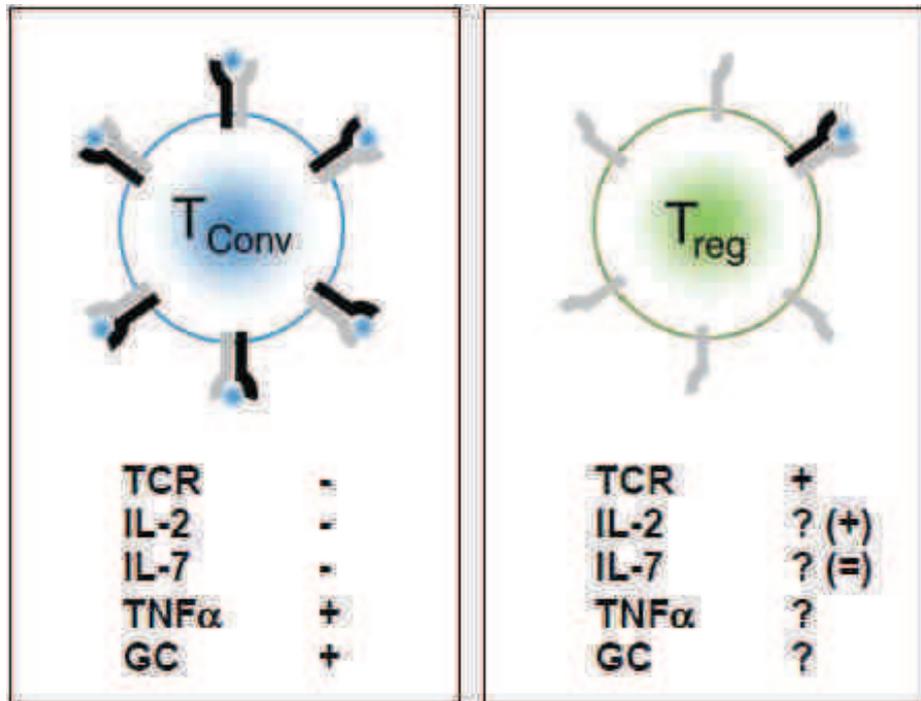


Figure 4.1 – Schematic summary of known and unknown effects of soluble factors on CD127 expression at conventional and regulatory T cells surface. GC : glucocorticoids. In brackets, effect suggested by preliminary results.

As resumed in section 1.1.2.3, IL-7 availability is one of the main factors regulating CD127 at conventional CD4 and CD8 T cells surface (Park et al., 2004). Bourgeois et al., showed in a mouse model of antibody CD4 T cell depletion that during lymphopenia, a condition in which, as mentioned, IL-7 availability is increased, naive and the memory CD4 T cells that remained following Ab-mediated depletion down-regulated IL-7R expression as compared with their counterparts in mice treated with control Abs (Bourgeois et al., 2006). In contrast, CD25⁺CD4⁺ regulatory T cells, maintained a lower but constant expression of CD127 during lymphopenia suggesting that regulation of CD127 expression at Treg surface is less affected by IL-7 signaling. Such a conclusion is supported by some preliminary *in vitro* results we obtained showing that exposure to low to moderate doses of IL-7 induced CD127

down-regulation at conventional but not regulatory T cell surface.

Another interesting aspect to investigate concerns the effects of inflammatory mediators on CD127 expression regulation. It has been shown that *in vitro* treatment of conventional CD4 and CD8 T cells with the inflammatory cytokine TNF α increases both CD127 gene transcription and surface protein expression (Park et al., 2004). Such a mechanism could be implicated in the proinflammatory effect IL-7. It would be therefore important to determine whether TNF α similarly affects CD127 expression at Treg surface or whether this phenomenon is limited to conventional T cell as this would affect the ability of Treg to efficiently compete for IL-7 consumption during inflammation. In an interesting report, Grinberg-Bleyer and coworkers recently showed that activated conventional effector T cells boost the expansion and suppressive function of Tregs *in vivo* by a TNF α -dependent mechanism (Grinberg-Bleyer et al., 2011). We may speculate that TNF α could interfere with Treg homeostasis directly and/or by modulating their reactivity to other soluble factors, such as IL-7.

Glucocorticoids, certainly the most widely clinically employed immuno-modulatory agents, have been shown to induce up-regulation of CD127 on both human (Frachimont et al., 2002) and murine (Lee et al., 2005) T cells in a process which is dependent on the presence of a glucocorticoid response element (GRE) on the IL7Ra gene (Lee et al., 2005). Whether glucocorticoids exert a similar action at Treg surface remains to be evaluated. If that is case it would be fascinating to imagine that glucocorticoids could exert their immuno-suppressive function, at least in part, by affecting Treg homeostasis and/or function through modulation of their reactivity to IL-7.

4.2 - CD127 on Treg: implications for Treg cells identification

Treg identification and isolation has been a major challenge since the discovery of this

crucial cell subset. If in mice the FOXP3 transcription factor seems to be absolutely specific of this cell subset and nicely correlates with CD25 at steady state in lymphoid organs, Treg identification by Foxp3 in humans is much more complex. Every surface marker that has been proposed as identifying Treg can be expressed on conventional CD4 T cells under some conditions, mainly upon activation. As a consequence, several gating strategies to increase cell purity for Treg identification and isolation have been proposed.

The observation that the Treg population as a whole express lower surface levels of CD127 both in human and mice (Cozzo et al. 2003; Seddiki et al., 2006; Liu et al., 2006) lead to the usage of CD127 as a marker of exclusion for Treg identification and isolation. However such an approach presents two major drawbacks.

First, as already mentioned, low levels of CD127 are expressed at the surface of conventional T cells during activation. CD127 down-regulation together with the up-regulation of CD25 render activated T cells indistinguishable from Treg identified on this basis. In human such a bias has been pointed out in condition of chronic immune activation such as systemic autoimmune diseases (Aerts et al., 2008) and HIV infection (del Pozo-Balado et al., 2010).

The second problem, which was already evident in the two papers describing this gating approach (Seddiki et al., 2006; Liu et al., 2006), is that exclusion of CD127 expressing CD25+FOXP3+ cells leads to the exclusion of a small but discrete population representing about 10% of CD4+CD25+FOXP3+ T cells, whose significance is still unclear.

In the first part of this work we showed in a murine system that CD127 expression on Treg cells is associated to the expression of CD103 and ICOS, two markers identifying highly suppressive, activated Tregs. Our results suggest that excluding CD127 expressing cells from phenotypic and functional Treg analysis would therefore limit the conclusions obtained to the resting Treg compartment excluding the most functionally effective one.

Baecher-Allan and coworkers conducted a detailed analysis of the CD127 expressing

fraction of Treg in the human system (Baecher-Allan et al., 2011). By analyzing the CD4+FOXP3+CD45RO+ population for HLADR and CD127 expression, these authors identified a CD127+HLA-DR- Treg subset which represented about 15% of the whole population. Interestingly, these cells were able to suppress conventional T cells proliferation or to secrete IL-17 depending on the co-stimulation provided during activation. Looking at Treg subset distribution in cord blood, the authors demonstrated that the Foxp3+ CD25+CD127+HLA-DR- cells are completely absent at birth, suggesting that the CD127+HLA-DR- Tregs are induced during immune system maturation. Based on these results, the authors speculated that the FOXP3+ cells contained in the CD25hiCD127+ population are indeed adaptively induced Tregs. However we can speculate, in agreement with results we obtained in the murine system, that CD25hiCD127+ Treg cells represent Treg in an activated state.

Independently from their ontogeny, CD127+ Treg cells represent a suppressive Treg subset excluded from most recent analysis and which could be especially relevant in chronic immune response and pathological contexts. This may notably explain some discrepancies observed between Treg cells analysis performed so far in autoimmune or infectious context. In multiple sclerosis patients, defective suppressive capacity of regulatory T cells has been described when unseparated CD25high Treg cells were tested (Viglietta et al., 2004; Michel et al., 2008), whereas no defect was detectable when selecting CD127low Treg cells (Michel et al., 2008). This discrepancy suggests that the CD127+ cell subset or mechanisms leading to CD127 up-regulation could indeed be responsible for defective Treg function in MS patients. Baecher-Allan et al. formally addressed this issue and demonstrated that the CD127+HLA-DR- population from MS patients and healthy donors exhibited no differences in their ability to suppress responder T cells proliferation (Baecher-Allan et al., 2011). Whether a defect in Treg activation is present in MS patients remain to be further investigated.

In conclusion, excluding CD127+ cells from Treg analysis leads to the exclusion of a

peculiar subset whose relevance in pathological contexts remains to be fully defined.

4.3 - CD127 on Treg : implications for Treg homeostasis

The demonstration of functional CD127 expression at Treg surface formally identify Treg as potential target of both endogenous and therapeutic IL-7.

Given IL-7 role in T cells homeostasis we first focused our attention on the potential effects of IL-7 on Treg homeostasis. Our results demonstrate that IL-7 signaling regulates Treg homeostasis similarly with what has already been described for other T cell compartments.

We showed that IL-7 treatment in normal mice induced an increase in conventional CD4 T cell numbers that was strictly paralleled by an increase in Treg cell numbers. Our murine data perfectly corroborate results obtained in humans during clinical trials investigating the effects of IL-7 therapy on immune-reconstitution in lymphopenic settings. Indeed, three published reports analyzed the proportion of Treg cells in peripheral blood from patients treated with IL-7 (Rosenberg et al., 2006; Sportes et al., 2008; Sereti et al., 2009) and detected either slight reduction or no difference in the percentage of Treg cells among CD4 T cells after IL-7 treatment. As total CD4 T cell count increased in IL-7 treated patients, we can imagine that, even in human studies, Treg changes in absolute numbers paralleled those detected in conventional CD4 T cells. Collectively, both human and mice studies reveal that IL-7 levels equally affect the size of conventional and Treg subsets thus maintaining the physiologic balance between these two compartments.

Most informations about factors regulating T cell homeostasis *in vivo* derive from studies using transfer of T cells sub-populations into empty hosts which were either competent or deficient for the investigated factor (Schluns et al., 2000; Tan et al., 2001). Results obtained with these models give a mixed picture of the effect of a factor on both cell proliferation and survival.

We used this model to assess the role of IL-7 on Treg expansion. It was already known that

Treg adoptively transferred into Rag-deficient lymphopenic mice undergo intense proliferation (Setoguchi et al., 2005; Zhang et al., 2005). Interestingly, Setoguchi et al showed that this phenomenon was independent from the availability of IL-2 as injection of IL-2 blocking antibodies had no effect of Treg expansion in this system (Setoguchi et al., 2005).

We were therefore able to show that Treg cells transferred into Rag-deficient mice lacking IL-7 proliferated and accumulated less than Treg transferred into IL-7 competent Rag-deficient mice. While demonstrating that IL-7 plays a major role in Treg expansion during lymphopenia, the residual proliferation still present in the absence of IL-7 indicates that other factors participate to the phenomenon. Treg are believed to be continuously activated by tissue self-antigens (Fisson et al., 2003). Reduction of cell competition for MHC-self antigen complexes in lymphopenic environments could increase proliferation. Another potential explanation is that accumulated bacterial products in lymphopenic hosts could drive Treg expansion via, for example, Toll-Like Receptors triggering (Caramalho et al., 2003). Moreover, Muller and coworkers showed that, in streptozotocin induced lymphopenia, Treg proliferation increased in parallel with TGF β seric concentrations, suggesting that such a classical Treg homeostatic factor could be implicated in Treg expansion in lymphopenic settings (Muller et al., 2011). These hypotheses warrant further testing.

Although several gamma-chain cytokines, including IL-2, IL-4, IL-7, IL-15, IL-21, have been reported to increase Treg survival *in vitro* (Pandyian et al., 2008), only IL-2 has been clearly established as an essential soluble factor governing Treg cells peripheral homeostasis (see section 1.3.4.2) (Fontenot et al., 2005; D’Cruz et al., 2005; Setoguchi et al., 2005; Bayer et al., 2005; Zhang et al., 2005; Almeida et al., 2006). Despite not being absolutely necessary for Treg expansion in lymphopenic setting, IL-2 has been shown to increase Treg expansion after transfer into both lymphorepleted and lymphopenic hosts (Zhang et al., 2005). Moreover, Zhang et al. have suggested that IL-2 and lymphopenia

could somehow synergize to induce Treg expansion. As lymphopenia is typically characterized by increased levels of IL-7 and having identified IL-7 as a key factor for Treg expansion during lymphopenia, we speculated that an interplay between IL-2 and IL-7 could exist. We showed that IL-7 availability affects CD25 expression on murine Treg surface both *in vitro* and *in vivo*. More importantly, such an effect on CD25 expression had functional relevance. By using *in vitro* IL-2 binding assay as well as by looking at STAT5 phosphorylation, we were able to show that IL-7 affects Treg capacity to bind IL-2 and to perceive and transduce IL-2 signaling. Moreover we demonstrated that during IL-2 induced *in vivo* Treg expansion, IL-7 availability influences Treg accumulation. Based on these results we can speculate two potential scenarios. IL-2 and IL-7 could act independently at different though complementary levels as IL-2 could induce Treg proliferation while IL-7 could be responsible for Treg survival and accumulation. On the other hand, as IL-2 is a well established survival factor for Treg, we can imagine that IL-7 acts mainly in synergy with IL-2 by increasing Treg reactivity to this cytokine.

Collectively, we thus demonstrated that IL-7 acts both as an IL-2 independent and IL-2 dependent factor on Treg survival (Figure 4.2). Such strategy may reveal extremely powerful to preserve Treg homeostasis: at the steady state, when limiting amounts of IL-2 are available, IL-7 can sustain CD25 maintenance optimizing IL-2 consumption by Treg, while providing at the same time IL-2 independent survival signals.

In addition to studying peripheral blood and secondary lymphoid organs, in our work we extended our analysis to peripheral tissues as these are an important localization for activated Treg. Depending on the organs studied, we observed strikingly different CD127 expression profile on Treg isolated from bone marrow and skin compared to Treg isolated from LN. PB and secondary lymphoid organs analysis revealed low CD127 expression on Treg *ex vivo*. Contrasting profiles were observed in mucosal sites: high CD127 expression on Treg was predominant in the skin but barely detectable in the intestine. Such heterogeneity among various mucosal sites suggests high CD127 expression is not a ubiquitous marker of activated Treg.

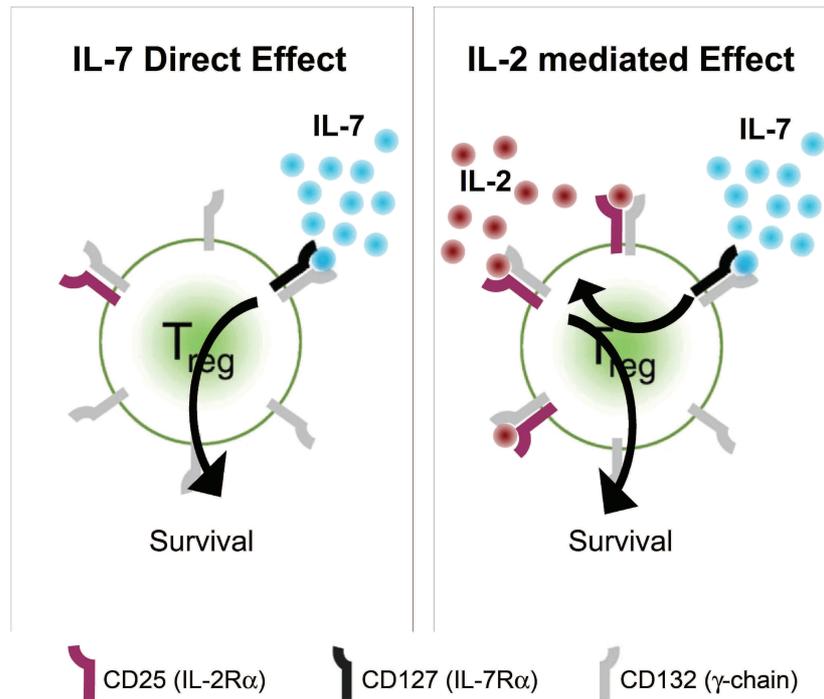


Figure 4.2 – Schematic representation of IL-7 effects on Treg homeostasis. IL-7 direct effect (left panel) ; IL-2 mediated effect through CD25 up-regulation (right panel).

Difference in CD127 expression depending on the tissue considered may rely on various mechanisms: differential antigenic load, differential kinetics of activation, differential activation/differentiation pathways and/or specific signals provided in specific sites, differential migration capacities of high CD127-expressing Treg, differential persistence depending on IL-7 production on sites (Funk et al., 1995). These latter hypotheses may also stand for the high percentage of CD127-expressing Treg in the BM and in the skin. We tried to experimentally investigate this hypothesis by comparing Treg proportions and cell numbers in different organs isolated from wild type, IL-7RKO, IL-7KO and IL-7Tg mice. Our experiments could not reveal any significant differences in Treg proportion among CD4 T cell in those organs between WT and IL-7RKO, IL-7KO and IL-7Tg mice (data not shown).

4.4 - CD127 on Treg : implications for Treg function

An aspect that we did not develop during this work is the potential effect of IL-7 on Treg cells suppressive functions.

The only available data come from *in vitro* experiments demonstrating that addition of high doses of IL-7 during *in vitro* suppressive assays reduced the suppressive activity of Treg on responder T cells proliferation (Ruprecht et al., 2005; Pandiyan et al., 2007). Because of higher expression of CD127 at conventional T cells surface authors speculate that IL-7 acted on responder T cells by reducing their susceptibility to Treg mediated suppression, eventually by preventing their apoptosis (Pandiyan et al., 2007) or by interfering with TGF β signaling (Pellegrini et al., 2009).

Demonstration of high levels of functional CD127 at Treg surface during activation allows to imagine several different potential scenarios.

We demonstrated that during activation the opposite regulation of CD127 on Treg cells and effector cells led to equivalent CD127 expression on Treg and effector T cells. We can therefore hypothesize that Treg and conventional T cells may compete for IL-7 during activation (Figure 4.2). This would lead to a model of “IL-7 deprivation” similarly to what has been suggested for IL-2.

Such IL-7 deprivation during immune-responses could act at several levels. By reducing IL-7 availability for conventional T cells, Treg could inhibit their proliferation and survival. In that respect, IL-7 deprivation could participate to Treg induction of conventional T cell apoptosis which has been until now linked to IL-2 deprivation (Pandiyan et al., 2007).

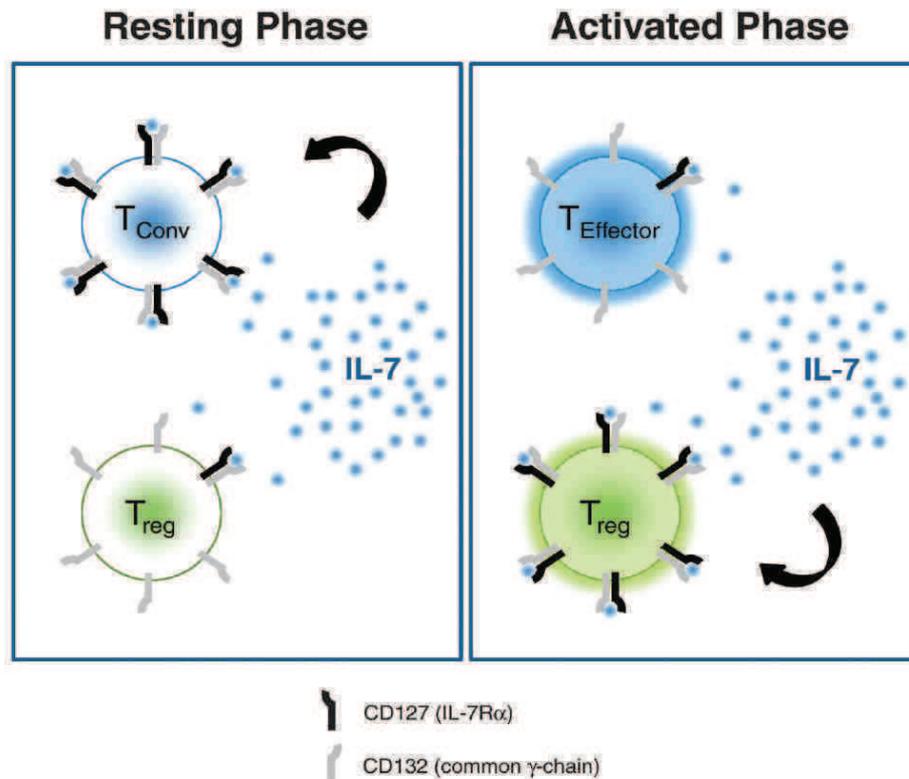


Figure 4.2 – Schematic representation of IL-7 deprivation model of suppression. Consequences of cytokine deprivation of effector T cells caused by Treg cells could include I) induction of apoptosis, II) interference with conventional T cell activation III) inhibition of synthesis effector molecules IV) modulation of memory T cell generation.

As mentioned, IL-7 signaling increases CD25 expression at conventional T cells surface during activation. By competing for IL-7, Treg could reduce CD25 levels at conventional T cell surface, thus interfering with their activation.

Otherwise, as IL-7 availability affects activated conventional T cells production of effector molecules, such as IFN γ and granzyme B (Pellegrini et al., 2011), IL-7 deprivation by Treg could reduce the synthesis of these molecules during immune responses.

Finally, Treg have been shown to interfere with CD8 T cells priming and to reduce the ensuing frequency of memory CD8 T cells (Chappert et al., 2010). As IL-7 is involved in

memory T cell formation, it is fascinating to speculate that Treg could modulate the memory T cell generation by competing for IL-7.

In addition, it has been previously shown that IL7RKO Tregs present impaired suppressive activity both *in vitro* (Mazzuchelli et al., 2008) and *in vivo* (Bourgeois et al., 2006). In particular, Bourgeois et al. have previously shown *in vivo* that, in contrast to WT Treg, IL7RKO Treg failed to prevent lymphopenia-induced proliferation of TCR-transgenic CD4 T cells co-transferred into empty hosts, suggesting that Treg cells and conventional T cells were competing for IL-7 during lymphopenia-induced proliferation (Bourgeois et al., 2006).

Another potential mechanism by which IL-7 could affect Treg cells suppressive capacity come from our results demonstrating that IL-7 signaling modulates CD25 expression at Treg surface. High CD25 expression allows Treg cells to efficiently bind and compete for IL-2 produced by conventional T cells during the early phases of immune responses (O’Gorman et al., 2009). We have seen how competition for IL-2 represents one of the potential mechanisms employed by Treg for suppressing activated conventional T cells (De la Rosa et al., 2004; Barthlott et al., 2005; Pandiyan et al., 2007). By increasing CD25 expression at Treg surface IL-7 could increase the Treg IL-2 competition potential and therefore suppressive activity.

Moreover, Treg cells exploit IL-2 for the induction of inhibitory molecules necessary for their suppressive activity such as IL-10 and TGF β (Barthlott et al., 2005; Fontenot et al., 2005). We showed that impaired IL-7 signaling led Treg to less efficiently bind IL-2 and perceive IL-2 signaling. We can therefore speculate that IL-7 deprived Treg would be less effective in suppressing conventional T cell responses because of this defect.

Finally we cannot exclude that IL-7 could modulate other Treg suppressive mechanisms such as the production of suppressive cytokines. It has been reported that IL-7 inhibits

TGF β synthesis in macrophages (Dubinett et al., 1993), murine fibrosarcoma cells (Dubinett et al., 1995) and fibroblast (Huang et al., 2002). We can imagine that IL-7 could interfere with TGF β production by Treg. Together with the aforementioned effect of IL-7 on TGF β signaling in responder cells (Pellegrini et al., 2009) this mechanism could participate to the observed inhibition of Treg suppression by IL-7.

CD127 is also part of the receptor for thymic stromal-derived lymphopoietin (TSLP), an IL-7-like cytokine expressed by epithelial cells, including keratinocytes, and important in allergic inflammation (Soumelis et al, 2002; He et al., 2008). The role of TSLP in Treg biology remains to be fully elucidated. In our work we assessed the capacity of Treg cells to transduce TSLP triggered signals and we showed that TSLP induced a dose-dependent increase in STAT5 phosphorylation in both conventional and, to a lesser extent, Treg. However we did not reveal any association between CD127 expression on Treg and TSLP signal transduction, failing to reproduce for TSLP the CD127 effect we detected for IL-7. Nevertheless, one may hypothesize that activated Treg could compete with effector T cells for TSLP and this may be one of the mechanisms by which Treg dampen inflammation at tissue sites. Such a mechanism could be particularly relevant for tissues where important TSLP synthesis takes place such as the skin (Soumelis et al, 2002; He et al., 2008). Otherwise, TSLP signaling could modulate Treg function by similar mechanisms as those discussed for IL-7.

During this thesis work, a major experimental limit to the investigation of the effects of IL-7 on Treg function has been represented by the impossibility to restrict the evaluation of IL-7 effects to the Treg compartment while formally excluding any influence on responder conventional T cells. IL7RKO mice represent the only available source of T cells non-responsive to IL-7 exposure. However, as already mentioned, these mice contain very low numbers of T cells, being therefore really hard to obtain sufficient cell numbers to perform *in vitro* and *in vivo* experiments. More importantly, given the major role of IL-7 in T cell development and homeostasis, the physiological relevance of remaining cells is

questionable. Proportions of cell subsets among T cells compartments are completely altered with a shift to the effector/memory compartment.

The only way to address the question of IL-7 effects on Treg would have probably been to employ an inducible knock-out murine system. Two models of inducible CD127 expression currently exist (Buentke et al., 2006; Jacobs et al., 2010). Unfortunately both of these systems fail to present physiological CD127 levels at cell surfaces in the periphery, being therefore useful to obtain IL-7 unresponsive cells developed in the presence of a functional IL-7 signaling, but not allowing studies which depend on subtle variations of the expression levels.

A system which could potentially be developed and eventually allow to answer to the mentioned questions is a Cre-lox system. By generating mice presenting a floxed CD127 allele (CD127 fl/fl) and crossing them with mice expressing Cre under control of the Mx promoter that is inducible by type I IFN, we could obtain a system in which T cells would develop and survive in the presence of a completely physiological IL-7 signaling. By then inducing CD127 gene depletion, we could obtain otherwise normal Treg lacking CD127 to be employed for *in vitro* and *in vivo* studies. Moreover, by generating bone-marrow chimera using FOXP3^{-/-} scurfy mice and CD127 fl/fl MxCre mice donors we could obtain, upon CD127 gene depletion, a system in which Treg would be the only cells deprived of CD127 signaling. Such a system would allow us to evaluate IL-7 contribution to Treg function *in vivo* both at steady state and in immune-pathological models.

A second model which could be generated employing the Cre-lox technology would be to cross CD127 fl/fl mice with mice presenting the Cre recombinase knocked into the *Foxp3* gene locus. As IL-7 signaling has been shown to be dispensable for FOXP3 induction and Treg development in the presence of physiological IL-2 signaling (Bayer et al., 2007), we would obtain a system in which CD127 deficiency is restricted to FOXP3 expressing Treg cells. This model would give us *in vivo*

informations concerning Treg homeostasis and function. However, given the role of IL-7 in Treg peripheral survival, it is difficult to predict whether in this model adequate numbers of CD127^{-/-} Treg for *in vitro* or adoptive transfer *in vivo* experiment could be obtained.

4.5 – HIV infection and Treg

Study of Treg homeostasis in the context of HIV infection offered us a unique occasion to investigate how human Treg subsets are affected in a condition of CD4 T cell lymphopenia.

Several studies have investigated the role of Treg in patients with HIV infection and still no consensus has been reached concerning their alterations as the result of the infection and their role in the pathogenesis of the disease.

Treg quantification in HIV infection remains controversial in part due to the already mentioned lack of reliable specific markers to identify human Treg. Similarly to what we did for murine studies, we tried to overcome this problem by taking into account Treg heterogeneity. Taking advantage of the novel consensus gating strategy recently described by Miyara and coworkers, we were able to demonstrate that a reduction in Treg cell numbers was present in all groups of HIV infected patients studied and that this Treg decay was essentially due to effector Treg decay, whereas naïve Treg were mildly affected. Whether this selective reduction is the result of increased sensitivity of the effector Treg subset to HIV infection and to cell depletion remains to be formally investigated.

A second aspect complicating Treg analysis in HIV infection and partially responsible for the contrasting published results derives from the enormous differences among patients populations which can be investigated. We have seen how, depending on the patient population examined, the published literature has presented extremely different results (see introduction). In our work we tried to overcome this problem by studying several different

patients cohorts ranging from viremic patients, including primary infected patients and chronically infected viremic patients, to aviremic patients, including ART treated patients and HIC. We were able to figure out that primary infected patients exhibited significant decrease of both naïve and effector Treg. In contrast, during chronic stages, decreased Treg count essentially relies on effector Treg decline, whereas naïve Treg were mildly affected. Based on these results we concluded that HIV infection induces an early and long lasting alteration of effector Treg homeostasis. We will hopefully formally confirm our results by completing our analysis with a longitudinal study, analyzing samples from the same patient at different time points after HIV infection. A second limit of our study concern the chronically infected viremic group of patients. While all presenting high level of viremia, patients presented relatively preserved levels of CD4 T cells, thus not being completely representative of HIV infection in chronic stage. Such a problem derives from a patient enrollment bias : by selecting viremic patients not treated with antiretrovirals we implicitly oriented patients selection to patient having no absolute indication to treatment and therefore higher CD4 count levels. We hope to complete our investigation studying lymphopenic untreated viremic patients, obtaining samples from subjects refusing therapy or diagnosed at late stages.

An aspect we did not developed in our work, mainly as a consequence of the limited amount of blood samples obtained, concerns the eventual effects of HIV infection of Treg. We demonstrated that HIV infection mainly affects the effector Treg compartment in terms of cell numbers. However we do not know whether residual effector Treg still possess all their functional activity or whether HIV infection alters their function. Moreover, even if not quantitatively affected, naïve Treg could be functionally affected by HIV infection, as a result for example of an impairment to full differentiation into effector Treg. Such an hypothesis could explain at least in part, the quantitative effect on effector Treg numbers.

In our work we tried to answer the major question which still remains open : are Treg cells friends or foes in HIV infection ? For doing so, we correlated Treg cell numbers with

both global and HIV specific T cell activation. We found that although no correlation existed between Treg cell numbers and the activation profile of global CD4 and CD8 T cells, the percentage of activated cells among HIV specific T cells inversely correlated with effector Treg cell numbers. These results suggest that Treg exert no or low control of the potentially deleterious chronic immune-activation, while actively inhibiting HIV-specific T cell responses, thus participating to viral persistence. This is, to the best of our knowledge, the first time that correlations between Treg numbers and the activation profile of HIV-specific T cells have been performed, thus providing *ex vivo* a picture of the action exerted by Treg of the antiviral response. Importantly, similar conclusions were drawn when effector Treg numbers were correlated with results obtained by ELISPOT. Collectively our data indicate that effector T reg, by inhibiting HIV specific responses, could represent an obstacle to viral eradication while not being capable to control the potentially harmful chronic immune-activation.

An important area of investigation in our lab is represented by “HIV controllers”. This group of patients, also known as “elite controllers”, represent a group of rare HIV infected individuals who maintain undetectable plasma HIV RNA loads without antiretroviral treatment (Lambotte et al., 2005). Immunological mechanisms responsible for viral control have been a subject of intensive investigation. In particular, HIV-specific CD8 T cell responses have been shown to play a crucial role in viral control. HIV-specific CD8 T cells have been reported to be present at higher frequencies in HIC (Migueles et al., 2002), to possess higher proliferation and perforin expression capacity (Migueles et al., 2002) and to present *ex vivo* a peculiar activation profile (Sàez-Ciri3n et al., 2007). Moreover, Sàez-Ciri3n and coworkers have shown that CD8 T cells from HIC patients strongly suppress *ex vivo* HIV infection of autologous CD4 T cells (Sàez-Ciri3n et al., 2007). However, the same authors demonstrated an immunological heterogeneity among HIC as some of those patient fail to present effective CD8 responses while still being able to control the

virus (Sàez-Ciri3n et al., 2009). They thus proposed to distinguish strong responder HIC from weak responder HIC based on *in vitro* functional analysis. Having demonstrated relatively high numbers of effector Treg in HIC patients and our results suggesting some degree of heterogeneity in HIC in respect of Treg counts, we are investigating whether lower HIV-specific CD8 T cell responses in weak responder HIC could be due to higher levels of Treg mediated suppression. In a first phase, we will compare Treg subsets numbers from weak and strong responder HIC. Depending on results at this stage, our analysis will eventually continue with functional studies in which the capacity of CD8 T cells from weak responder HIC to suppress *ex vivo* HIV infection of autologous CD4 T cells will be determined before and after Treg depletion. In addition to give further insights to mechanisms of viral suppression in HIC patients, such a study could pave the way to Treg targeted immuno-modulatory strategies in HIV infection.

4.6 - Concluding Remarks

We have seen how, both in human and mice, the Treg compartment is indeed an extremely heterogeneous cell population constituted by several different subsets. This thesis work took advantage from the analysis of such an heterogeneity to better investigate some important issues which still remained elusive from studies addressing the Treg population as a whole. In the murine system, we defined the peculiar mechanisms regulating CD127 expression at the Treg surface and we identified IL-7 as an essential cytokine regulating Treg homeostasis. In humans, we showed that HIV infection lead to early and long lasting alterations of Foxp3⁺ Treg homeostasis while providing supportive evidence for a role of Treg in HIV infection pathogenesis.

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6. APPENDIX

Chapter Number

Animal Models of Contact Dermatitis

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1. Introduction

Contact dermatitis is an inflammatory disease of the skin resulting from direct contact with foreign substances. Understanding the immunological processes that cause the disease is therefore essential for the development of new therapeutic strategies.

Murine models of chemically induced dermatitis have played an essential role in our understanding of the pathophysiology of contact dermatitis, unraveling the role played by inflammatory mediators and identifying potential targets for therapeutic interventions.

In the present chapter we review data obtained in animal models of allergic and irritant contact dermatitis and provide basic protocols to reliably induce contact dermatitis.

A major intent of this chapter is to highlight the respective role of innate and adaptive immune cells in contact dermatitis pathogenesis as revealed by murine studies. Through genetic ablation of single molecules or depletion of specific cell subsets, murine studies provide novel insight on the role of different components of the immune system in the development of contact dermatitis. We review the experimental evidence revealing the role of different T cell subsets in contact dermatitis development, focusing our attention on mechanisms responsible for maintenance or disruption of immune-tolerance. Our analyses will focus on molecular pathways which are promising candidates as targets of future biological therapies.

2. Allergic contact dermatitis

Most of our knowledge on the pathophysiology of contact dermatitis is derived from murine models of Allergic Contact Dermatitis (ACD), a T cell mediated delayed-type hypersensitivity reaction also referred to as contact hypersensitivity (CHS). In this model, skin inflammation is induced by topical application on the epidermis of sensitizing chemical agents which act as haptens (Eisen HN et al., 1952).

Haptens can be defined as low molecular weight chemicals which are not immunogenic by themselves and that generate new antigenic determinants by binding to epidermal proteins. The reactive haptens commonly employed for ACD induction in murine models include oxazolone (OX), dinitrochlorobenzene (DNCB), dinitrofluorobenzene (DNFB), trinitrochlorobenzene (TNCB), picryl chloride (PI), and fluorescein isothiocyanate (FITC).

Such widely employed haptens are all strong contact allergens exhibiting high proinflammatory capacities which therefore differ from the vast majority of chemicals

1 responsible for human ACD. Importantly recent studies have reproduced the conclusions
2 drawn with strong allergens by using weak haptens (Vocanson et al., 2006; Vocanson et al.,
3 2009) which are more close to those encountered in clinical practice.

4 **2.1 Phases of ACD**

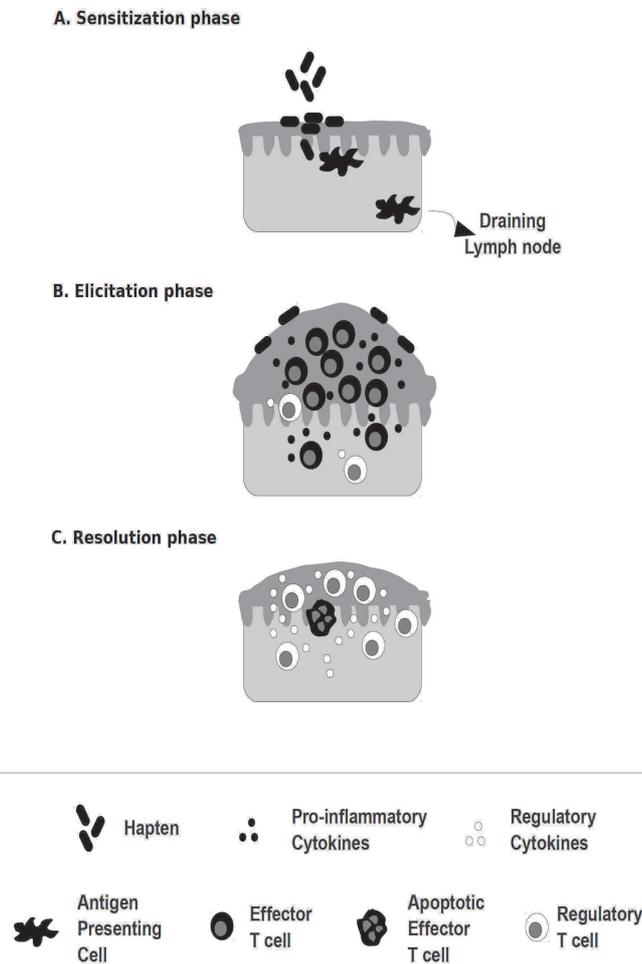
5 Three temporally distinct phases can be identified in ACD: the **sensitization phase**, the
6 **elicitation phase** and the **resolution phase**.

7 The **sensitization phase**, also referred to as the afferent phase or induction phase, occurs
8 at the first skin contact with a hapten (Fig. 1A). This phase lasts 10–15 days in humans and
9 5–7 days in mice. Most of employed haptens induce local inflammation by acting on
10 innate immunity receptors (see section 4.1). Activation of the skin innate immunity induce
11 the production of mediators (IL-18, IL-1 β , TNF- α , ATP, PGE2, LTB4, ROS, histamine,
12 CCL20) by resident skin cells. These mediators are able to induce the recruitment,
13 migration and activation of cutaneous antigen presenting cells (APC). The skin contains a
14 dense network of APC constituted by two phenotypically and spatially distinct subsets:
15 Langerhans cells (LC), which are located in the epidermal layer of the skin, and dermal
16 DCs (dDC). In addition to skin resident APC, circulating CCR2⁺ monocytes are rapidly
17 recruited from blood to skin and locally differentiate into dendritic cells. Once activated,
18 APC phagocyte haptenated proteins and migrate from the skin to the para-cortical area of
19 draining lymph nodes (Kripke et al., 1990). There, APC present haptenated peptides
20 through MHC classes I and II molecules at their cell surface (Weltzien et al., 1996) to
21 hapten-specific CD8⁺ and CD4⁺ T lymphocytes thus inducing their priming and
22 expansion (Bour et al., 1995 ; Xu H et al., 1996 ; Krasteva et al., 1998). Primed hapten-
23 specific T cells can then emigrate from the lymph nodes and enter the blood from which
24 they recirculate to different tissues including the skin.

25 The **elicitation phase**, also referred to as the efferent or challenge phase, occurs after
26 subsequent challenges with the same hapten to which a host has been previously
27 sensitized (Fig. 1B). This efferent phase of CHS takes place within 72 hours upon exposure
28 to the allergen in humans and 24 to 48 hours in mice. Once more, haptenated peptides are
29 uptaken by skin APC which present to hapten specific primed T cells patrolling in the
30 skin. As this process directly takes place on the site of inflammation and as T cells
31 activated at this stage are already primed cells, the elicitation phase develops extremely
32 rapidly.

33 Activated T cells produce effector cytokines, notably IFN- γ and IL-17 (see section 4.2), which
34 activate skin resident cells which in turn secrete cytokines and chemokines pertaining cell
35 recruitment at exposure or contact or tissue site. Continuous recruitment of circulating cells
36 then leads to polymorphous cellular infiltrate and persistence of the inflammatory reaction
37 over several days.

38 The **resolution phase**, during which the inflammatory response progressively disappears,
39 then follows. This phase presumably results from down-regulating mechanisms including
40 passive processes, such as the progressive disappearance of the hapten from the
41 epidermis, as well as active cellular processes, such as the intervention of regulatory T
42 cells (see section 5).

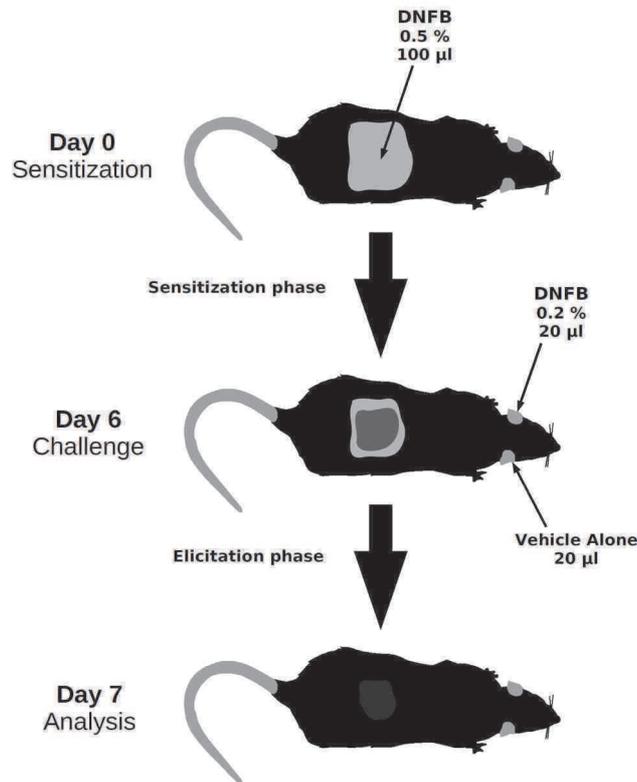


1
2 Fig. 1. Schematic representation of the pathophysiology of the different phases of allergic
3 contact dermatitis. (A) the sensitization phase (B) the elicitation phase (C) the resolution
4 phase.

5 2.2 Experimental protocol

6 Experimental protocol for ACD induction is graphically summarized in Figure 2. For
7 sensitization, mice are painted at day 0 on the shaved back (or abdomen) with 100 μ l of
8 DNFB 0.5% in a 4:1 mixture of acetone and olive oil. Mice are challenged 6 days later by
9 application of 20 μ l DNFB 0.2% in olive oil (10 μ l to each side of one ear). Ear thickness is
10 measured with a digital calliper before challenge and at 24-48 hr after treatment. Ear
11 swelling is calculated by subtracting the value recorded for vehicle-control ear from the
12 hapten-applied ear. Histological examination can be performed in order to confirm changes
13 in ear thickness and to quantify cellular infiltration. Moreover, lymphocytes infiltrating

1 inflamed skin can be investigated by FACS analysis after isolation. In figure 4 we provide a
 2 detailed protocol to isolate lymphocytes from skin.



3
 4 Fig. 2. Allergic contact dermatitis experimental protocol.

5 **3. Irritant contact dermatitis**

6 Clinical evidence indicates that a primary, irritative form of contact dermatitis can develop
 7 upon a single first contact with chemicals in previously unsensitized patients.

8 Irritant contact dermatitis (ICD), also known as “primary ACD” or “primary CHS” develop
 9 upon skin exposure to strong haptens such as urushiol, primine, DNFB and DNCB (Kanerva
 10 et al., 1994).

11 ICD is the result of the liberation of proinflammatory mediators upon skin exposed to
 12 haptens which in turn promote recruitment from blood of inflammatory cells to tissue sites.
 13 The magnitude of ICD is similar to that of classical ACD but presents a different kinetic
 14 since the onset of the skin inflammation is delayed in ICD by 5 days, a time period needed
 15 to achieve T cell priming .

16 The eczematous lesions of ICD are morphologically indistinguishable from those present in
 17 conventional ACD, suggesting that common mechanisms could be involved in the two

1 processes. Importantly, using an experimental murine model of primary CHS induced by a
2 single DNFB or FITC painting on mice ears without subsequent challenge, Saint-Mezard
3 and colleagues were able to show that the pathophysiology of one-step ICD is identical to
4 that of two-step classical ACD, involving the same effector and down-regulating immune
5 mechanisms (Saint-Mezard et al., 2003). Remarkably, ICD responses were strictly dose-
6 dependent and reproducibly more important in C57B/6 mice than in BALB mice
7 (Bonneville et al., 2007). More importantly, Bonneville et al. demonstrated an inter-
8 relationship between ICD and ACD and showed that upon hapten rechallenge the intensity
9 of ACD reaction is proportional to the magnitude of the former ICD response (Bonneville et
10 al., 2007).

11 **3.1 Experimental protocol**

12 Experimental protocol for ICD induction is graphically summarized in Figure 3. Naive mice
13 are exposed at day 0 to a single application of 20 μ l of DNFB 0.5% in a 4:1 mixture of acetone
14 and olive oil applied on the left ear, while the same volume of vehicle alone is applied on
15 the right ear as a control. Six days after ear sensitization, ear thickness is measured with a
16 digital calliper. Analysis can be performed similarly to ACD model (see section 2.1).

17 **4. Effector mechanisms in allergic contact dermatitis**

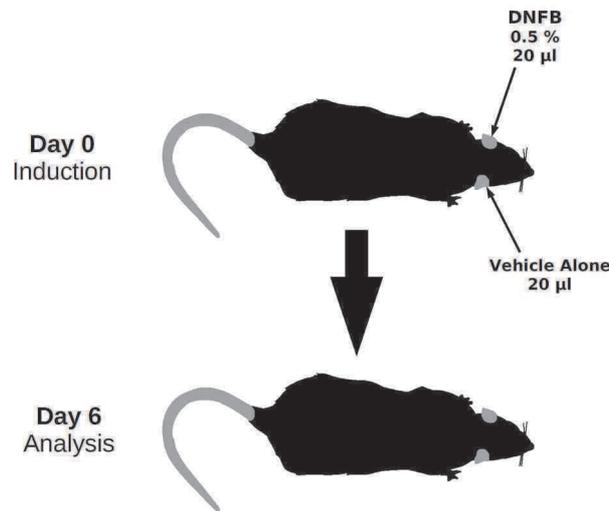
18 **4.1 Role of innate immunity**

19 ACD is a delayed type hypersensitivity reaction. Despite being a prototypical T cell
20 mediated response, a role of innate immunity which has been pointed out since early
21 studies has been recently better elucidated. A common feature of contact allergens
22 employed in experimental studies is their local irritancy and their capacity to act as
23 adjuvants. This feature depends on their ability to activate the innate immune system.

24 Innate immune system cells express pattern recognition receptors (PRR), germ-line encoded
25 receptors that recognize so-called pathogen-associated molecular patterns (PAMPs),
26 microbial molecular structures such as bacterial or fungal cell wall components, microbial
27 nucleic acids, proteins or sugars. Depending on their localization we can distinguish
28 transmembrane PRRs, such as Toll-like receptors (TLR) which recognize PAMPs in the
29 extracellular space and/or in phagosomes or endosomes, and cytosolic PRRs, such as
30 nucleotide-binding oligomerization domain containing (Nod)-like receptors (NLR).

31 Investigating the involvement of TLRs in the development of CHS to contact allergens,
32 Martin and coworkers first revealed a crucial role for TLR2 and TLR4 (Martin et al., 2008).
33 Mice lacking both TLR4 and TLR2 were resistant to TNCB induced CHS, thus establishing a
34 link between hapten-induced inflammation and innate immune-responses. Accordingly,
35 mice deficient MyD88, a molecule centrally involved in TLRs signaling transduction, failed
36 to mount CHS responses to DNFB (Klekotka et al., 2010). Further evidence for a role of TLRs
37 in cutaneous ACD comes from a study on Nickel (Ni(2+)), by far one of the most relevant
38 contact allergens in terms of incidence of contact eczema and sensitization rates. Schmidt et
39 al., identified human TLR-4 as the crucial mediator of the innate immune response to Ni(2+)
40 (Schmidt M et al., 2010). After having provided in vitro evidence for the need of TLR-4
41 expression for Ni(2+) induced activation, the authors demonstrated that transgenic
42 expression of hTLR4 in TLR4-deficient mice confers sensitivity of naturally resistant mice to

1 Ni(2+)-induced CHS. Those results formally demonstrate that Ni(2+) employs TLR4, a
 2 signaling component of the antibacterial defense system, to elicit its allergic reactions.
 3



4
 5 Fig. 3. Irritant contact dermatitis experimental protocol.

6 NLRP3, also known as NALP3/Cryopyrin/CIAS1/PYPAF1, belongs to the NLR family of
 7 PRRs and is activated by a variety of pathogen- and host-derived “danger” signals including
 8 : whole pathogens (*Candida albicans*, *Saccharomyces cerevisiae*, *Staphylococcus aureus*,
 9 *Listeria monocytogenes*); pathogen-associated molecules (bacterial pore-forming toxins and
 10 malarial hemozoin); environmental irritants (silica, asbestos, ultraviolet light); host-derived
 11 “danger-associated molecular patterns” (ATP, glucose, monosodium urate, calcium
 12 pyrophosphate dihydrate, amyloid β , hyaluronan); and immune adjuvants (aluminum salt).
 13 NLRP3 forms a multi-protein complex, known as the NLRP3 inflammasome, together with
 14 the adaptor protein apoptosis-associated specklike protein (ASC) and caspase-1.
 15 Inflammasome activation leads to the proteolysis to bioactive form of the proinflammatory
 16 cytokines IL-1 β and IL-18. Langerhans cells and keratinocytes can secrete IL-1 β and IL-18
 17 upon exposure to sensitizing agents (Sauder et al., 1984 ; Enk et al., 1993 ; Nail et al., 1999)
 18 and these cytokines are crucial for Langerhans cells migration to the draining lymph nodes
 19 (Cumberbatch et al., 2002). It is therefore not surprising that NLRP3 inflammasome and IL-
 20 1/IL-1R signaling are required for ACD development. Shornick and colleagues first
 21 demonstrated that IL-1 β deficient mice showed defective CHS responses to topically applied
 22 TNCB and that this defect could be overcome by local intradermal injection of recombinant
 23 IL-1 β immediately before antigen application (Shornick et al., 1996). Accordingly, as NLRP3
 24 inflammasome activation is necessary for active IL-1 β production, mice lacking either
 25 NLRP3, the adaptor protein ASC or caspase-1 showed impaired CHS responses to TNCB
 26 and DNFB (Sutterwala et al., 2006 ; Watanabe et al., 2007 ; Antonopoulos et al., 2001).
 27 Finally, IL-1R deficiency (Klekotka et al., 2010) or treatment of mice with the IL-1R
 28 antagonist (Kondo et al., 1995) efficiently prevented CHS development.

1 Production of IL-18, a cytokine with structural similarities to IL-1 β , is also regulated by
2 NLRP3 inflammasome activation. In vivo studies showed that CHS responses to oxazolone
3 and DNFB were significantly inhibited in mice treated with neutralizing IL-18 Ab (Wang et
4 al., 2002) or which were deficient for either IL-18 (IL-18^{-/-} mice) (Antonopoulos et al., 2008)
5 or IL-18R (Klekotka et al., 2010). Interestingly CHS could be rescued by local intradermal
6 administration of IL-18 prior to sensitization, in agreement with an implication of IL-18 in
7 the afferent phase of the disease.

8 Trying to dissect the relative contribution of IL-1 β and IL-18 in ACD development,
9 Antonopoulos et al., further showed that IL-1 β but not IL-18 administration was able to
10 rescue the defective CHS response observed in caspase-1^{-/-} mice, which have no functional
11 IL-1 β or IL-18 (Antonopoulos et al., 2008). Therefore IL-1 β appears to be the main caspase-1-
12 dependent inducer of inflammation in CHS.

13 **4.2 Role of adaptive immunity**

14 ACD is a cellular immune reaction which has been identified since early studies as mediated
15 primarily by T cells. Adoptive transfer of T cells from sensitized mice into non-sensitized
16 recipients results in the transfer of sensitization (Moorhead et al., 1978 ; van Loveren et al.,
17 1983). Moreover, T cell depletion before the elicitation phase results in complete abolition of
18 the reaction (Gocinski and Tigelaar, 1990).

19 As ACD is a classical DTH reaction and since cutaneous infiltrates in humans show a clear
20 preponderance of CD4⁺ T cells, ACD has first being considered to be mediated primarily by
21 CD4 T cells. However, based on contrasting experimental results, the role exerted by
22 different T cell subsets in the physiopathology of the disease has longly been debated.

23 In some experimental models CD4 T cells have indeed been shown to mediate the allergic
24 response (Miller and Jenkins, 1985 ; Gocinski and Tigelaar 1990 ; Kohler et al., 1995 ; Wang B
25 et al., 2000). However, most studies agree in identifying CD8⁺ T cells as the main effector
26 compartment in ACD to different haptens (Gocinski and Tigelaar, 1990 ; Bour et al, 1995 ; Xu
27 et al, 1996 ; Bouloc et al, 1998 ; Kehren et al., 1999 ; Martin et al., 2000 ; Akiba et al., 2002 ;
28 Dubois et al., 2003 ; Saint-Mezard et al., 2004).

29 Such evidence pointing to a role of CD8 T cells in ACD has been obtained through several
30 experimental approaches such as in vivo depletion of normal mice with anti-CD4 and anti-
31 CD8 mAbs, use of MHC class I^{-/-} CD8⁺ T cell-deficient mice and MHC class II^{-/-} CD4⁺ T
32 cell-deficient mice or adoptive transfer of purified primed CD4⁺ and/or CD8⁺ T cells from
33 sensitized mice into naïve recipients.

34 Using the antibody depletion model, Gocinski and Tigelaar showed that CD8 depletion prior
35 to DNFB sensitisation led to a substantial reduction of ear swelling upon rechallenge, for the
36 first time pointing to CD8 T cells as major players in ACD. Even more surprisingly, the authors
37 demonstrated that when CD4 T cells were depleted prior to sensitization with DNFB, rather
38 than observing a reduction in ear swelling upon rechallenge, responses were augmented
39 suggesting that CD4 T cells were indeed behaving as down-regulatory cells. These conclusions
40 were subsequently confirmed by Bour et al. studying CHS in MHC class I and MHC class II
41 knockout mice, which are deficient in CD8⁺ and CD4⁺ T cells, respectively. In this system,

1 Class I deficient mice failed to develop DNFB-induced CHS responses while class II deficient
2 mice developed an enhanced CHS reaction (Bour et al., 1995).

3 An explanation to these phenomena came from the demonstration that upon sensitization
4 with DNFB or oxaloxone hapten-loaded APC migrated to draining lymph nodes from the
5 skin induce the differentiation of IFN- γ -producing effector CD8⁺ T cells and IL-4/IL-10-
6 producing CD4⁺ T cells that negatively regulate the response (Xu et al., 1996). Since CD8⁺
7 effector T cells primarily exert their function through cytotoxicity, the demonstration of a
8 central role of CD8 T cells in CHS leads to investigate whether cytotoxicity mediated skin
9 inflammation. Kehren et al. showed that mice double deficient in perforin and FasL were
10 able to develop hapten-specific CD8⁺ T cells in the lymphoid organs but did not show CHS
11 reaction in the skin, thus demonstrating that the CHS reaction is dependent on CD8⁺ T cells
12 cytotoxic activity (Kehren et al., 1999). Using immunohistochemistry and RT-PCR analysis
13 Akiba and colleagues extended those results by demonstrating that epidermal keratinocytes
14 were the target cells of hapten-specific CD8⁺ T cells cytotoxicity (Akiba et al., 2002).

Tissue Preparation	1 - Sacrifice mice 2 - Isolate ears 3 - Separate the two sheets of ear skin with forceps 4 - Finely cut the tissue with scissors
Tissue Digestion	5 - Incubate tissue fragments in Liberase (Roche) 400 μ g/ml freshly diluted in PBS for 1 hour at 37°C while shaking
Cells Isolation	6 - Vortex vigorously for 1 min 7 - Pass the suspension through a 70- μ m cell strainer 8 - Press digested tissue through the cell strainer 9 - Add PBS 10 - Centrifuge 10 min at 1800 rpm 11 - Carefully remove the supernatant 12 - Repeat steps 9-11 13 - Resuspend in a 35% Percoll solution (Sigma-Aldrich) 14 - Centrifuge 25 min at 2500 without brake 15 - Carefully discard supernatant 16 - Resuspend the cell pellet in PBS 17 - Wash twice

15

16 Fig. 4. Skin infiltrating lymphocytes preparation for FACS analysis

17

18

In addition to the aforementioned role of perforin and FasL, IFN- γ has been shown to play
an important role in CHS responses and a defect in IFN- γ signals as a result of the genetic

1 disruption of the IFN- γ R2 gene (Lu et al., 1998) or of the IFN- γ encoding gene (Wakabayashi
2 et al., 2005) suppresses CHS responses. However other reports failed to confirm impaired
3 CHS responses in mice with IFN- γ signaling disruption (Saulnier et al., 1995 ; Reeve et al.,
4 1999). Moreover, neutralization of IFN- γ in *DNFB* sensitized mice before challenge failed to
5 suppress the elicitation of CHS (He et al., 2006).

6 Searching for other mediators of CHS, Nakae et al. reported that CHS was reduced in IL-17
7 knockout mice compared with wild-type controls (Nakae et al., 2002). Subsequently, IL-17
8 neutralization in *DNFB* sensitized mice before challenge has been shown to suppress the
9 elicitation of CHS (He et al., 2006). IL-17 is a well known chemotactic factor and absence of
10 IL-17 signaling inhibited the infiltration of T cells, monocytes/macrophages and
11 granulocytes into hapten-challenged skin tissues. Interestingly, He and coworkers
12 demonstrated that in the *DNFB* induced CHS model CD8+ T cells represents the major
13 source of IL-17 at the inflammation site. Moreover, CD8+ IL-17-producing T cell
14 subpopulation is distinct from CD8+ IFN- γ -producing Tc1 cells and is important in effector
15 functions during the elicitation of CHS. Subsequently the same group extended those results
16 and demonstrated that IL-17 and IFN- γ signaling are both required for optimal elicitation of
17 CHS by probably acting through distinguished mechanisms (He et al., 2009).

18 Globally, murine studies allowed to identify CD8+ T cells as the major cellular player in
19 ACD pathogenesis and pointed out several molecular pathways which can be taken into
20 account for the development of immuno-therapeutic strategies.

21 **5. Regulatory mechanisms in allergic contact dermatitis**

22 Both sensitization to chemicals and the effector phase of contact allergy are highly regulated
23 events. This task is guaranteed by multiple mechanisms, including antigen-presenting cells
24 and effector T cells apoptosis, production and release of anti-inflammatory mediators and
25 action of a specialized subset of T lymphocytes with down-regulatory properties known as
26 regulatory T cells.

27 Regulatory T cells (Treg) are a critical CD4 T cell subset involved in the control of immune
28 homeostasis and in regulation of inflammation (Sakaguchi et al., 2008). Treg subset
29 represent about 5-10% of the whole T cell compartment and are characterized by the
30 preferential expression of several molecules including CD25, the α chain of IL-2 receptor
31 (Sakaguchi 1995), and FOXP3, a transcription factor which is necessary for Treg
32 development and function (Fontenot 2003).

33 CD4+CD25+ Treg cells have been implicated in the control CHS responses to haptens in
34 mice. The first evidence of such a role for Treg cells came from a study investigating the
35 mechanisms responsible for the "oral tolerance" phenomenon (Dubois et al., 2003). It was
36 known that *DNFB* oral administration prior to sensibilisation could induce a tolerant state
37 and prevent the development of ACD (Garside et al., 2001). Desvignes and coworkers
38 showed that tolerance induction was dependent on the presence of CD4 T cells (Desvignes
39 et al., 1996 ; Desvignes et al., 2000) suggesting the implication of a CD4 T cell subset with
40 regulatory characteristics. Using in vivo models of adoptive transfer and antibody depletion
41 of CD4+CD25+ cells, Dubois et al. demonstrated that naturally occurring CD4+CD25+ T
42 cells are instrumental for orally induced tolerance and control hapten-specific CD8+ T cell
43 responses mediating skin inflammation (Dubois et al., 2003).

1 Further studies confirmed the role of Treg cells in classical ACD reaction. Depletion of CD4+
2 CD25+ T cells by in vivo treatment of mice with an anti-CD25 mAb at the time of
3 sensitization led to an increased CD8+ T cell priming and an enhanced ACD reaction (Kish
4 et al., 2005). Conversely, IL-2-IgG2b fusion protein treatment of mice induces a decreased
5 ACD reaction associated with an increase in the CD4+ CD25+ Treg cell numbers (Ruckert et
6 al., 2002).

7 Importantly, a role for CD4+ CD25+ Treg cells in maintaining immune tolerance to skin
8 allergens has been confirmed in humans. Cavani et al. showed that CD4+ T cells isolated
9 from the peripheral blood of six healthy nonallergic individuals showed a limited capacity
10 to proliferate in response to nickel in vitro, but responsiveness was strongly augmented by
11 CD25+ Treg depletion (Cavani et al. 2003).

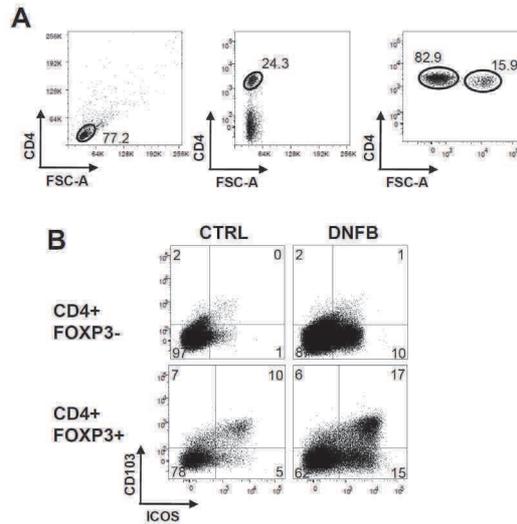
12 Mechanisms involved in Treg maintenance of skin tolerance still remain to be fully
13 elucidated. As IL-10 has been shown to mediate in vivo Treg suppression in other murine
14 models of disease, it has been evaluated in the context of ACD. IL-10 has been shown to
15 participate in restoring oral tolerance to haptens induced by CD4+ T cells (Dubois et al.,
16 2003). Subsequently Ring et al. showed that Treg cells injection into sensitized mice at the
17 time of local hapten challenge significantly inhibited influx of effector T cells into inflamed
18 skin tissue, but that this effect was abrogated when CD4+CD25+ Treg cells isolated from IL-
19 10-/- mice were transferred (Ring et al., 2006). More recently, Rudensky and coworkers
20 generated mice in which Treg cell-specific ablation of a conditional IL-10 allele was induced
21 by Cre recombinase knocked into the *Foxp3* gene locus (Rubtson et al., 2008). These mice
22 were more prone to skin hypersensitivity reaction induced by DNFB, thus formally
23 demonstrating the implication of IL-10 in Treg mediated control of CHS.

24 Another potential mechanisms through which Treg could inhibit effector migration to
25 inflamed skin site, has been proposed by Ring and coworkers who found that Treg cell-
26 derived adenosine plays a major role in preventing the elicitation of CHS reactions by
27 blocking the interaction of effector T cells with the vascular endothelium (Ring et al., 2009).

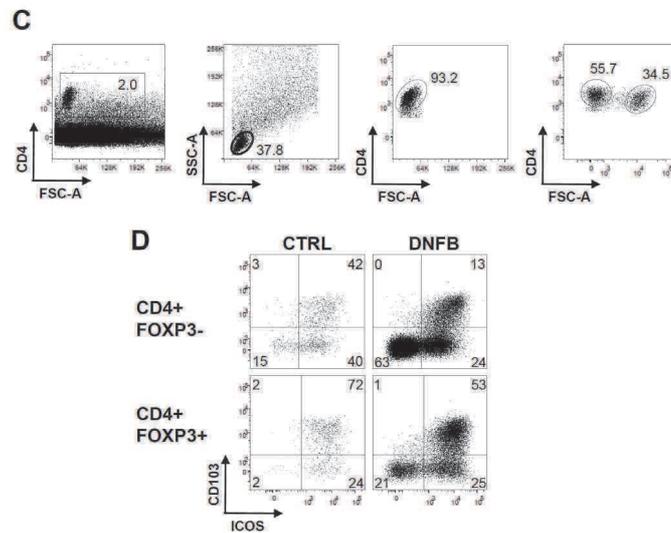
28 Independently from the mechanism involved, CD4+CD25+Foxp3+ regulatory T cells
29 require to be activated in order to develop their full suppressive capacity. Recently, studies
30 from our and other groups better defined Treg activation in murine models of CHS.
31 Analysing the expression kinetics of CD62L, CD69 and CD44 expression at Treg surface,
32 Ring et al. showed that during the sensitization phase of CHS reactions, Tregs get activated
33 in the draining lymph nodes while Tregs get activated in the blood during the elicitation
34 phase. (Ring et al., 2010). Employing the isolation protocol resumed in Fig. 4, we were able
35 to extend the analysis of Treg activation to the skin tissue site in a model of primary allergic
36 dermatitis (Simonetta et al., 2010). We confirmed that Treg were activated in lymph nodes
37 after skin application of a strong hapten, as revealed by the surface upregulation of the
38 activation molecules ICOS and CD103 (Fig. 5). Surprisingly when we extended the analysis
39 to the skin tissue, we found high levels of ICOS and CD103 expression at surface of skin
40 infiltrating Treg of both primed and non-primed animals (Fig. 5), indicating that skin Treg
41 under normal physiological conditions are already in an activated state. A study performed
42 by Vocanson and coworkers further extended our results on ICOS expression on Treg
43 revealing that these cells present superior suppressive activity and express IL-10, IL-17, and
44 IFN- γ (Vocanson et al., 2010). More importantly for ACD comprehension, the authors

1 showed that ICOS expressing Treg during ACD were hapten-specific, activated Treg cells
 2 proliferating in response to their cognate antigen in vivo.

Draining Lymph Nodes



Ear Skin



3
 4 Fig. 5. FACS analysis of skin infiltrating CD4+ FOXP3+ Treg cells in a murine model of
 5 primary irritative contact dermatitis. (A, C) Gating strategy for Treg identification in
 6 draining lymph nodes (A) and ear skin (C). (B, D) ICOS and CD103 expression on
 7 CD4+FOXP3- conventional T cells and CD4+FOXP3+ Regulatory T cells from (B) draining
 8 lymph nodes and (D) ear skin. FSC-A, forward scatter; SSC-A, side scatter; CTRL, control.

1 6. Conclusions

2 Murine models of allergic and irritant skin inflammation have shed light into the
3 pathogenesis of human contact dermatitis. They have highlighted the role of innate and
4 adaptive immunity in allergic reactions to epicutaneously introduced allergens. More
5 importantly, they allowed an in-depth dissection of cellular and molecular mechanisms
6 involved in the development of the disease and will hopefully lead to new potential
7 therapeutic interventions for this common dermatologic disorder.

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◆ Summary

Regulatory T cells (Treg) represent a crucial CD4 T cells subset involved in maintenance of immune-tolerance. The aim of this thesis work was to better define mechanisms involved in governing Treg homeostasis and to investigate the eventual contribution of perturbation of Treg homeostasis in human disease. In the first part of this work we defined in the murine system the role played by interleukin-7 in governing Treg homeostasis. In the second part, we studied Treg homeostasis in the context of HIV infection.

◆ Résumé

Les cellules T régulatrices Foxp3+ (Treg) représentent une sous-population T CD4 cruciale pour le maintien de l'immuno-tolérance. L'objectif de ce travail de thèse était de mieux définir les mécanismes impliqués dans le contrôle de l'homéostasie Treg et d'évaluer l'éventuelle contribution des perturbations de l'homéostasie Treg en pathologie humaine. Dans la première partie de ce travail de thèse nous avons essayé de finalement définir dans le modèle murin le rôle joué par l'interleukine-7 dans le contrôle de l'homéostasie Treg. Dans la deuxième partie, nous avons étudié l'homéostasie Treg dans le contexte de l'infection par le VIH.

◆ Mots clés

Cellules T régulatrices, Regulatory T cells, Treg, FOXP3, CD25, CD127, interleukin-7, IL-7, HIV, AIDS

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