PD-L1 blockade: Impact on regulatory T cells from HIV-infected individuals

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TESI DOCTORAL UPF / 2015

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A la meva mare,

ACKNOWLEDGEMENTS

Em semblava impossible fa dos mesos, però per fi s'acosta el moment de dipositar la tesi, i ha arribat l'hora d'escriure els agraïments.

I want to start thanking my supervisor Andreas for giving me the opportunity to work in his lab. Thank you for giving me freedom to work, for your trust in me, and for your enthusiastic support.

En segon lloc vull agrair al Jordi la seva paciència i confiança. Gràcies per ensenyar-me i guiar-me al llarg d'aquests anys. Gràcies per donar-me suport moral i encoratjar-me per a seguir lluitant (sé que no ha estat fàcil); i tenies raó, al final tot ha sortit bé!

També vull donar les gràcies a la resta dels meus companys de laboratori, presents i passats, al Javier, la Sandra, la Isi, l'Ana i la Mireia; especialment a l'Eric i la Irene, vosaltres heu fet aquesta experiència molt més agradable. Gràcies per les bones estones i per aguantar el meu mal humor :)

También quiero agradecer a todas aquellas personas fuera del Infection Biology lab, que han contribuido a mi formación científica, ya sea porque me han enseñado un protocolo, como usar un aparato, como escribir un artículo o como hacer una presentación oral.

A Carmen, Hernando y Alfred por haberme acogido y enseñado en mi periodo de formación anterior a la tesis. A Valerie, Núria, Lorena, Maria, Luis, Mireia, Rui y Pep.

A Tom Banks, Clay, Dustin, Genna Bocharov, Jordi G.Ojalvo, Elena, Bernat y Grace por la aventura matemática.

A Robert, Berta, Felipe, Montse, Carmen, Pilar y Hernando por su colaboración y implicación en esta tesis.

A Elinor Thompson y Carolyn Newey.

A los miembros del Juana lab (y a Juana), por el feedback en los seminarios, por los buenos ratos y por dejarnos siempre material ;) Gràcies Gemma, que sempre ho saps tot i ajudes a tothom amb un somriure. Gràcies Bernat, Marc, Vero, Georgios, Jenni i Alba.

A l'Eva, l'Erika, l'Òscar i la Cris Xufré, pel suport tècnic i per haver fet més entretingudes les hores davant del citòmetre.

I per acabar, vull donar les gràcies a les persones que fora de l'àmbit científic m'han acompanyat en aquesta etapa de la meva vida.

Vull agrair a la meva família (biològica i política) els missatges de recolzament.

Vull agrair al meus amics els sopars, els cafès i les cerveses. A la Irene, la Cèlia, la Roussie, el Carlos, l'Ares, el Guifré, el Mendoza i el Javi; i en especial al Pau, a l'Ada i la Roser, per la vostra fantàstica companyia.

A l'Alba, pels bons moments i per donar-me consell científic. Saps que per a mi sempre has estat un exemple i una guia a seguir.

A la Berta, per acompanyar-me en aquesta aventura i en les que vindran. Gràcies pels teus ànims, per confiar en mi més que ningú, pel nostre dia-dia virtual.

A ti papa, por estar a mi lado, apoyarme y preocuparte siempre por mi.

A ti mama, porqué sin ti no hubiese depositado esta tesis. Gracias por ayudarme a relativizar, por transmitirme serenidad y por estar siempre a mi lado.

I per últim a tu, Arnau, gràcies per la teva paciència, per guiar-me, per ajudar-me; per estar cada dia al meu costat.

Cris

Barcelona, 12 de setembre del 2015

SUMMARY

Blocking the PD-1/PD-L1 pathway has emerged as a potential therapy to restore impaired immune responses in human immunodeficiency virus (HIV)-infected individuals. Most reports have studied the impact of the PD-L1 blockade on effector cells and neglected possible effects on regulatory T cells (Treg cells), which play an essential role in balancing immunopathology and antiviral effector responses. In this thesis I investigated PD-1 and PD-L1 expression on Treg cells and the impact of *ex vivo* PD-L1 blockade on Treg cells from HIV-infected individuals. We observed that HIV infection led to an increase in PD-1+ and PD-L1+ Treg cells. This upregulation correlated with disease progression and decreased under antiretroviral treatment. In comparison with Treg cells from individuals under antiretroviral treatment, Treg cells from viremic individuals had a particularly high PD-1 expression and impaired proliferative capacity. PD-L1 blockade restored the proliferative capacity of Treg cells from viremic individuals but did not change their suppressive capacity. Moreover, it increased viral production in cell cultures from viremic individuals. This increase in viral production correlated with an augmented percentage of Treg cells and reduced CD4/Treg and CD8/Treg cell ratios. In contrast, we did not observe a significant effect on the proliferative capacity of Treg cells from individuals in whom viremia was controlled (either spontaneously or by antiretroviral treatment). However, PD-L1 blockade did increase the proliferative capacity of HIV-specific-CD8 T cells in all HIV-study groups. Taken together, our findings suggest that manipulating PD-L1 *in vivo* can be expected to influence the net gain of effector function depending on the subject's plasma viremia. PD-L1 blockade might skew the effector to regulatory T cell ratio in favour of effector cells only in patients in whom viremia is controlled. In patients with uncontrolled viremia, PD-L1 blockade might not favour effector-T cells over regulatory-T cells, and rather boost virus reactivation. In conclusion, this thesis supports the rationale to combine a PD-L1 blockade with antiretroviral treatment to restore effector responses in HIV-infected individuals.

RESUM

La infecció amb el virus de la immunodeficiència humana (VIH) causa el deteriorament progressiu de la resposta immune efectora. Una estratègia prometedora per a restaurar i potenciar la resposta immune efectora es basa en bloquejar la via de senyalització PD-1/PD-L1. Malgrat l'extensa recerca duta a terme en aquest camp, actualment es desconeixen els efectes que aquesta immunoteràpia pot tenir en les cèl·lules T reguladores (Treg). Les cèl·lules T reguladores tenen un paper fonamental en el control de les cèl·lules efectores per tal d'evitar immunopatologia. Per tant, possibles canvis en la funció de les cèl·lules Treg tindrien un efecte directe en la funció efectora. En aquesta tesi he investigat l'expressió de les molècules PD-1 i PD-L1 així com l'impacte de bloquejar PD-L1 sobre les cèl·lules Treg de pacients infectats amb el VIH. Hem observat que la infecció pel VIH indueix l'augment de cèl·lules Treg que expressen PD-1 i PD-L1. Aquest augment correlaciona amb la progressió de la malaltia i disminueix amb el tractament antiretroviral. En comparació amb les cèl·lules Treg de persones sota tractament antiretroviral, les cèl·lules Treg d'individus virèmics presenten percentatges d'expressió de PD-1 particularment elevats així com deficiències en la capacitat de proliferar. Bloquejar PD-L1 en cultius de cèl·lules mononuclears de sang perifèrica d'individus virèmics ha restaurat la capacitat de proliferar de les cèl·lules Treg sense alterar la seva capacitat supressora. D'altra banda, bloquejar PD-L1 ha causat un increment en la reactivació de virus. Hem observat que l'increment en la producció de virus en cultiu correlaciona amb un augment en el percentatge de cèl·lules Treg i una reducció de les proporcions de cèl·lules T CD4/Treg i cèl·lules T CD8/Treg. Al contrari, bloquejar PD-L1 en cultius de cèl·lules d'individus infectats amb el VIH que són capaços de controlar la virèmia (ja sigui de forma espontània o degut al tractament antiretroviral) no ha augmentat significativament la proliferació de les cèl·lules Treg. No obstant això, bloquejar PD-L1 sí que ha augmentat la capacitat de proliferar de les cèl·lules T CD8 específiques per al VIH en tots els grups de pacients estudiats. En conjunt, bloquejar PD-L1 augmenta diferencialment la capacitat de proliferar de les cèl·lules T reguladores i T efectores depenent de la virèmia de l'individu infectat amb el VIH, i per tant pot influir en el guany net de la funció efectora. Els nostres resultats suggereixen que bloquejar PD-L1 pot afavorir les cèl·lules efectores sobre les reguladores en pacients amb la virèmia sota control, mentre que en pacients virèmics, bloquejar PD-L1 podria no potenciar la resposta efectora i, a més, impulsar la reactivació de virus. En conclusió, els resultats d'aquesta tesi donen suport a la lògica establerta de combinar la immunoteràpia dirigida a PD-L1 amb tractament antiretroviral per tal de restaurar les respostes efectores en els pacients infectats amb el VIH.

PROLOGUE

Human immunodeficiency virus (HIV) infection causes a progressive impairment of effector immune responses contributing to virus persistence. The restoration of these responses is essential to achieve a drug-free control over HIV. One strategy that could restore effector immune responses is the relief of the inhibitory signal displayed by the PD-1/PD-L1 pathway on effector cells. Several studies showed that PD-L1 blockade restores impaired effector CD4- and CD8- T cell responses in HIV-infection. In addition, it has been described that the PD-1/PD-L1 pathway also plays a role in the biology of regulatory T cells (Treg cells).

Treg cells are a suppressive T cell subset that maintains self-tolerance and immune homeostasis. During HIV-infection, Treg cells have both, beneficial and detrimental roles. Treg cells control excessive immune activation that limits immunopathology and the availability of HIV target cells. On the contrary, Treg cells contribute to the destruction of the lymphatic tissue architecture, and inhibit HIV-specific immune responses promoting virus persistence. Thus, alteration of Treg cells by immunotherapy may directly influence the balance between immunopathology and viral control.

As for conventional CD4- and CD8- T cells, PD-1 was found up-regulated on Treg cells from HIV-infected individuals compared with healthy controls. Nonetheless, as most reports have focused on effector cells, possible effects from PD-L1 blockade on Treg cells have been neglected. In this thesis I have investigated PD-1 and PD-L1 expression and the impact of *ex vivo* PD-L1 blockade on regulatory T cells from HIV-infected individuals. The presented results provide new insights into the biology of Treg cells in HIV infection as well as unexplored PD-L1 blockade effects on the HIV immune response.

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INTRODUCTION

1. An introduction to HIV infection

The human immunodeficiency virus (HIV) is a lentivirus that was discovered in 1983 as the cause of the acquired immune deficiency syndrome (AIDS) (Barre-Sinoussi et al. 1983; Gallo et al. 1984; Popovic et al. 1984). After 30 years, HIV still represents a great medical and social worldwide challenge. The World Health Organization has estimated that since the beginning of the epidemic, almost 39 million people have died from HIV infection. In 2014, there were 37 million people living with HIV, with the highest prevalence in developing countries (UNAIDS/WHO, 2014, www.unaids.org).

In developed countries where there is access to therapy, current antiretroviral treatment considerably reduced HIV-transmission, AIDS-related morbidity and AIDS-related mortality (Bertozzi et al. 2005). However, the virus can not be eradicated and interruption of treatment rapidly leads to re-emergence of virus from latent reservoirs (Finzi et al. 1999; Mylvaganam et al. 2015). This implies a need of a life long therapy that is associated with side effects. These limitations, together with other factors such as need of treatment adherence, viral escape and high cost highlight the need of new therapeutic approaches to treat HIV infection. The ability to accomplish such a challenging goal will be facilitated by the study of the immune response that leads to infection and disease progression (Mylvaganam et al. 2015).

1.1. HIV infection course and immunopathogenesis

HIV is mainly transmitted via unprotected sex (Askew & Berer 2003; Hladik & McElrath 2008). Less frequently HIV is transmitted through contaminated blood and from mother to child during pregnancy, delivery or breastfeeding (Senturia et al. 1987; Bertozzi et al. 2005; Lindholm et al. 2011). In a sexually transmitted infection the first step is the transmission of cell-free or cell-associated virus particles across a mucosal barrier through microabrasions. Early propagation of the virus occurs in activated CD4 T cells, followed by dissemination to peripheral lymphoid tissue. The transmitted virus grows exponentially and causes a profound and irreversible depletion of CCR5+ CD4 T cells especially in the gut associated lymphoid tissue (GALT) (Lim et al. 1993; Pantaleo et al. 1993; Guadalupe et al. 2003; Brenchley et al. 2004; Mehandru et al. 2004). Depletion of CCR5+ CD4 T cells in the GALT results in microbial translocation that cause hyperactivation of the immune system and overall immunological dysfunction (Brenchley et al. 2006; Moir et al. 2011).

The **acute phase** of the infection is characterized by high circulating levels of virus (Figure I1). During the 3rd and 4th week, typically plasma viremia peaks, reaching levels of 10^7 vRNA copies/mL. Then, the immune response partially controls the infection reducing plasma viremia for several months until it reaches a particular set point (Moir et al. 2011). This set point is highly variable and is a good predictor for disease progression together with CD4 T cell counts (Mellors et al. 1996; McMichael et al. 2010).

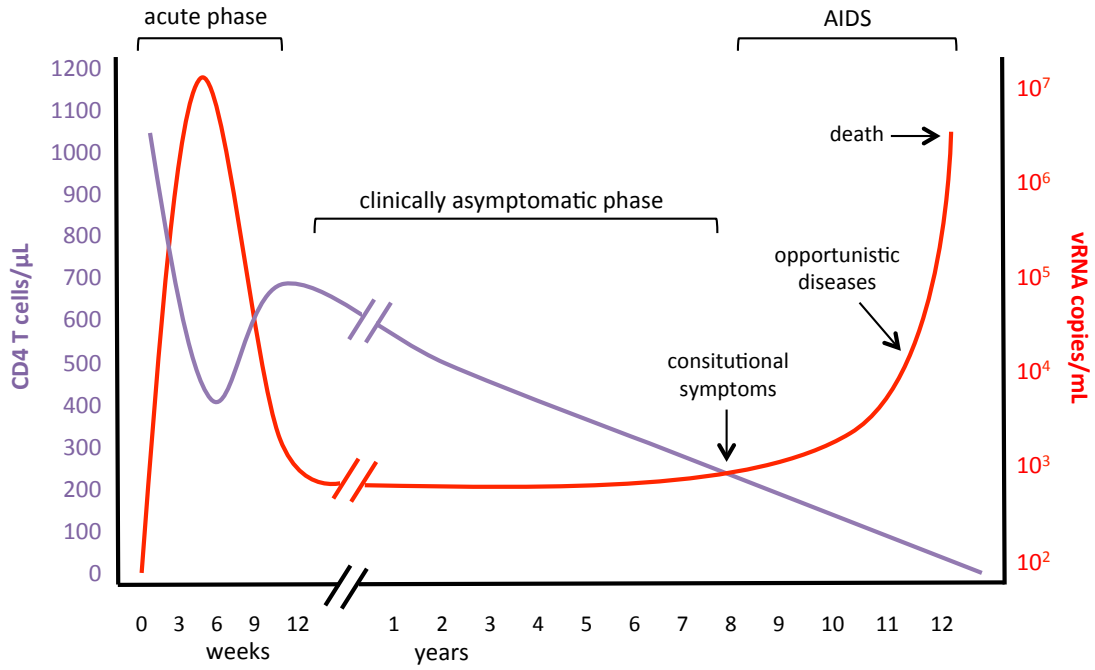

Figure I1. Graphic representation of HIV infection course. Representation of HIV disease progression on the basis of CD4 T cell count/mL and viral RNA copies/µL of blood over time. Adapted from Pantaleo et al. 1993 and Wikipedia (www.wikipedia.org/wiki/HIV).

The acute phase of HIV infection is followed by a chronic phase, which is first a clinically **asymptomatic phase.** This phase is characterized by controlled levels of circulating virus and progressive CD4 T cell decline while immune activation progressively increases. In the absence of antiretroviral treatment (ART), the asymptomatic phase typically lasts around 8 years (Pantaleo & Fauci 1996). However, the rate of disease progression is very variable and depends on virus and host factors.

The asymptomatic phase of HIV infection is followed by a symptomatic phase, named the acquired immune deficiency syndrome (AIDS). **AIDS** is characterized by fewer than 200 CD4 T cells/µL of blood and increased viral load (http://www.cdc.gov/hiv/statistics/surveillance/terms.html). In this phase, the immune system is compromised and this is manifested by poor immune responses against HIV and other infections. Opportunistic infections and cancers characterize the AIDS phase. The most common

are (1) bacterial infections e.g with *Mycobacterium tuberculosis* and *Mycobacterium avium*; (2) protozoan infections e.g. with *Strongyloides, Cryptosporidium, Toxoplasma, Candida* and *Cryptococcus*; (3) virus infections e.g. with cytomegalovirus, herpes zoster virus and herpes simplex virus; and (4) cancers e.g. Kaposi sarcoma and non-Hodgkin lymphoma (Bertozzi et al. 2005). AIDS eventually causes the death of the HIV-infected individual.

1.2. Immunological abnormalities and immune activation during HIV infection

The depletion of CD4 T cells is a hallmark of HIV infection. However, other leukocyte subsets are also affected, for instance CD8 T cells, B cells, natural killer (NK) cells, macrophages and dendritic cells (DC). Abnormalities include increased cell turnover, apoptosis, immune senescence and overall altered functionality. Among CD8 T cells, for example, there is a preferential loss of naïve and central memory cell subsets, with an increased short-lived effector cell subset. Among B cells, there are abnormal increased fractions of immature transitional B cells, activated B cells and plasmablasts. NK cells present defects in cytokine production, cytotoxicity and homing properties. Macrophages, for instance, show reduced phagocytosis, and among DC there are abnormal levels of plasmocytoid and myeloid DC, as well as loss of mucosal DC (Klatt et al. 2013).

Several of these immunological abnormalities including the progressive CD4 T cell depletion occur due to (1) direct virus infection, (2) chronic immune activation and (3) altered homeostasis caused by changes in lymphoid tissue architecture (Haase 1999; Brenchley et al. 2004; Klatt et al. 2013; Phetsouphanh et al. 2015). The **direct virus infection** causes death by several mechanisms. These include direct killing of infected cells via CTL responses, death due to HIV budding or induction of pro-apoptotic proteins together with repression of anti-apoptotic proteins (Westendorp et al. 1995; Xu et al. 1997; Appay et al. 2002). In addition, death occurs in uninfected cells as a consequence of the contact with infected cells (Perfettini et al. 2005) or due to chronic immune activation (Février et al. 2011). **Immune activation** is associated with disease progression, morbidity and mortality (Hunt 2012). It has several causes: (1) viral stimulation of HIV-specific T cells and innate cells through TLR7/8; (2) reactivation of other viruses that are usually controlled such as Epstein-Barr virus or cytomegalovirus; (3) secretion of inflammatory cytokines such as IFNα, TNFα, IL-1, IL-6, and IL-18; and (4) loss of gut epithelial integrity leading to microbial translocation. The latter is considered one of the major factors leading to immune activation (Klatt et al. 2013). Another factor promoting immunological abnormalities is the **disruption of the**

lymphoid tissue architecture. This alteration is caused by collagen deposition, it impairs immune responses and leads to a decrease in the supply of thymic emigrants, limiting the restoration of CD4 T cells (Schacker et al. 2006).

1.3. Effects of viral and host factors in HIV infection

The rate of disease progression is very variable among HIV-infected individuals in the absence of ART. The majority of HIV-infected individuals develop AIDS after approximately 8 years (Figure I1). This is associated with viral replication, chronic immune activation and severe decline of CD4 T cells. However, in around 5-10% of HIV-infected individuals progression to AIDS occurs rapidly in only 2-3 years after infection (Pantaleo & Fauci 1996) (Figure I2). Contrarily, it is estimated that another 5-15% of HIV-infected individuals, named slow progressors or **long-term non progressors**, are able to control virus replication and maintain CD4 T cells counts for more than 10 years. Within this group, **elite controllers** are able to maintain viral loads below the limit of detection and remain healthy for periods of over 35 years without progressing to AIDS (reviewed in Deeks & Walker 2007; Walker & Yu 2013). The study of those individuals who maintain low viral replication has helped to gain insight into the effects of host and viral factors involved in HIV disease progression.

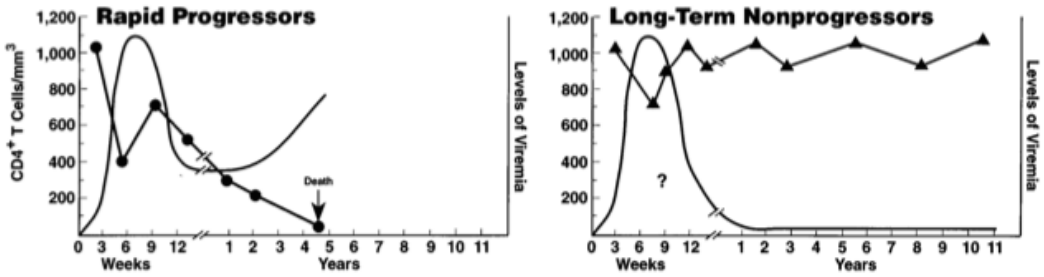

Figure I2. Graphic representation of HIV disease progression. Representation of HIV disease progression in a rapid progressor (left) and a long-term non progressor (right) on the basis of CD4 T cell counts and levels of viremia. Modified from Pantaleo et al. 1996.

Several viral and host factors are thought to influence HIV disease progression. Among the **viral factors**, there are viral replicative properties and the ability to escape immune responses through genetic variation. Among the host factors, there are genetic and immunological factors. Examples of **host genetic factors** are: (1) polymorphisms in the HLA class I locus in particular HLA-B and HLA-C; (2) polymorphisms in CCR5 and CCR2; and (3) polymorphisms in other HLA class I-binding molecules such as killer cell immunoglobulin-like receptors (KIR) and leukocyte immunoglobulin-like receptors (LILR). Examples of **host immunological factors** are: (1) quality of HIV-specific CD4- and CD8- T cell responses; (2) neutralizing antibodies and antibodies that facilitate cell-

mediated killing (ADCC); and (3) unique properties of the innate immune system such as antigen presenting properties of myeloid DC or maintained γδ T cells that express Vγ2Vδ2 TCR (reviewed in Moir et al. 2011; Walker & Yu 2013).

1.3.1. The quality of the T cell response

Strong and effective HIV-specific CD4 and mainly CD8 T cell responses are considered one of the main factors contributing to viral control. It has been reported that the immune response from elite controllers and non-controller individuals differ in the quality rather than the number of virus-specific immune cells. Several quality measures have been associated with protection against disease progression. But it remains unknown whether some of these quality parameters are a cause or a consequence of HIV control. For CD8 T cells quality parameters are (1) their proliferative capacity (McKinnon et al. 2011), (2) their degree of cytotoxicity (Migueles et al. 2008), (3) their avidity i.e. antigen sensitivity (Almeida et al. 2007), (4) their breath of recognition (Geldmacher et al. 2007), (5) their susceptibility to regulatory T cell inhibition (Elahi et al. 2011) and (6) their polyfunctionality, i.e. the ability to simultaneously execute several functions such as production of IFNγ, TNFα, IL-2, CD107a and MIP1b (Kannanganat et al. 2007; Betts et al. 2006). For CD4 T cells quality parameters are (1) their avidity (Vingert et al. 2010), (2) their ability to support CD8 T cell activity by secretion of IL-2 and IL-21 (Lichterfeld et al. 2004; Tilton et al. 2007); and (3) their ability to support B cell production of antibodies (Cubas et al. 2013).

2. Exhaustion

2.1 Introduction to Exhaustion

Exhaustion is a state of dysfunction that occurs due to antigen persistence (reviewed in Wherry 2011; Pauken & Wherry 2015). It is a common feature of several persistent infections and cancers; and mainly studied in CD8 T cells in chronic viral infections. Exhaustion is associated with an altered metabolism and a unique transcriptional program compared with functional effector T cells and memory T cells. However, no master transcription factor of the exhausted transcriptional program has been identified to date (Wherry et al. 2007; Utzschneider et al. 2013; Crawford et al. 2014).

During exhaustion, CD8 T cell dysfunction develops in a progressive manner (Figure I3). Some functions are lost in an early stage of exhaustion, for instance a high proliferative

capacity and IL-2 production. Other functions are lost in a more advanced stage of exhaustion, such as cytotoxicity and TNFα and IFNγ production (Virgin et al. 2009; Wherry 2011). During exhaustion, T cells also lose their ability to proliferate in response to IL-7 and IL-15, thus depending on TCR signals for their maintenance (Shin et al. 2007). This process ultimately ends with the apoptosis of the exhausted cell (Wherry 2011; Kahan et al. 2015).

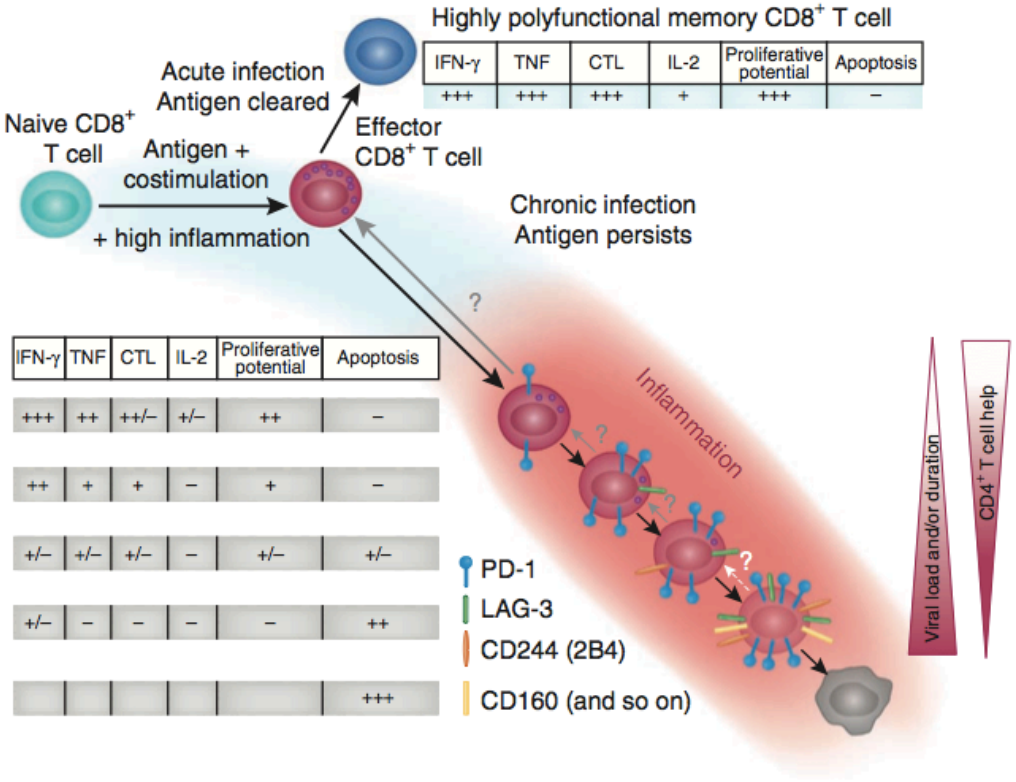

Figure I3. T cell exhaustion. During infection, naïve T cells are primed and differentiate into effector T cells. Following the clearance of antigen, most effector T cells die but a pool of cells differentiates into memory T cells (up). When re-stimulated, memory T cells can proliferate, produce several cytokines and become cytotoxic. In addition, they can be maintained in an antigen-independent way by IL-7 and IL-15. In contrast, when antigen is not cleared (down), this differentiation process is altered and T cells become exhausted. The severity of exhaustion is associated with inflammation, antigen exposure and CD4 T cell help. The capacity to exert a particular function is presented on a scale from high (+++) to low (-). From Wherry et al., 2011

During chronic infections, exhaustion has been described as a causative factor for the lack of pathogen control. However, exhausted T cells still contribute to the containment of chronic infections (Kahan et al. 2015). Therefore, exhaustion might be an adaptation to reduce the sensitivity of virus-specific T cells to antigen and promote its survival in a persistence environment. By these means, the immune response could balance effector control of the virus and immunopathology, while at the same time maintaining the adaptability of T cell responses to subsequent viral bursts (Radziewicz et al. 2009; Barnaba & Schinzari 2013; Pauken & Wherry 2015).

2.2. Intrinsic and extrinsic regulatory pathways of exhaustion

Exhaustion is associated with prolonged and high expression of **inhibitory receptors**. The number and type of inhibitory receptors determine the degree of dysfunction. The pattern of inhibitory receptors differs between CD4- and CD8- T cells, as well as the type of infection. Inhibitory receptors involved in exhaustion include PD-1 (which plays a major role), Lag-3, Tim3, CD244 (2B4), CD160, TIGIT, CTLA4, BTLA, KLRG1 and others. However, none of these receptors are exclusive markers for exhausted T cells, because activated T cells can transiently express them (Crawford & Wherry 2009; Blackburn et al. 2009; Nguyen & Ohashi 2015).

In addition to inhibitory receptors, multiple extrinsic factors influence T cell exhaustion, such as altered antigen presentation, immunosuppressive cytokines and several cell subsets (reviewed in Wherry 2011; Jin et al. 2011; Kahan et al. 2015). Effector responses are influenced by **deletion or alteration of dendritic cells**, such as decrease in MHC molecules and co-stimulatory ligands, and increase in co-inhibitory ligands (Jin et al. 2011). Likewise, effector responses are influenced by suppressive cytokines such as **TGFβ** and **IL-10**; and inflammatory cytokines such as type I interferons (**IFNs**). For example, in LCMV (lymphochoriomeningitis virus) mouse model the blockade of TGFβ (Tinoco et al. 2009) or IFN α/β during the first days of infection (Teijaro et al. 2013) prevents from severe exhaustion, and the blockade of IL-10 improves T cell functionality and promotes viral control (Brooks et al. 2006; Richter et al. 2013). In the case of HIV-infected individuals, as a result of PD-1 triggering, monocytes produce IL-10, which has been described as a major factor influencing effector impairment (Said et al. 2010). In addition, **depletion of CD4 T cells** is another important factor influencing exhaustion. CD4 T cells provide help to CD8 T cells and are major producers of IL-21, which influences CD8 T cell and B cell differentiation and restricts regulatory T cell expansion (Matloubian et al. 1994; Zajac et al. 1998; Lichterfeld et al. 2004; Elsaesser et al. 2009; Fröhlich et al. 2009; Yi et al. 2009; Konforte et al. 2009; Aubert et al. 2011; Schmitz et al. 2013). On the contrary, **NK cells** and immunoregulatory cells such as **myeloid-derived suppressor cells** and **regulatory T cells** have a detrimental impact on virus-specific CD8 T cells further contributing to exhaustion (Dittmer et al. 2004; Dietze et al. 2011; Waggoner et al. 2012; Schmitz et al. 2013; Norris et al. 2013; Waggoner et al. 2014; Penaloza-MacMaster et al. 2014).

2.3. Reversibility of the exhausted phenotype

The exhausted phenotype of lymphocytes can be partially reversed. This was first shown in the LCMV model, where the blockade of the PD-1/PD-L1 pathway showed a

restoration of impaired effector T cells and a reduction of viral loads (Barber et al. 2006). This observation was quickly extended (1) to important human diseases such as HIV- and hepatitis C virus (HCV)- infections *in vitro* (Day et al. 2006; Trautmann et al. 2006; Urbani et al. 2006); (2) *in vivo* to distinct infection models such as simian immunodeficiency virus (SIV) in macaques, HCV in chimpanzees and HIV, hepatitis B virus (HBV) or *Plasmodium yoelii* in mice (Velu et al. 2009; Shetty et al. 2012; Palmer et al. 2013; Seung et al. 2013; Fuller et al. 2013; Maier et al. 2007; Butler et al. 2011); and (3) to clinical trials in the case of HCV infection and several cancers (Yao et al. 2013; Nguyen & Ohashi 2015). Collectively, these results indicate that the PD-1/PD-L1 pathway plays a major role in exhaustion and represents a promising therapeutic target.

Restoration of exhausted T cells by the blockade of PD-1/PD-L1 pathway is heterogeneous. In the LCMV model, it has been reported that exhausted CD8 T cells with intermediate levels of PD-1 expression can be reinvigorated, whereas CD8 T cells with high levels of PD-1 cannot (Blackburn et al. 2008). This heterogeneity can be partly explained by the expression of multiple co-inhibitory receptors. Several studies showed that the simultaneous blockade of multiple co-inhibitory receptors increases considerably the restoration of impaired effector responses. Some of these combinations are PD-1 and Lag3, PD-1 and CTLA4, PD-1 and Tim 3 or PD-1 and 2B4. (Blackburn et al. 2009; Nakamoto et al. 2009; Kaufmann et al. 2007; Jin et al. 2010; Yamamoto et al. 2011).

2.4. Introduction to the PD-1/PD-L1 pathway and T cell dysfunction

2.4.1. PD-1 and PD-Ls expression

Programmed cell death-1 (**PD-1**, CD279) (Ishida et al. 1992) is an inhibitory receptor of the B7:28 family that regulates the threshold of immune responses. It has a role in peripheral tolerance and autoimmunity (Nishimura et al. 1999; Nishimura et al. 2001; Keir et al. 2006), as well as in cancer and infectious diseases, where it regulates the balance between effector immune responses and tissue damage (Iwai et al. 2002; Barber et al. 2006; Okazaki et al. 2013; Pauken & Wherry 2015). PD-1 is expressed on activated T cells, B cells, natural killer cells and natural killer T cells, as well as on monocytes, macrophages and dendritic cells (Agata et al. 1996; Nishimura et al. 1996; Yamazaki et al. 2002; Keir et al. 2006; Moll et al. 2009; Keir et al. 2008). PD-1 binds to two ligands: PD-L1 (B7-H1 or CD274) (Dong et al. 1999; Freeman et al. 2000) and PD-L2 (B7-DC or CD273) (Latchman et al. 2001). Both PD-L1 and PD-L2 interact with additional receptors: PD-L1 with CD80 (B7-1) (Butte et al. 2007), which in turn interacts

with CD28 and CTLA4; and PD-L2 with RGMb (repulsive guidance molecule family member b) (Xiao et al. 2014).

The PD-1 ligands have distinct patterns of expression. **PD-L1** is constitutively expressed on T cells, B cells, DC and macrophages and it is upregulated upon activation. It is also expressed on a wide range of non-haematopoietic cells such as endothelial, epithelial or muscle cells, hepatocytes, and astrocytes in the brain, as well as in immunologically privileged sites, such as placenta and eyes (reviewed in Sharpe et al. 2007). PD-L1 is also highly expressed on tumour cells and virus-infected cells. PD-L1 can be upregulated in response to several stimuli such as IFNγ, IL-2, IL-7, IL-15, IL-21, type I IFNs and hypoxia (Eppihimer et al. 2002; Schreiner et al. 2004; Kinter et al. 2008; Noman et al. 2014). **PD-L2** has a more restricted expression than PD-L1. PD-L2 is expressed on some B cell subsets and it can be inducibly expressed in DC, monocytes and macrophages. PD-L2 can be upregulated in response to several stimuli such as IFNα, IFNβ, IFNγ, IL-13, IL-4 and granuolcyte macrophage colony-stimulating factor (GM-CSF) (Liang et al. 2003; Yamazaki et al. 2002; Loke & Allison 2003; Zhong et al. 2007; Keir et al. 2008). Thus the expression of PD-1 ligands is broad and very sensitive to the context varying in response to inflammation.

2.4.2. PD-1 regulation

Recent reports identified mechanisms controlling PD-1 expression involving NFAT, FOXO1, T-bet, Blimp-1 and Eomes. Following TCR engagement, NFAT translocates to the nucleus and induces PD-1 expression (Oestreich et al. 2008). Moreover, TCR engagement promotes demethylation of the PD-1 promoter *Pdcd1,* contributing to its overexpression. When antigen stimulation is prolonged, *Pdcd1* promoter fails to be remethylated even when levels of antigen decrease (Youngblood et al. 2011). Additionally, TCR engagement dowregulates the expression of T-bet, which is a negative regulator of PD-1 (Kao et al. 2011). It has also been described that activation of AKT and mTOR are impaired during exhaustion and this impairment results in increased activity of FoxO1 that promotes PD-1 activation (Staron et al. 2014). Furthermore, other transcription factors like Blimp-1 and Eomes are also associated with high PD-1 expression (Shin et al. 2009; Paley et al. 2012). Blimp-1 has been described to regulate PD-1 through epigenetic mechanisms while the mechanism of PD-1 regulation by Eomes remains to be determined (Pauken & Wherry 2015).

2.4.3. PD-1 signalling and mechanisms of inhibition

PD-1 is a protein composed of an N-terminal immunoglobulin domain, a transmembrane domain and a C-terminal intracellular domain containing both an ITIM

(immunoreceptor tyrosine-based inhibitory motif) and an ITSM (immunoreceptor tyrosine-based switch motif). PD-1 exists as a monomer on the surface and it functions by dampening TCR signalling, which can be rescued by CD28 co-stimulation and IL-2 (Freeman et al. 2000; Carter et al. 2002). PD-1 inhibits TCR signalling mainly by recruiting phosphatases (SHP-2 and to a lesser extent SHP-1). This prevents LCK-mediated phosphorylation and activation of Zap70 in T cells (Chemnitz et al. 2004; Yokosuka et al. 2012; Sheppard et al. 2004).

Other mechanisms for PD-1-mediated inhibition have been described. First, PD-1 impacts survival, proliferation and metabolism through the PI3K/AKT/mTOR pathway and the Ras pathway (Staron et al. 2014; Parry et al. 2005; Patsoukis et al. 2012). PD-1 also represses effector genes by inducing the expression of BATF (Quigley et al. 2010) and it also mediates motility paralysis in T cell-antigen presenting cell (APC) interactions (Fife et al. 2009; Zinselmeyer et al. 2013). Finally, the binding of PD-1 to PD-L1 can induce reverse signalling of inhibitory nature on the PD-L1-expressing cells (Hirano et al. 2005; Kuipers et al. 2006; Azuma et al. 2008).

2.5. PD-1/PD-L1 pathway in HIV infection

2.5.1. PD-1/PD-L1 in HIV-specific CD4- and CD8- T cells

In HIV infection, PD-1 is upregulated on HIV-specific CD4- and CD8- T cells, as well as on total CD4- and CD8- T cells. Both the level of PD-1 expression and the percentage of PD-1-expressing cells correlate with parameters of disease progression: negatively with CD4 T cell counts and positively with viral load (Day et al. 2006; Trautmann et al. 2006; Souza et al. 2007). Consistently, the percentage of PD-1-expressing cells is lower in long-term non-progressors than in typical progressors (Zhang et al. 2007) and control of viremia by ART reduces its expression (Day et al. 2006; Trautmann et al. 2006; Souza et al. 2007). PD-1 expression is also reduced on CTL specific for epitopes that have undergone mutational escape (Streeck et al. 2008). Altogether this indicates that antigen-specific TCR stimulation is determinant for PD-1 expression. Nevertheless, other mechanisms also contribute to up-regulation of PD-1, including HIV proteins (Muthumani et al. 2008) and inflammatory cytokines (Keir et al. 2008; Kinter et al. 2008).

PD-1 expression is associated with an impaired proliferative capacity and cytokine production in HIV-specific T cells (Day et al. 2006; Trautmann et al. 2006). In addition, PD-1 expression is associated with a higher susceptibility to apoptosis (Petrovas et al. 2006) and a low telomerase activity (Lichterfeld et al. 2008). The relief of PD-1

inhibition by *ex vivo* blockade of PD-L1 results in increased effector function; including increased proliferation, IFNγ, TNFα, granzyme B and lymphotoxin A production in CD8 T cells (Day et al. 2006; Trautmann et al. 2006; Zhang et al. 2007); as well as increased proliferation, IFNγ, IL-2, IL13 and IL-21 production in CD4 T cells from HIV-infected individuals (Souza et al. 2007; Porichis et al. 2011). Collectively these data indicate that the level of PD-1 expression and the percentage of PD-1-expressing cells are related with the degree of T cell exhaustion in HIV infection.

As PD-1, PD-L1 is also significantly upregulated in several cell subsets in HIV infection. These include DC, macrophages, B cells, T cells and neutrophils (Trabattoni et al. 2003; Rosignoli et al. 2007; Sachdeva et al. 2010; Bowers et al. 2014). Some reports show that PD-L1 expression correlates positively with viral load and negatively with CD4 T cell counts (Trabattoni et al. 2003). Likewise, a reduction in PD-L1 expression in some cell subsets from individuals under ART has been reported (Trabattoni et al. 2003; Bowers et al. 2014). However, whether there are differences in PD-L1 expression on PBMC among different groups of HIV-infected individuals is controversial. PD-L1 is upregulated upon *in vitro* HIV exposure or infection in monocytes, DC, CCR5+ T cells and neutrophils (Meier et al. 2008; Boasso et al. 2008; Rodríguez-García et al. 2011; Planès et al. 2014; Bowers et al. 2014). Several of these reports (Boasso et al. 2008; Rodríguez-García et al. 2011; Bowers et al. 2014) did not find differences in PD-L1 upregulation when using competent or inactivated virus, which suggests that HIV exposure is sufficient for PD-L1 upregulation. Distinct mechanisms for this upregulation have been identified, including signalling through TLR7/8 (Meier et al. 2008), IFNγ-dependent mechanisms (Boasso et al. 2008) and a direct effect of the HIV-Tat protein (Planès et al. 2014). In addition, IFNα and LPS, which are associated with immune activation in HIV infection, can also induce PD-L1 (Boasso et al. 2008; Bowers et al. 2014). Taken together, these data suggest that during HIV infection both the virus itself and the pro-inflammatory milieu induce PD-L1 in several cell subsets and further contribute to the suppression of PD-1-expressing T cells.

2.5.2. PD-1/PD-L1 in HIV infection: Beyond effector T cells

Besides HIV-specific T cells, the PD-1/PD-L1 pathway might also play a role in other cell subsets during HIV infection. **B cells** from HIV-infected individuals express a higher percentage of PD-1 compared with healthy controls, which has been linked to an exhausted B cell phenotype (Nicholas et al. 2013; Kardava et al. 2011). In SIV-infected macaques, PD-1 blockade enhances humoral immune responses and promotes B cell survival by decreasing Fas-mediated apoptosis (Velu et al. 2015). In addition, Cubas and co-authors showed that PD-1 engagement on **T follicular helper cells** (Tfh) causes B cell impairment in HIV infection (Cubas et al. 2013). PD-1 trigger on Tfh cells reduced

Tfh activation, proliferation and IL21 production (needed for B cell survival, proliferation and plasma cell differentiation). As a consequence, Tfh cells failed to produce the adequate help to germinal center B cells. In addition, this study showed that *in vitro* PD-L1 blockade enhances HIV-specific immunoglobulin production, suggesting that PD-L1 blockade may enhance HIV-specific humoral immune responses (Cubas et al. 2013; Phetsouphanh et al. 2015).

As for conventional CD4 T cells, the percentage of PD-1-expressing **regulatory T cells** (Treg cells) is also high in HIV-infected individuals compared with healthy control individuals (Lim et al. 2009; Rueda et al. 2013; Cho et al. 2014). In individuals under successful ART, the percentage of PD-1-expressing CD4+CD127loFOXP3+ Treg cells decreases (Lim et al. 2009; Rueda et al. 2013). However, the percentage of PD-1+ Treg cells remains high in individuals that do not recover CD4 T cell counts after ART (Rueda et al. 2013). In mucosal samples, the percentage of PD-1-expressing Treg cells was even higher compared with peripheral blood, and was not normalized under ART (Rueda et al. 2013). These data suggest that PD-1/PD-L1 could have a role in Treg cells from HIV-infected individuals.

2.6. PD-1/PD-L1 pathway in regulatory T cells

Regulatory T cells (Treg cells) express both PD-1 and PD-L1 (Keir et al. 2008). Several roles of the PD-1/PD-L1 pathway have been described for this cell subset. First, the PD-1/PD-L1 pathway is essential in the **induction of Treg cells** in the periphery. The PD-L1 expressed in several cell types (i.e. antigen presenting cells, endothelial cells or tumor cells) promotes differentiation of PD-1+ CD4 T cells to Treg cells (Aramaki et al. 2004; Krupnick et al. 2005; Wang et al. 2008; Francisco et al. 2009; Amarnath et al. 2011; Periasamy et al. 2011; Trinath et al. 2012). Second, the PD-1/PD-L1 pathway plays a role in Treg cell **proliferative capacity**. Similarly to its role in conventional CD4 T cells, PD-1 has been described as a negative regulator of Treg cell proliferation. This regulation is mediated through the interference with STAT 5 phosphorylation (Franceschini et al. 2009). Third, it has been described that, as for conventional CD4 T cells (Fife et al. 2009; Honda et al. 2014; Zinselmeyer et al. 2013), PD-L1 mediates **motility paralysis** in Treg cells (Dilek et al. 2013). Finally, the PD-1/PD-L1 pathway seems to play a role in Treg cell **suppressive capacity,** although this relationship remains controversial. Some authors reported that engagement of PD-1 on Treg cells maintains FOXP3 expression and increases the suppressive capacity of Treg cells (Francisco et al. 2009; Zhou et al. 2010). In line with these data, PD-1 signalling maintains functional Treg cells and prolongs their survival (Wong et al. 2013). However, other authors reported that PD-1 trigger decreases Treg cell suppressive

capacity (Franceschini et al. 2009). In addition, it has been shown that Treg cells can inhibit its targets when (1) PD-L1 from the Treg cell binds to PD-1 from the target cell (Gotot et al. 2012) and when (2) PD-1 from the Treg cell binds to PD-L1 from the target cell (Park et al. 2015).

When PD-1/PD-L1 pathway is blocked by PD-L1 antibodies, opposite effects on Treg cells have been observed. Some authors show that PD-L1 blockade reduces the numbers and the suppressive capacity of Treg cells in different mouse models of cancer (Wang et al. 2008; Ni et al. 2011; Zhou et al. 2010; Curran et al. 2010). However, other authors show that *in vitro* PD-L1 blockade increases the proliferation and the suppressive capacity of Treg cells from HCV-infected individuals (Franceschini et al. 2009). Likewise, *in vivo* PD-L1 blockade increased Treg cell numbers in the friend virus mouse model (Joedicke et al. 2014). Whether these contradictory data result from differences in PD-1/PD-L1 role in Treg cells from cancers and infectious diseases it is not known. Nonetheless, these findings suggest that Treg cells may be influenced in either way by PD-1/PD-L1 blockade immunotherapy.

3. Regulatory T cells

Regulatory T cells (Treg cells) are a suppressive T cell subset mediating self-tolerance and immune homeostasis. They play a crucial role in human diseases such as autoimmunity, allergy, cancer and infection (reviewed in Sakaguchi et al. 2008; Oleinika et al. 2013; Belkaid & Tarbell 2009). Treg cells can originate from the thymus (tTreg, former natural Treg) or be induced in the periphery from conventional T cells under certain conditions (pTreg, former induced Treg) (reviewed in Dhamne et al. 2013). Treg cells were first identified as CD4+ T cells with constitutive high CD25 expression and with the capacity to prevent autoimmunity upon thymectomy of mice (Sakaguchi 1982; Sakaguchi 1985; Sakaguchi et al. 1995; Powrie 1990; Smith et al. 1991; Morrissey et al. 1993; Suri-Payer et al. 1998). Subsequently, FOXP3 (Forkhead box P3 or scurfin) was identified as a master regulator for Treg cell development and function (Fontenot et al. 2003; Hori et al. 2003; Khattri et al. 2003). FOXP3 deficiency was shown to cause lymphocyte proliferation and multiorgan autoimmunity in mice and the IPEX syndrome (immunodysregulation polyendocrinopathy and enteropathy X-linked) in human patients. Altogether, these discoveries confirmed the existence of an independent lineage of T cells with suppressive capacity (reviewed in Wing & Sakaguchi 2010; Benoist & Mathis 2012).

3.1. Treg cell diversity and phenotype

In recent years, it has been widely recognized that Treg cells are very heterogeneous. In addition to classical CD4+CD25hiFOXP3+ Treg cells, other subsets have been defined. These include, for instance, FOXP3+CD8 regulatory T cells and even non-FOXP3 regulatory T cells with suppressive activity (Tr1 and Th3 cells). Treg cells comprise a wide range of cells with different antigen-specificities, origins, tissue-tropisms and specialized functions (reviewed in Gratz & Campbell 2014). In addition to their diversity, Treg cells show plasticity allowing them to adapt to the local microenvironment (reviewed in Sakaguchi et al. 2013). These, together with the lack of consensus markers, highlight the complexity in the characterization and study of Treg cells (reviewed in Dhamne et al. 2013; Morikawa & Sakaguchi 2014).

3.1.1. Backbone markers of Treg cells

Common markers to identify Treg cells are: **CD4**, high expression of **CD25** (IL-2 receptor), low expression of **CD127** (IL-7 receptor) and intracellular **FOXP3**. FOXP3 can not be used for sorting Treg cells for functional assays. For this reason, the use of CD127 has supposed a major advantage as it negatively correlates with FOXP3 (Seddiki et al. 2006; Liu et al. 2006). However, none of these four markers are exclusively expressed by Treg cells. Conventional non-Treg CD4 T cells can, upon activation, upregulate CD25 and FOXP3, and downregulate CD127. As a result of this limitation, several alternative markers to identify *bona fide* Treg cells have been investigated. The use of **CD45RA** in combination with FOXP3 (or alternatively CD25 for sorting experiments) has proven helpful. This strategy allows the exclusion of activated FOXP3+ T cells without suppressive capacity (CD4+CD25+CD45RA-FOXP3lo) that might be included when using the classical Treg cell gating (CD4+CD25hiCD127loFOXP3+) (Simonetta & Bourgeois 2013). Additionally, the use of CD45RA allows, as with conventional CD4 T cells, to differentiate resting and effector Treg cells (Miyara et al. 2009) (Figure I4):

- **Resting Treg cells** (rTreg cells) (or naïve or central) are identified as CD4+CD45RA+ FOXP3lo. These rTreg cells are considered the major population of circulating Treg cells and they have mainly thymic origin. rTreg cells express CCR7 and high CD62L, which direct their recirculation through lymphoid tissues. Even though they express CD45RA, these rTreg cells cannot be considered strictly naïve (Brod et al. 1989; Sakaguchi et al. 2010), as they exert suppressive capacity at baseline and show a history of antigen exposure. Upon activation, rTreg cells can proliferate and convert to effector Treg cells.

- **Effector Treg cells** (eTreg cells) (or activated) are identified as CD4+CD45RA-FOXP3hi (together with low CCR7 and CD62L). It is unclear whether effector Treg cells might comprise a mixture of Treg cells with thymic and peripheral origin, and whether they are terminally differentiated or they are capable of returning into the resting state (Sakaguchi et al. 2010; Liston & Gray 2014). Effector Treg cells are highly proliferative and suppressive, and susceptible to apoptosis (Miyara et al. 2014). As helper CD4 T cells, effector Treg cells can also differentiate into different subsets that colocalize and regulate Th1, Th2, Th17, Th22 and Tfh respectively (Duhen et al. 2012; Cretney et al. 2013).

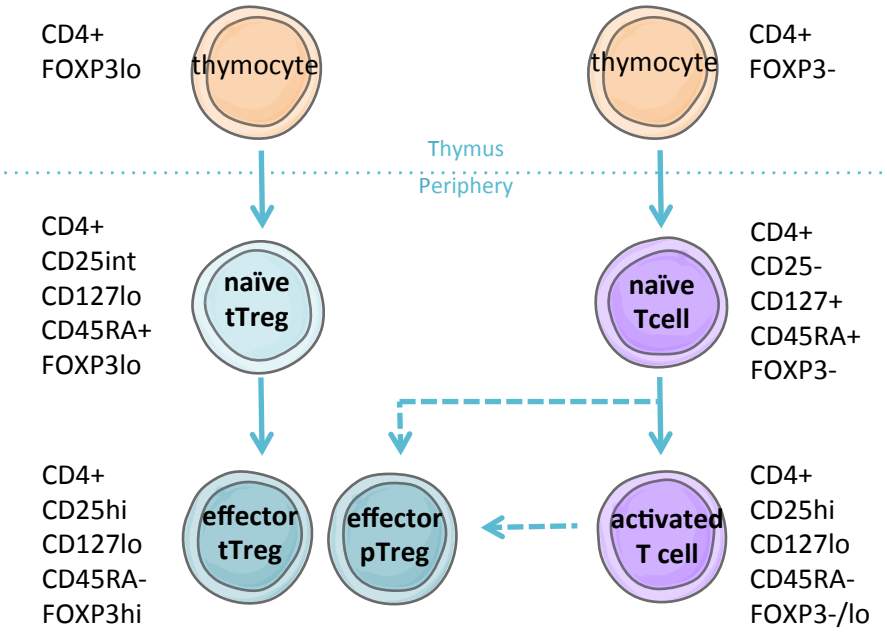

Figure I4. Treg cell differentation. Treg cells can be differentiated in 2 subpopulation by the expression of CD45RA and FOXP3: naïve or resting Treg cells and effector or activated Treg cells. Dashed line indicates unknown differentiation pathway. tTreg: thymic Treg cell, pTreg: peripheral Treg cell. hi: high; int: intermediate; lo: low. Modified from Miyara et al. 2013 and Servier Medical Art.

3.2. Mechanisms of Treg cell-mediated suppression

Treg cells suppress effector cells through different mechanisms. These mechanisms include: metabolic disruption, modulation of antigen presentation, cytokine production, cytolysis and apoptosis. Altogether these different mechanisms support the idea that Treg cells can suppress in an antigen-specific and a bystander manner (Figure I5). These non-redundant mechanisms of suppression probably occur in different Treg cell subsets and at different times and locations (reviewed in Vignali et al. 2008; Wing & Sakaguchi 2012; Schmidt et al. 2012).

Treg cell-mediated suppression by metabolic disruption includes **IL-2 deprivation**. This has been considered one of the core mechanisms in Treg cell-mediated suppression. Treg cells cannot produce IL-2 and present high expression of IL-2 receptors (CD25). For this reason, Treg cells act as IL-2 sinks, depriving effector T cells from the IL-2 that they require for T cell growth and survival (de la Rosa et al. 2004). In addition, metabolic disruption can be mediated by **CD39** and **CD73** expressed on the surface of Treg cells. CD39 together with CD73, generate adenosine by breakdown of ATP (Deaglio et al. 2007; Borsellino et al. 2007; Kobie et al. 2006). The resulting **adenosine** promotes the generation of more Treg cells and inhibits proliferation and IFNγ/IL-2 production in effector T cells (Zarek et al. 2008; Ohta & Sitkovsky 2014). These effects are mediated through adenosine receptor A2AR and increased cAMP. Additionally, this **cAMP** can also be directly transferred through gap junctions from Treg cells to effector T cells (Bopp et al. 2007).

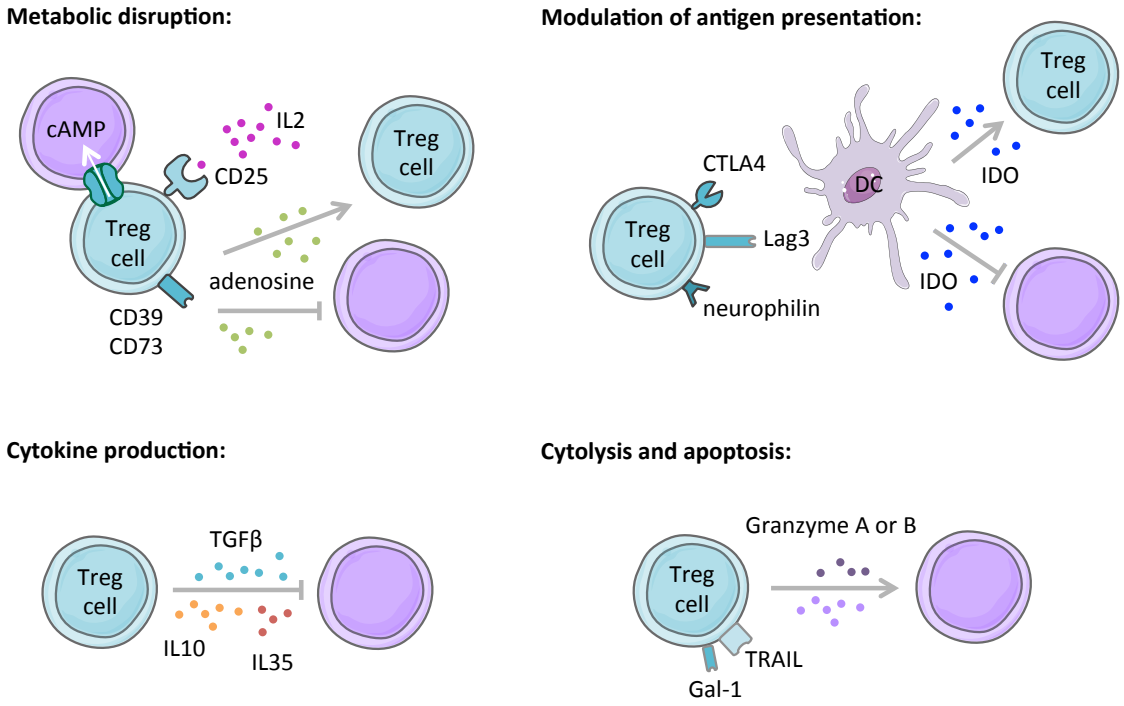

Figure I5. Treg cell-mediated suppression. Treg cells suppress effector cells through different mechanisms. These include metabolic disruption, modulation of antigen presentation, cytokine production, cytolysis and apoptosis. Modified from Vignali et al. 2008 and Servier Medical Art

Treg cell suppression can also be mediated through the modulation of antigen presentation. One mechanism by which Treg cells can modulate antigen presentation is through the surface receptor **CTLA4**. CTLA4 dampens the capacity of DC to activate T cells because CTLA4 competes with CD28 for CD80/86 in DC and can also engulf CD80/86 from DC (Wing et al. 2008; Qureshi et al. 2011). Additionally, CTLA4 can

induce IDO expression in DC (Indoleamine 2,3-dioxygenase) (Grohmann et al. 2002; Fallarino et al. 2003). **IDO** catabolizes tryptophan to kynurenine leading to effector T cell suppression and Treg cell generation (Opitz et al. 2011; Mezrich et al. 2010). Treg cells can also modulate antigen presentation by **Lag 3**, which blocks DC maturation (Liang et al. 2008), or by **neurophilin**, which promotes prolonged Treg/DC interactions (Sarris et al. 2008).

In addition to the metabolic disruption and the modulation of DC, other mechanisms of Treg cell suppression include (1) secretion of immunosuppressive cytokines such as **IL-10, TGFβ** and **IL-35** that can either inhibit effector T cells or DC (Asseman et al. 1999; Nakamura et al. 2001; Collison et al. 2007), (2) cytolysis of effector cells through **granzyme A** and **B** (Gondek et al. 2005; Zhao et al. 2006), and (3) apoptosis of effector cells through the **TRAIL-DR5** pathway and **galectin-1** (Ren et al. 2007; Garín et al. 2007).

3.3. Treg cells in HIV infection

3.3.1. Treg susceptibility to HIV infection

HIV can infect Treg cells (Oswald-Richter et al. 2004), but *ex vivo* analysis showed that circulating Treg cells are not preferentially infected compared with effector T cells (Moreno-Fernandez et al. 2009). Interestingly, *in vitro* studies showed that rTreg express CXCR4 and are more susceptible to X4-HIV strains. On the contrary, eTreg express more CCR5 and are more susceptible to R5-HIV strains (Antons et al. 2008; Arruvito et al. 2012). Hence, regulatory T cells contribute to maintain the latent HIV reservoir and viral replication. However, FOXP3 is associated with downregulation of HIV transcription and, therefore, HIV replication in this subset of cells might be attenuated (Grant et al. 2006; Selliah et al. 2008; Chevalier & Weiss 2013).

Whether Treg cells preserve their function during HIV infection is unclear. While several studies showed that Treg cells retain their suppressive capacity (Angin et al. 2012; Phetsouphanh et al. 2015), others reported that Treg cells are impaired during HIV infection (Tsunemi et al. 2005; Kared et al. 2008; Seddiki et al. 2009; Mendez-Lagares et al. 2012). *In vitro* infection of Treg cells from healthy controls with X4-HIV strains (but not R5-HIV strains) downregulates FOXP3, which is followed by loss of their suppressive capacity (Pion et al. 2013). Recently, similar experiments have reported that also R5-HIV infection impairs the Treg cell suppressive function by dowregulating genes related with Treg cell function (Angin et al. 2014). Although *in vitro* infection of Treg cells impacts its suppressive function, the authors suggested that this impairment

is unlikely to have an effect at Treg population level, considering the low percentage of infected Treg cells in HIV-infected individuals (<0.7% of Treg cells in peripheral blood). Nevertheless, HIV infection might provoke Treg cell impairment in other tissues were viral burden is enhanced.

3.3.2. Treg cell dynamics in HIV infection

During HIV infection, Treg cell numbers are reduced. However, Treg cells are **preferentially preserved** compared with conventional CD4 T cells both in blood and gut mucosa (reviewed in Moreno-Fernandez et al. 2012; Simonetta & Bourgeois 2013; Chevalier & Weiss 2013). In the primary phase of HIV infection, a decrease in Treg cell counts and percentages has been described (Kared et al. 2008; Simonetta et al. 2012). In the chronic phase, Treg cell counts progressively decrease in parallel to total CD4 counts, whereas Treg cell percentatges increase (Bi et al. 2009; Angin et al. 2012; Simonetta et al. 2012). In line with these observations Treg cell counts and percentages recover in patients under ART (Lim et al. 2007; Montes et al. 2011). Moreover, most studies report that the percentage of Treg cells correlates negatively with CD4 counts and positively with plasma viral load (reviewed in Chevalier & Weiss 2013; Simonetta & Bourgeois 2013). Likewise, studies of the gut mucosa showed a decrease in Treg cell counts but preferential preservation of Treg cells in comparision to Th17 (Favre et al. 2009; Falivene et al. 2015).

In spite of these general dynamics of Treg cells, different dynamics of rTreg and eTreg cell subsets have been reported. rTreg cell counts are significantly reduced in primary HIV-infected individuals compared with healthy control individuals. During HIV progression, the rTreg cell subset is recovered and preserved (no differences have been observed when comparing chronic viremic and aviremic HIV-infected individuals). Contrarily, eTreg cell counts decrease during primary infection and do not recover during HIV progression, not even under ART (Simonetta et al. 2012; Simonetta & Bourgeois 2013; Zhou et al. 2013). Regarding the percentage of these subsets during HIV infection, there is an increased proportion of eTreg compared with rTreg cells (Zhou et al. 2013). In addition, evaluation of CD31 indicates impaired thymic output and increased generation of eTreg from rTreg cells. Analysis of Ki67 (marker of proliferation) indicates hyper-proliferation in both subsets in HIV-infected individuals compared with healthy control individuals (Zhou et al. 2013). In fact, during HIV infection, Ki67+ total Treg cell percentages correlate negatively with CD4 T cell counts and positively with viral loads (Bi et al. 2009; Xing et al. 2010).

3.3.3. Mechanisms for preferential accumulation of Treg cells

Two mechanisms have been proposed to explain the increased Treg cell frequency in comparison to conventional CD4 T cells: 1) increased production and 2) increased survival of Treg cells. It has been reported that during HIV there is increased production of Treg cell in the thymus (Kolte et al. 2009; Kolte 2013) as well as the periphery. In the periphery Treg cells increase by different mechanisms. First, Treg cells increase due to immune activation (Chevalier & Weiss 2013). Second, the virus itself through HIV-gp120 interaction can activate Treg cells and promote their survival (Becker et al. 2009; Nilsson et al. 2006; Ji & Cloyd 2009). Third, HIV-exposed DC induce Treg cells through IDO production (Manches et al. 2008; Chen et al. 2008). In addition, it has been reported that Treg cells are relatively more resistant to cell death induced by activation, in comparison to conventional CD4 T cells (Fritzsching et al. 2005).

3.3.4. Dual role of Treg cells in HIV infection

During HIV infection, Treg cells act as a double edge sword, having both beneficial and detrimental roles (Figure I6). Some authors have hypothesized that the role of Treg cells might be predominantly beneficial during acute HIV infection and predominantly detrimental during chronic HIV infection (Moreno-Fernandez et al. 2012). However, increasing evidence points at a high diversity in Treg cell function. This could explain different roles of Treg cells at different times and locations during HIV infection (Simonetta & Bourgeois 2013).

Treg cells are associated with the **control of excessive immune activation** (Eggena et al. 2005), which limits tissue damage and bystander cell death. Moreover, this control reduces the availability of HIV targets (as virus predominantly infects activated CD4 T cells), preventing further virus production (Kared et al. 2008; Petitjean et al. 2012; Moreno-Fernandez et al. 2012). Treg cells can also reduce HIV dissemination by limiting the frequency and quality of DC:T cell interactions (Moreno-Fernandez et al. 2014) and by reducing virus replication in macrophages (Liu et al. 2009). Therefore, Treg cells might have a beneficial role during acute infection by influencing the viral set point (Kared et al. 2008). In addition, a recent report suggests that Tr1 (IL-10 producing Treg cells) might have a beneficial role in early HIV infection. In contrast to conventional Treg cells, Tr1 inversely correlated with immune activation but they were not associated with lower effector responses (Chevalier et al. 2015). However, the role of Treg cells in control of immune activation remains a matter of debate. Several studies indicate that an increased proportion of Treg cells in relation to Th17 cells in the gut is associated with a loss of epithelial integrity leading to microbial translocation and immune activation (Favre et al. 2009; Klatt et al. 2013; Falivene et al. 2015).

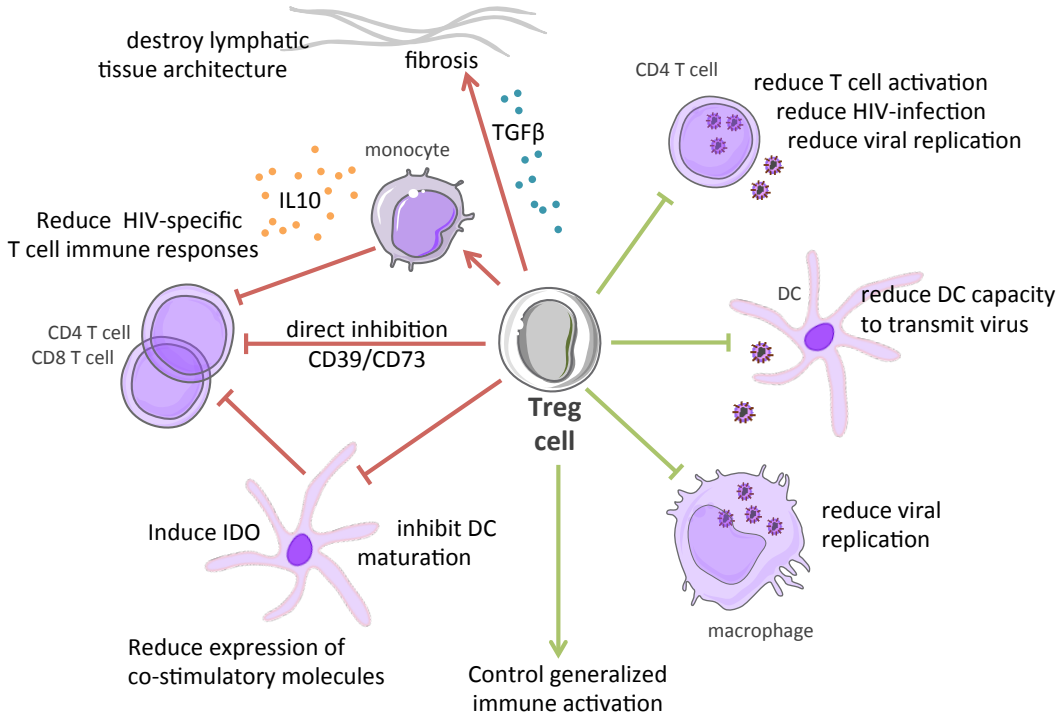

Figure I6. Beneficial and detrimental roles of Treg cells during HIV infection. On one hand Treg cells control immune activation and limit viral replication. On the other hand Treg cells reduce HIV-specific T cell immune responses and destroy lymphatic tissue architecture contributing to T cell dysfunction. Adapted from Moreno-Fernández et al. 2012 and Servier Medical Art.

Treg cells are associated with a **negative impact on HIV-specific T cells**, which is supported by several *in vitro* studies (Kinter et al. 2004; Aandahl et al. 2004; Weiss et al. 2004; Nilsson et al. 2006). Treg cells can reduce CD4- and CD8- HIV-specific immune responses by direct or indirect mechanisms, such as direct CD39/CD73 inhibition, alteration of antigen presentation, induction of IL-10 production by monocytes or IDO induction on DC (Schulze zur Wiesch et al. 2011; Nikolova et al. 2011; Kwon et al. 2012; Manches et al. 2008). Moreover, a recent study reported that after a DC-based HIV vaccine, a reduction in HIV-specific-Treg cells in comparison to HIV-specific-effector T cells correlates with low viral replication following treatment interruption (Brezar et al. 2015). It has also been reported that CD8 T cells restricted by protective alleles (HLAB27 and HLAB57) evade Treg cell suppression (Elahi et al. 2011). These reports support that Treg cell play a relevant role in the impairment of protective HIV effector T cell responses (Moreno-Fernandez et al. 2012). In addition, it has been reported in SIV-infected macaques (non-human primate model for HIV) that Treg cells secrete TGFβ that **destroys the lymphatic tissue architecture** and prevents CD4 T cell reconstitution (Estes et al. 2007). Altogether these indicate that Treg cells promote immune exhaustion and viral persistence (Penaloza-MacMaster et al. 2014; Phetsouphanh et al. 2015).

4. Towards an HIV cure

Current **antiretroviral therapy** (ART) has shown success in controlling HIV infection, dramatically reducing both AIDS and non-AIDS-related deaths, and increasing life expectancy of HIV-infected individuals (Mylvaganam et al. 2015; Walensky et al. 2006; Bertozzi et al. 2005). ART profoundly represses virus replication maintaining plasma viremia below the limit of detection by current clinical assays. But it **fails to eradicate the virus** from the reservoir of long-lived infected cells, resulting in re-emergence of viremia when ART is interrupted. For this reason, current ART must be life-long (Finzi et al. 1997; Finzi et al. 1999). Moreover, although ART recovers CD4 T cells counts and reduces T cell activation, it **fails to restore immune responses**, especially in individuals that initiate therapy in a late stage of HIV infection (Corbeau & Reynes 2011; Mylvaganam et al. 2015). For these reasons, there is a need of new therapeutic approaches that could complement current ART in order to reach an HIV cure.

Two potential strategies to cure HIV have been described: a sterilizing cure and a functional cure. The **sterilizing cure** involves the total permanent elimination of the virus in absence of ART. There is just a single example of a sterilizing cure, referred as the Berlin patient. He became cured after a bone marrow transplant from a donor who lacked CCR5 expression (a coreceptor needed for HIV infection). Thus, repopulated CD4 T cells were resistant to HIV infection. The Berlin patient provided a proof-of-concept that HIV eradication might be possible. However, the use of this procedure cannot be generalized because it is excessively complex and it constitutes health risks for the patient (Hutter et al. 2009; Yukl et al. 2013; Chun et al. 2015). In view of this, a more feasible approach for HIV cure is the functional cure. The **functional cure** involves the control of viral replication in absence of ART. This approach aims to mirror the viral control achieved in elite controllers, were the hosts' HIV-specific immune response is capable of controlling viral replication. In this regard, a functional cure of HIV requires reducing the size of HIV reservoirs and enhancing HIV-specific immune responses (Chun et al. 2015).

4.1. Reducing HIV reservoirs

The HIV viral reservoir consists of a pool of infected cells that are not eliminated by ART or antiviral immune responses. This HIV reservoir includes both **latent HIV-infected cells** and other yet to be defined sources of persistent virus (Archin et al. 2014). The main known latent HIV reservoirs are resting CD4 T cells. Other cell populations might contribute, such as haematopoietic stem cells, macrophages,

dendritic cells, γδ T cells, astrocytes or microglia (Massanella et al. 2013; Archin et al. 2014). The establishment of a latent reservoir in resting CD4 T cells mainly occurs when highly susceptible activated CD4 T cells get infected and then reverse to the resting memory state (Siliciano et al. 2003). However, resting CD4 T cells can also be directly infected. In these long-lived cell subsets, HIV provirus can remain integrated within the host-cell DNA, transcriptionally silent and hidden from the immune system. Under certain circumstances, reactivation occurs and virus is produced (Finzi et al. 1999; Archin et al. 2014; Dahabieh et al. 2015).

HIV reservoirs can be maintained by T cell survival, low-antigen driven proliferation and homeostatic proliferation (Chomont et al. 2009; Margolis & Bushman 2014). In addition, residual viral replication might be responsible for the continuous replenishment of the latent reservoir. This residual replication might occur under ART because of a suboptimal penetration of antiretroviral drugs to different anatomical locations, such as the gut mucosa or the central nervous system (Massanella et al. 2013; Chun et al. 2015).

Irrespective of the mechanisms involved in the establishment and maintenance of these HIV reservoirs, it has been shown that HIV reservoirs are established within days of infection and are not altered by current antiretroviral therapy (Chun et al. 1997; Finzi et al. 1997; Chun et al. 1998; Whitney et al. 2014; Archin et al. 2014). They represent the main obstacle for HIV eradication. To reduce HIV reservoirs, several approaches have been proposed, such as early initiation of ART, optimization and intensification of ART, therapeutic vaccines, the use of latency-reversing agents and immune-based therapies, which include administration of neutralizing HIV antibodies, cytokines or inhibitors of immune check-points such as PD-1 (Massanella et al. 2013; Chun et al. 2015).

4.2. Reversing exhaustion

It has been hypothesized that if the latent HIV reservoir is small enough, HIV-specific immune responses might be able to control viral replication without ART (Archin et al. 2014). However, due to antigen persistence, HIV-specific T cells are depleted or dysfunctional, they appear exhausted. Thus, an effective HIV cure is likely to require approaches that restore exhausted HIV-specific immune responses.

The most promising of such approaches is the blockade of PD-1/PD-L1 pathway. The blockade of the PD-1/PD-L1 pathway reverses the exhausted phenotype of T cells from HIV-infected individuals *in vitro* (Day et al. 2006; Trautmann et al. 2006; Souza et al.

2007; Porichis et al. 2011). *In vivo* experiments in HIV-infected humanized mice showed that the blockade of the PD-L1/PD-1 pathway can restore HIV-specific T cell responses and significantly reduce HIV replication (Palmer et al. 2013; Seung et al. 2013). In addition, experiments with SIV-infected macaques showed, upon PD-1 blockade: enhancement of both cellular and humoral SIV-specific immune responses, reduced viremia, reduced AIDS-related symptoms and prolonged survival (Velu et al. 2009; Titanji et al. 2010). A follow up report, showed reduced immune activation and plasma LPS levels, suggesting that upon PD-1 blockade there is an enhancement of gut immunity leading to a reduction in microbial translocation (Shetty et al. 2012). In addition, recent studies in the SIV model, showed that PD-1 blockade in combination with ART delays viral rebound after the cessation of ART (Amancha et al. 2013; Vargas-Inchaustegui et al. 2013; McGary et al. 2014). These studies suggest that the delayed viral rebound results from the restoration of exhausted antiviral responses. Although full suppression of virus production was not reported in any of these studies, altogether these studies support the blockade of the PD-1/PD-L1 pathway to enhance antiviral responses in new combination therapies that aim to cure HIV (McGary et al. 2014; Archin et al. 2014).

The blockade of PD-1/PD-L1 pathway not only might help to restore exhausted antiviral effector responses, but also might reduce the latent HIV reservoir. It has been shown, that within the resting memory CD4 T cell compartment, cells expressing high PD-1 are an important pool of latently-infected cells (Chomont et al. 2009). Therefore, the blockade of PD-1/PD-L1 pathway might help decreasing the threshold of TCR activation making it easier to reactivate the latent virus in this cell subset. In addition, it has been reported that the blockade of PD-1 can trigger HIV replication and could reactivate latent virus (DaFonseca et al. 2010; Massanella et al. 2013). Altogether these studies show a therapeutic potential for PD-1/PD-L1 blockade in HIV infection that is not exempt of risks. These will be tested in an ongoing phase I clinical trial that aims to evaluate the safety of an anti-PD-L1 antibody (BMS-936559) in HIV-infected individuals under ART with undetectable viremia (Archin et al. 2014; www.clinicaltrials.gov identifier NCT02028403).

The promising role of PD-1/PD-L1 blockade as a therapy component to cure HIV motivated the research presented in this thesis, where I studied PD-L1 blockade consequences for Treg cells.

OBJECTIVES

Blocking the PD-1/PD-L1 pathway has emerged as a potential therapeutic strategy to restore impaired immune responses in HIV-infected individuals. So far, most reports have focused on effector cells, and possible effects from PD-L1 blockade on regulatory T cells have been neglected. Regulatory T cells are key players balancing immunopathology and antiviral effector responses. Therefore, any alteration on regulatory T cells may directly influence the balance between immunopathology and viral control. In light of the upcoming therapeutic trials blocking PD-L1 in HIV-infected patients, this thesis has 3 objectives:

- To evaluate PD-1 and PD-L1 expression in regulatory T cells from HIV-infected individuals.

- To explore the consequences of *ex vivo* PD-L1 blockade on regulatory T cells from HIV-infected individuals.

- To generate hypotheses on patient selection criteria for PD-L1 blockade immunotherapy.

MATERIALS and METHODS

1. Medias, Buffers and Solutions

Freezing media:
10% dimethyl sulfoxide (DMSO) (Sigma), 90% heat inactivated fetal bovine serum (FBS) (Sigma).

R10 media:
RPMI-1640 media (Gibco), 10% FBS (Sigma), 1% penicillin/ streptomycin (Gibco)

R10 media for Treg cells suppressive assays:
R10 media supplemented with 50U/mL recombinant human Interleukin-2 (rhIL-2) (R&D Systems) and 1mM sodium pyruvate (Sigma).

FACS Buffer:
Phosphate-buffered saline (PBS) (Gibco), 5% FBS (Sigma), 0.5% Bovine serum albumin (BSA) (Sigma), 0.07% sodium azide (Sigma)

FACS Fix Buffer:
Deionized water water, 1% paraformaldehyde (Sigma), 150mM NaCl (Sigma), pH7.4

Microbeads Buffer:
PBS (Gibco), 0,5% BSA (Sigma), 2mM ethylenediaminetetraacetic acid (EDTA) (Sigma), pH7.2

Fixation/Permeabilization (Fix/Perm) Buffer 1x:
Diluted from Fix/Perm concentrate (4x) (eBioscience) in Fix/Perm diluent (eBioscience)

Permeabilization (Perm) Buffer 1x:
Diluted from Perm concentrate (10x) (eBioscience) in deionized water.

2. Blood samples

2.1. Ethics statement

Ethical committee approval and written informed consent from all subjects, in accordance with the Declaration of Helsinki, were obtained prior to study initiation. The study was approved by the institutions' ethical commitees: CEIC- Parc de Salut Mar, Barcelona, Spain (Protocol approval number: 2013/5422/I) and Comitè étic

d'investigació clínica, Hospital Clinic, Barcelona, Spain (Protocol approval number: 2013/8671).

2.2. Human subjects

Blood was obtained from HIV-uninfected volunteers (healthy controls) and HIV-infected individuals at the Hospital Clinic and the Hospital del Mar, both in Barcelona, Spain. HIV-infected individuals were categorized into 4 groups: (1) fewer than 500 CD4/µL and more than 2000 RNA copies/mL blood; (2) more than 500 CD4/µL and more than 2000 RNA copies/mL blood; (3) more than 500 CD4/µL and fewer than 2000 RNA copies/mL; (4) HIV-infected individuals under successful antiretroviral treatment (cART) for at least 2 years with more than 500 CD4/µL blood and viral loads below the limit of detection (40 RNA copies/mL). HIV-infected individuals from the cross-sectional study with the exception of the cART group were naïve to antiretroviral therapy at the time of testing (Table M1 & Table M2).

Table M1. Comparison of demographic characteristics of HIV-infected individuals and healthy controls used in Figure R1

	for PD-1 staining			for PD-L1 staining		
	HIV patients	**Healthy Controls**	**p-value**	**HIV patients**	**Healthy Controls**	**p-value**
	n= 46	n= 9		n= 23	n= 7	
age (years) mean ± SD	39.43± 8.65	36.11± 12.81	0,339 [a]	37.22± 8.92	39.71± 11.64	0,539 [a]
Sex, male: female	33:13	3:6	0,051 [b]	21:2	4:3	0,068 [b]

[a] p-values were calculated by Mann-Whitney test

[b] p-values were calculated by Fisher's exact test

Table M2. Characteristics of HIV-infected individuals

	< 500 CD4/µL > 2000 vRNA/mL n=11	**> 500 CD4/µL > 2000 vRNA/mL** n=11	**> 500 CD4/µL < 2000 vRNA/mL** n=10	**HAART** n=14	**p-value**
age (years) mean ± SD	32.55 ± 5.3	42.09 ± 8.5	39.3 ± 9.6	42.86 ± 7.6	0,037 [a]
Sex, male: female	10:1	7:4	8:2	8:6	0,245 [b]
CD4 count (cells/µL) Median, IQR (25-75%)	267 (150-365)	624 (543-696)^	704 (594-787)^	780 (606-869)^	<0.0001[a]
viral RNA (LOG10 copies/mL) Median, IQR (25-75%)	4.69 (4.32-4.9) [+]	4.48 (3.6-5.1) [+]	2.3 (1.89-2.48)[++]	1.57 (1.57-1.57)[++]	<0.0001[a]
Hepatitis C, positive: negative	3:8	0:11	0:10	4:10	0,074 [b]
Hepatitis B, positive: negative	01:10	00:11	00:10	01:13	0,584 [b]

[a] p-values were calculated by Kruskal-Wallis test to compare the 4 HIV study groups

[b] p-values were calculated by Chi-squared test to compare the 4 HIV study groups

^ non significant differences in CD4 count between the indicated groups (Kruskal Wallis test , p-value:0.3371)

+ non significant differences in viral RNA between the indicated groups (Mann Whitney test, p-value: 0.5326)

++ significant differences in viral RNA between the indicated groups (Mann Whitney test, p-value: <0.0001)

IQR: interquartile range

vRNA: viral RNA

In addition we studied two subgroups of patients longitudinally. A group of 5 individuals (group A) were followed before starting cART (pre-cART), during cART (on-cART), upon interruption of cART (off-cART), and after restarting cART (on-cART). A second group of 7 individuals (group B) were followed before cART (pre-cART) and after >2 years receiving cART (on-cART) (Table M3).

Table M3. Characteristics of HIV-infected individuals for longitudinal studies

Group A		**CD4 count** (cells/µL)	**viral RNA** (copies/mL)
Individual 1	pre-cART	1169	1850
	on-cART	1704	199
	off-cART	566	22984
	on-cART	1119	37
Individual 2	pre-cART	968	110000
	on-cART	941	19
	off-cART	962	4817
	on-cART	966	37
Individual 3	pre-cART	809	4347
	on-cART	1100	199
	off-cART	595	89300
	on-cART	337	37
Individual 4	pre-cART	462	976
	on-cART	1331	199
	off-cART	885	100000
	on-cART	1744	37
Individual 5	pre-cART	440	7160
	on-cART	577	199
	off-cART	427	25200
	on-cART	956	37

Group B		**CD4 count** (cells/µL)	**viral RNA** (copies/mL)
Individual 1	pre-cART	550	32061
	on-cART	524	37
Individual 2	pre-cART	605	4966
	on-cART	1006	37
Individual 3	pre-cART	552	33900
	on-cART	775	37
Individual 4	pre-cART	488	265000
	on-cART	434	37
Individual 5	pre-cART	574	309
	on-cART	1872	199
Individual 6	pre-cART	968	110000
	on-cART	941	19
Individual 7	pre-cART	1169	1850
	on-cART	1704	199

2.3. PBMC isolation

Blood samples were collected on sodium-heparin collection tubes (BD bioscience). Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll density centrifugation within 8 hours of blood collection. Briefly, blood was diluted 1:1 in R10 media and layered over Ficoll solution (Invitrogen) in a 3:1 ratio. Then centrifugation was performed at 600g for 20 minutes at room temperature without brake. After centrifugation, PBMC were transferred to a new tube with R10 media and washed twice. Isolated PBMC were gradually frozen in 10% DMSO 90% FBS using a Mr.Frosty freezing container (Thermo scientific). For subsequent analysis, PBMC were thawed at 37ºC and washed twice in warm R10 media. Thawed PBMC rested on an incubator at 37ºC 5% CO_2 for 1-2h hour prior to phenotyping; and 6-7 hours prior to the performance of functional assays (Figure M1).

3. Cell staining and Flow cytometry

PBMC were rinsed with PBS and stained with the Live/Dead fixable violet dye (Vivid)(Invitrogen) for 15 minutes at room temperature to exclude dead cells from the analysis. Then, PBMC were washed with FACS Buffer and centrifuged. Pelleted cells were then incubated for 25 minutes, at room temperature, in a total volume of 100 µL with fluorochrome-labelled monoclonal antibodies against: CD3, CD4, CD8, CD14, CD19, CD25, CD39, CD45RA, CD56, PD-1, PD-L1 and CTLA4. After surface antibody staining, cells were washed with FACS Buffer. For intracellular detection of FOXP3, Helios and Ki67, cells were fixed and permeabilized using the FOXP3 staining kit (eBioscience) according to manufacturer's instructions. Briefly, PBMC were incubated in Fix/Perm Buffer (eBioscience) for 30 minutes. Then, PBMC were washed twice with Perm Buffer (eBioscience) and centrifuged. Pelleted cells were then incubated 25 minutes, at room temperature, in a total volume of 100µL with fluorochrome-labelled monoclonal antibodies against: FOXP3, Helios and Ki67. After 25 minutes, cells were washed twice with Perm Buffer (eBiosciences) and finally resuspended in FACS Fix buffer.

Stained cells were acquired in a flow cytometer within 2 hours after staining. Prior to acquisition cells were kept protected from light at 4ºC. Flow cytometry data were collected on a LSR Fortessa (BD biosciences) and analysed with Flow Jo software (Tree Star). At least 200.000 lymphocytes were recorded. Panels containing the corresponding isotype controls were collected to set PD-1, PD-L1, CTLA4 and Ki-67 gates. Treg cells were identified as a joint population of effector Treg cells (CD4+CD45RA-FOXP3^{hi}) (eTreg) and resting Treg cells (CD4+CD45RA+FOXP3^{lo}) (rTreg) as previously described (Miyara et al., 2009) (shown in Figure R1A).

Table M4. Flow cytometry panels

PD-1 on Treg cells, CD4- and CD8- T cells:

Antigen	Fluorochrome	Company	Cat.nº	Clone	Volume
live/dead	Vivid	Invitro	L34955		dil 1:8000
CD4	PE Cy7	BD	557852	SK4	1.2µL
CD8	PE	BD	555367	RPA-T8	0.3µL
CD25	APCH7	BD	560244	M-A251	2.5µL
CD45RA	FITC	BD	555488	HI100	5µL
CD279 (PD-1)	PerCPCy5.5	BD	561273	EH12.1	4.8µL
FOXP3	Alexa 647	BD	560045	259D/C7	2.5µL

PD-1 on Treg cells:

Antigen	Fluorochrome	Company	Cat.nº	Clone	Volume
live/dead	Vivid	Invitro	L34955		dil 1:8000
CD3	BV605	BD	563217	SK7	2µL
CD4	APC Cy7	BD	561839	SK3	0.3µL
CD45RA	FITC	BD	555488	HI100	5µL
CD279 (PD-1)	PerCPCy5.5	BD	561273	EH12.1	4.8µL
FOXP3	Alexa 647	BD	560045	259D/C7	2.5µL

PD-L1 on Treg cells:

Antigen	Fluorochrome	Company	Cat.nº	Clone	Volume
live/dead	Vivid	Invitro	L34955		dil 1:8000
CD3	BV605	BD	563217	SK7	2µL
CD4	APC Cy7	BD	561839	SK3	0.3µL
CD45RA	FITC	BD	555488	HI100	5µL
CD274 (PD-L1)	PE	BD	557924	MIH1	5µL
FOXP3	Alexa 647	BD	560045	259D/C7	2.5µL

PD-L1 on T cells, B cells, NK cells and monocytes:

Antigen	Fluorochrome	Company	Cat.nº	Clone	Volume
live/dead	Vivid	Invitro	L34955		dil 1:8000
CD14	FITC	BD	555397	M5E3	0.8 µL
CD56	APC	eBio	17-0567	CMSSB	0.6µL
CD19	PECy7	BD	561742	HIB19	2.5µL
CD3	PerCPCy5.5	BD	332771	SK7	15µL
CD274 (PD-L1)	PE	BD	557924	MIH1	5µL

Proliferation (CFSE) of Treg cells, CD4- and CD8- T cells:

Antigen	Fluorochrome	Company	Cat.nº	Clone	Volume
	CFSE	Invitro	V12883		
live/dead	Vivid	Invitro	L34955		dil 1:8000
CD4	PE Cy7	BD	557852	SK4	1.2 µL
CD8	PE	BD	555367	RPA-T8	0.3µL
CD25	APCH7	BD	560244	M-A251	2.5µL
CD45RA or	PerCPCy5.5	BD	563429	HI100	1.25uL
CD45RA	eF605	eBio	93-0458	HI100	2.5µL
FOXP3	Alexa 647	BD	560045	259D/C7	2.5µL

Proliferation (Ki67) of Treg cells, CD4- and CD8- T cells:

Antigen	Fluorochrome	Company	Cat.nº	Clone	Volume
live/dead	Vivid	Invitro	L34955		dil 1:8000
CD3	PerCPCy5.5	BD	332771	SK7	15µL
CD4	PE Cy7	BD	557852	SK4	1.2µL
CD8	APC-Cy7	BD	557834	SK1	0.5µL
CD45RA	FITC	BD	555488	HI100	5µL
FOXP3	Alexa 647	BD	560045	259D/C7	2.5µL
Ki67	PE	BD	556027	B56	5µL

Phenotype of Treg cells:

Antigen	Fluorochrome	Company	Cat.nº	Clone	Volume
live/dead	Vivid	Invitro	L34955		dil 1:8000
CD4	APC Cy7	BD	561839	SK3	0.3µL
CD45RA	FITC	BD	555488	HI100	5µL
CD39	PE Cy7	eBios	25039942	eBiosA1	0.1µL
CTLA4 (CD152)	PE	BD	560939	BNI3	8µL
FOXP3	Alexa 647	BD	560045	259D/C7	2.5µL
Helios	PerCPCy55	BioLeg	137230	22F6	5µL

CD8 T cell proliferation for Treg cell suppressive assays:

Antigen	Fluorochrome	Company	Cat.nº	Clone	Volume
	CFSE	Invitro	V12883		
CD3	APC	BD	345767	SK7	0.25uL
CD8	PE	BD	555367	RPA-T8	0.3µL

Treg purity of isolated Treg cells:

Antigen	Fluorochrome	Company	Cat.nº	Clone	Volume
CD4	PE Cy7	BD	557852	SK4	1.2µL
CD25	APCH7	BD	560244	M-A251	2.5µL
CD45RA	eF605	eBio	93-0458	HI100	2.5µL
CD127	PE	BD	561028	HIL7R-M21	1µL
FOXP3	Alexa 647	BD	560045	259D/C7	2.5µL

eBio: eBiosciences; BD: BD Biosciences; Invitro: Invitrogen; dil: dilution

4. Cell culture and Functional assays

4.1. Proliferation assay and Treg cell phenotyping after PD-L1 blockade

Proliferation of T cells was determined by carboxyfluorescein succinimidyl ester (CFSE) dilution or Ki67 expression. For CFSE assays, PBMC were filtered through a 70µm nylon cell strainer (BD Biosciences) to remove clumps of cells and then stained with CFSE as described in (Quah et al., Nature Protocols, 2007). In brief, 10^7 PBMC/mL were incubated with CFSE (Invitrogen) at 5µM for 5 minutes at 37ºC in PBS containing 5% FBS. Labelling was quenched with 4ºC PBS containing 5% FBS and stained cells were washed twice with PBS containing 5% FBS.

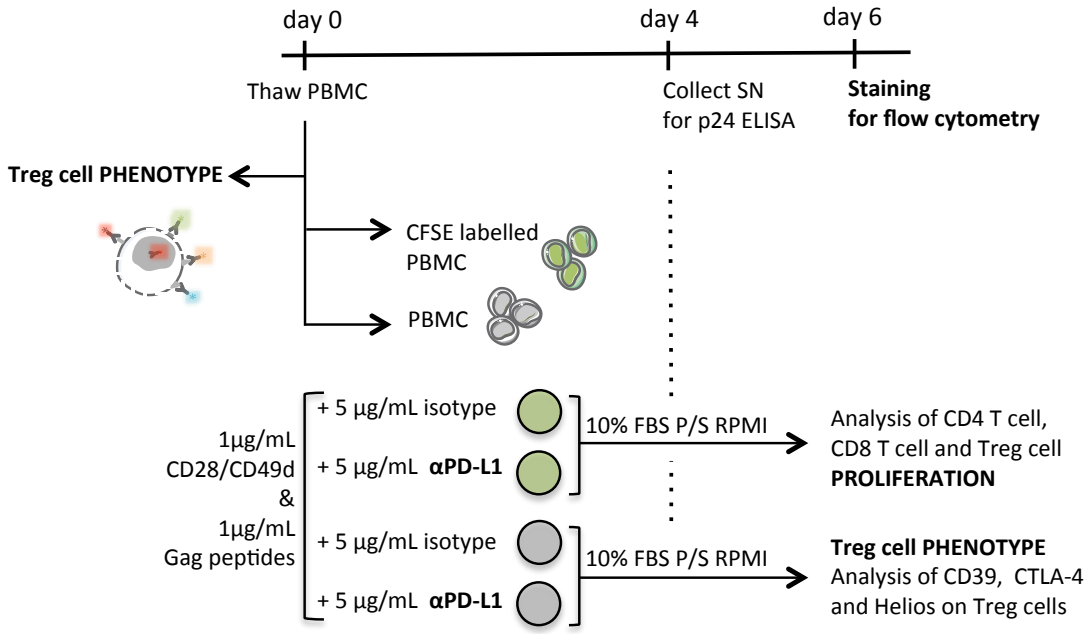

Figure M1. Graphical representation of the methodology

CFSE-labelled cells were seeded at $2 \cdot 10^6$ PBMC/well in 24-well plates (Greiner bio-one) in R10 media and 1µg/mL anti-CD28 and anti-CD49d antibodies (BD Biosciences). Cells were either left unstimulated or incubated with 1µg/mL Gag pool of overlapping peptides (Gag peptides; NIH AIDS Research and Reference Reagent Program, catalogue number 8117 and 8118) plus 5µg/mL anti-PD-L1 blocking or isotype control antibodies (eBioscience). Stimulation was performed in a total volume of 350µL/well for 14-16 hours and then 1150µL of R10 media were added to each well. After 6 days, cells were harvested and stained to analyse proliferation of Treg, CD4- and CD8- T cell subsets (Figure M1). Alternatively, to analyse proliferation in longitudinal samples for which cell numbers were limited, non-CFSE labelled PBMC were cultured for 6 days as

previously described, and stained with Ki67 or isotype control antibodies. Fold change in proliferation (FC proliferation) was calculated as a ratio of proliferation under PD-L1 blockade condition divided by proliferation under control condition.

To analyse the Treg phenotype, non-CFSE stained PBMC were stimulated with Gag peptides in the presence of anti-PD-L1 blocking antibody or isotype control antibody as described above. After 6-day-culture, cells were harvest and stained to analyse the percentage of CD39-, CTLA4- and Helios- expressing Treg cells (Figure M1). Fold change in CD39 or CTLA4 or Helios on Treg cells (FC) was calculated as a ratio of the percentage of CD39/CTLA4/Helios-expressing Treg cells under PD-L1 blockade condition divided by the percentage of CD39/CTLA4/Helios-expressing Treg cells under control condition.

4.2. *In vitro* suppression assay for functional assessment of Treg cells

PBMC were stimulated with Gag peptides in the presence of anti-PD-L1 blocking antibody or isotype control antibody as described above. After 6-day culture, PBMC were harvest and filtered through a 40µm nylon cell strainer (BD Biosciences) to remove clumps of cells. Then, Treg cells were isolated by magnetic beads using the CD4+CD25+CD127dim/- regulatory T cell isolation kit II (Mitenyi Biotec) (Figure M2). Briefly, PBMC were incubated with CD4+CD25+CD127dim T cell Biotin antibody cocktail for 10 minutes at 4ºC, and then anti-Biotin-microbeads where added and incubated for extra 15 minutes at 4ºC. PBMC were washed and placed into a LD column (Miltenyi Biotec). Total effluent (negative fraction) was collected and incubated with CD25 microbeads for 15 minutes at 4ºC. Then cells were washed and transferred to a MS column (Miltenyi Biotec). Magnetically labelled cells (positive fraction) were flushed out of the magnet and transferred to a second MS column to obtain higher purity. Finally, Treg cells were flushed out of the second MS column and resuspended in R10 media.

To assess purity, the isolated Treg cells were labelled with CD4, CD25, CD127, CD45RA and FOPX3 as previously described and acquired in a flow cytometer. Treg cells with a purity of over 80% FOXP3 were used to perform the suppressive assays.

For the suppressive assays, purified Treg cells were co-cultured with 50.000 CFSE-labelled PBMC at different ratios and stimulated with 0.5µg/mL anti-CD3 and 1µg/mL anti-CD28 antibodies (Figure M2). Cells were cultured in 96-U bottom well plates (Greiner bio-one) in R10 media supplemented with 50U/mL rhIL-2 (R&D Systems) and 1mM sodium pyruvate (Sigma). After a 3-day-culture, cells were harvested and stained to analyse proliferation of CD8 T cells by CFSE dilution. The Treg cell suppressive

capacity was determined by the percentage of inhibition of CD8 proliferation, calculated as: [(CD8 proliferation – CD8 proliferation in presence of Treg cells) / CD8 proliferation] x 100.

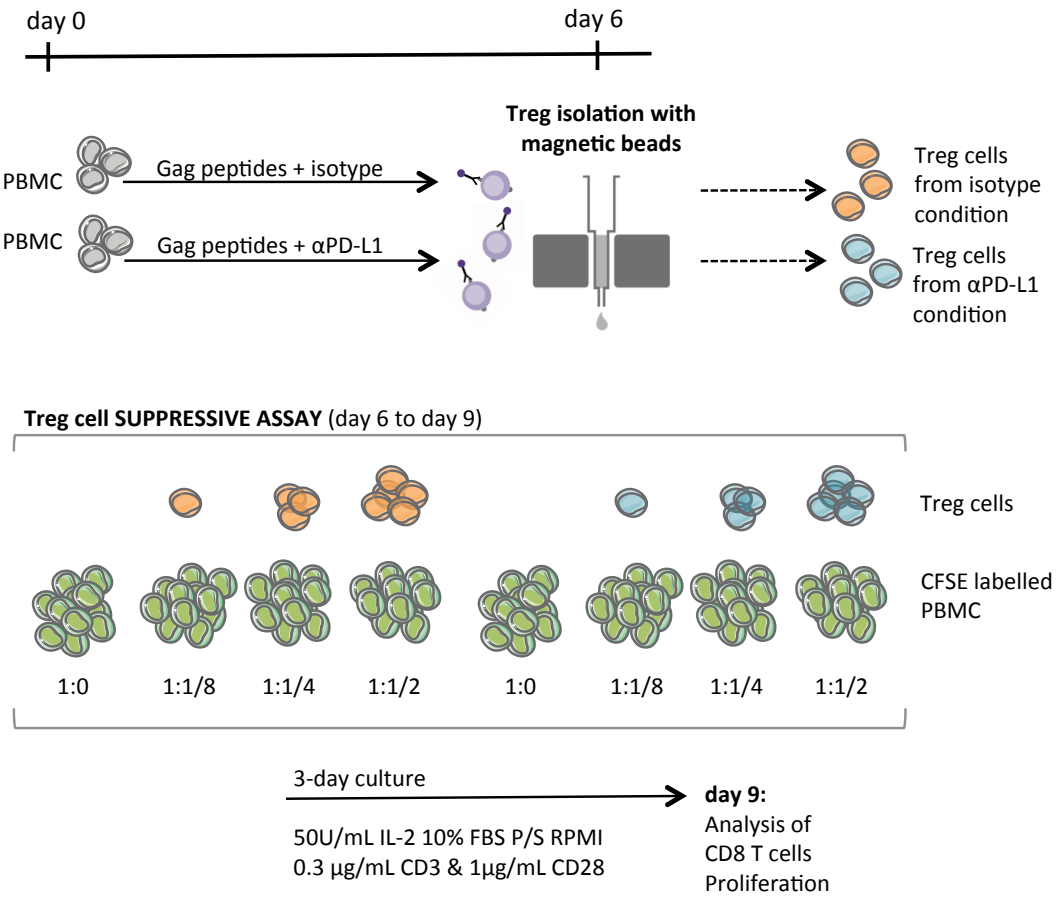

Figure M2. Graphical representation of *in vitro* suppression assay for functional assessment of regulatory T cells

5. PBMC exposure to HIV

HIV-1Bal was obtained from the Centre for AIDS Reagents NIBSC (repository reference: ARP118). Virus was propagated in phytohemagglutinin(PHA)-stimulated PBMC in RPMI-1640 media (Gibco) supplemented with 20% FBS (Sigma), 1% penicillin/ streptomycin (Gibco) and 10U/mL rhIL-2 (R&D Systems) for 7 days. As a mock control, supernatant from non-infected PBMC was also collected. Virus was titrated in TZM-bl cells (NIH AIDS Research and Reference Reagent Program, catalogue number: 8129) and stored at -80°C.

PBMC from healthy controls were cultured in the presence of HIV-1Bal (or mock) at a multiplicity of infection (MOI) of 0.3 and 0.03. To discard that PD-L1 upregulation requires HIV infection, PBMC were cultured in the presence of HIV-1Bal at 0.3 MOI, in the presence or absence of 5µM T20 (an HIV-entry inhibitor drug). After 4 hours at 37ºC, HIV-exposed cells were washed twice with warm R10 media to remove unattached virus. $0.5 \cdot 10^6$ PBMC/well were cultured in 48-well plates in R10 media in the presence or absence of 5µM T20. After 3 days, cells were harvested and stained to analyse PD-1 and PD-L1 expression on Treg cells by flow cytometry (Figure M3).

The efficacy of HIV-1Bal inhibition by T20 treatment was controlled by stimulating the virus-exposed cells from above with 5µg/ml PHA (Sigma) at day 3 and culturing them for further 7 days (Figure M3). The presence or absence of virus production was determined by a p24 HIV core antigen ELISA kit (Innogenetics). T20 treatment completely blocked HIV-1Bal infection under these conditions.

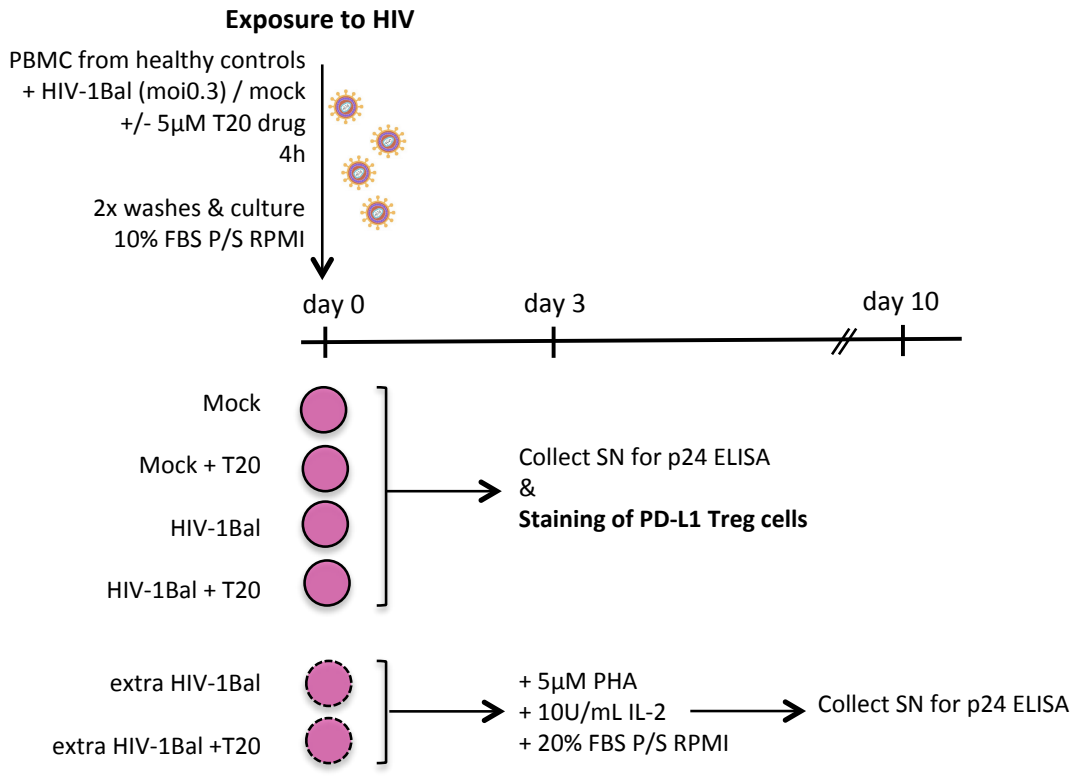

Figure M3. Graphical representation of PBMC exposure to HIV

6. p24 HIV core antigen quantification

ELISA

p24 HIV core antigen was quantified by a commercially available ELISA kit (Innogenetics). Culture supernatants after a 4-day culture in the presence of Gag peptides and anti-PD-L1 blocking or isotype control antibodies (as described in previous section, see Figure M1) were collected, centrifuged at 400g 5 minutes and stored frozen at -80ºC. ELISA was performed according to manufacturer's instructions and colorimetric reaction was measured at 450nm of wavelength.

7. Statistical analysis

Comparisons between two groups were performed using the Mann-Whitney *U* test, between more than two groups using Kruskal-Wallis test and within the same patient using Wilcoxon matched pairs test. Correlation coefficients (r) were calculated using the Spearman rank correlation test. Categorical variables between study groups were compared using Chi-squared and Fisher's exact test.

Statistical analyses were performed using GraphPad Prism 5.0 (San Diego, CA, USA) and SPSS 15.0 statistical software (Chicago, IL, USA). *p*-values (P) below 0.05 were considered significant and were indicated by asterisks: * $p<0.05$; ** $p<0.01$; *** $p<0.001$. Non-significant differences were indicated as "ns".

RESULTS

1. HIV infection induces PD-1 and PD-L1 on Treg cells

Previous studies have shown that (1) PD-1 is overexpressed on CD4- and CD8- T cells in several persistent infections and cancers, and (2) that this overexpression plays a key role in the exhausted phenotype of these cells (reviewed in Kim & Ahmed 2010). To first evaluate the expression of PD-1 and its ligand PD-L1 on Treg cells from HIV-infected individuals, we used the gating strategy of Miyara and colleagues (Miyara et al. 2009). It distinguishes between effector Treg cells (eTreg, CD4+CD45RA-FOXP3^{hi}) and resting Treg cells (rTreg, CD4+CD45RA+FOXP3^{lo}) (Figure R1A). The advantage over the traditional Treg cell characterization by CD4+CD25^{hi}CD127^{lo}FOXP3+ is that conventional CD4 T cells with an up-regulated CD25 and FOXP3 expression due to the generalized immune activation are excluded from the analysis (Simonetta & Bourgeois 2013). PBMC from HIV-infected individuals and healthy controls were isolated (Table M1), stained with fluorescence-labelled antibodies and characterized by flow cytometry.

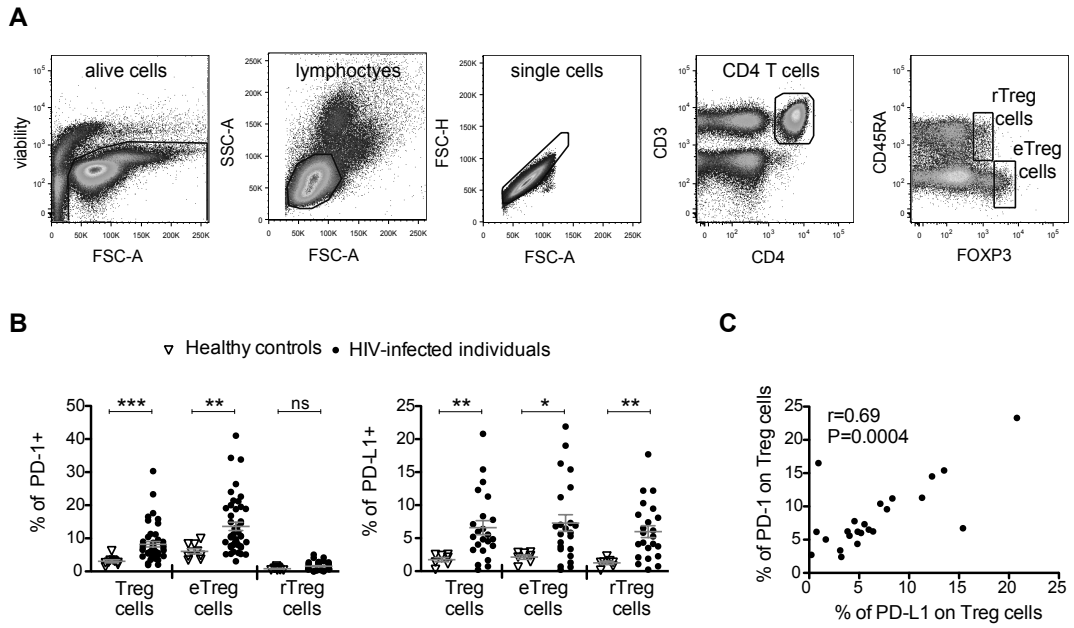

Figure R1. The percentage of PD-1- and PD-L1- expressing Treg cells is increased in HIV-infected individuals. (A) A representative flow cytometry plot showing the gating of effector Treg cells (eTreg: CD4+CD45RA-FOXP3^{hi}) and resting Treg cells (rTreg: CD4+CD45RA+FOXP3^{lo}). (B) Percentages of PD-1+ (left panel) and PD-L1+ (right panel) Treg (including eTreg and rTreg), eTreg and rTreg cell populations in HIV-infected individuals (black circles) and healthy controls (empty triangles). The mean ± SEM (standard error of the mean) is shown. Significant differences were determined by a Mann-Whitney U test and indicated by asterisks (*p <0.05; **p <0.01; ***p <0.001; ns: non significant). (C) Correlation between PD-1 expression and PD-L1 expression on Treg cells from HIV-infected individuals. Each dot represents the result from one individual. The Spearman's rank correlation coefficient (r) and the p value (P) are indicated.

A significantly higher percentage of PD-1+ Treg cells was observed for HIV-infected individuals (8.2% ± 0.8 SEM) compared with healthy controls (3.0% ± 0.4 SEM) (Figure R1-B left). This difference in PD-1 expression was due to PD-1 on effector Treg cells (13.6% ± 1.2 SEM) since very little PD-1 was expressed on resting Treg cells (1.5% ± 0.2 SEM). These observations are concordant with previous data (Lim et al. 2009; Rueda et al. 2013; Cho et al. 2014) and fit to the current understanding of PD-1 upregulation induced by T cell stimulation (Agata et al. 1996). We also found a higher percentage of PD-L1+ Treg cells for HIV-infected individuals (6.59% ± 1.1 SEM) compared with healthy controls (1.75% ± 0.33 SEM) (Figure R1-B right). However, in contrast to the expression pattern of PD-1, an increased percentage of PD-L1-expressing cells was observed for both, effector and resting Treg cells. In addition, the expression of PD-1 and PD-L1 on Treg cells from HIV-infected individuals correlated positively (Figure R1-C).

The differential distribution of PD-1 and PD-L1 on resting and effector Treg cells from HIV-infected individuals and healthy controls suggested that the virus itself could induce PD-L1 upregulation on Treg cells. To test this hypothesis, PBMC from healthy controls were isolated and exposed to HIV-1Bal containing supernatants without additional stimuli or additional interleukin-2. As controls, we used supernatants from non-infected PBMC that have been cultured under similar conditions as the infected cells. While culture supernatants from non-infected PBMC increased the frequency of PD-L1+ effector and resting Treg cells, virus exposure dramatically augmented this effect in a dose-dependent manner (Figure R2-B and C). In contrast, virus exposure had no effect on PD-1 expression (Figure R2-A).

To evaluate whether PD-L1 upregulation occurred without infection, we cultured PBMC with competent HIV-1Bal in presence and absence of T20 (an HIV-entry inhibitor drug). As shown in Figure R2-D, comparable percentages of PD-L1+ Treg cells were induced by the virus in presence and absence of T20. These data are in line with previous studies that demonstrate a PD-L1 upregulation upon HIV exposure in different cell populations including monocytes, macrophages, dendritic cells, neutrophils and CCR5+T cells (Meier et al. 2008; Boasso et al. 2008; Rodríguez-García et al. 2011; Bowers et al. 2014). When taken together, our results show an upregulation of PD-1 and PD-L1 on Treg cells of HIV-infected individuals that may be mediated by different routes and suggest that the Treg cell compartment is likely to be influenced by immunotherapy targeting the PD-1/PD-L1 pathway.

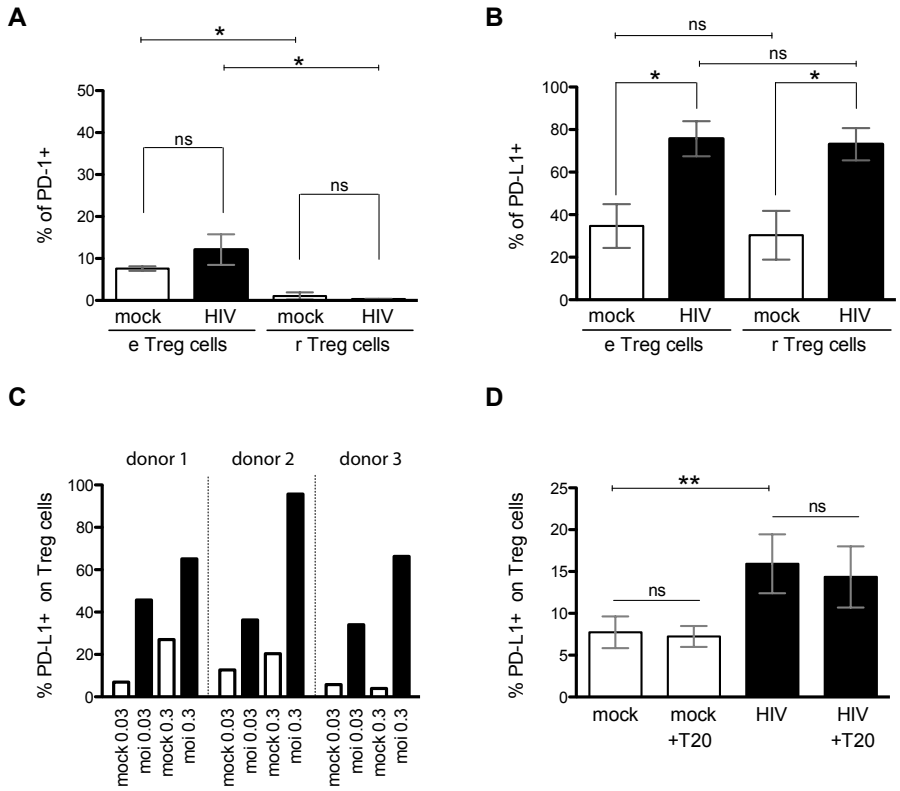

Figure R2. HIV exposure induced PD-L1 expression but not PD-1 on Treg cells. Panel A and B, expression of PD-1 (A) and PD-L1 (B) on Treg cells from healthy control's PBMC exposed to HIV-1 Bal (black bars) at 0.3 multiplicity of infection, compared with mock control (white bars). Bars represent the mean ± SEM from 7 different donors. (C) Expression of PD-L1 in Treg cells from 3 healthy control's PBMC (indicated as donor 1, donor 2, donor 3) exposed to HIV-1 Bal at 0.03 and 0.03 multiplicity of infection. (D) Expression of PD-L1 in Treg cells from healthy control's PBMC exposed to HIV-1 Bal at 0.3 multiplicity of infection in presence and absence of T20 (an HIV-entry inhibitor drug). Bars represent the mean ± SEM from 7 extra donors. In panels A, B and D significant differences were determined by Wilcoxon matched pairs test and indicated by asterisk (*p <0.05; **p <0.01). ns, non significant.

2. PD-1 expression on Treg cells is associated with disease progression

PD-1 expression on CD4- and CD8- T cells correlates with HIV disease progression (Zhang et al. 2007; Porichis et al. 2011; Day et al. 2006). To test whether the same is true for Treg cells, we analysed PD-1 expression on these cells from HIV-infected individuals categorized into 4 groups according to CD4 count and viral load (Table M2). The highest PD-1 expression on Treg cells was found in the HIV study group with the lowest CD4 T cell counts and the highest viral loads (Figure R3-B). As many as 13.6% ± 2.3 SEM of Treg cells where PD-1+ in this group whereas only 4.7% ± 0.4 SEM of Treg cells where PD-1+ in the group of individuals under combination antiretroviral therapy (cART). Although PD-1 expression dropped with cART we still found higher PD-1 on

Treg cells from treated patients compared with healthy controls (3.1% ± 0.4 SEM). In contrast, PD-1 expression on total CD4- and CD8- T cells was normalized in patients under cART (Figure R4-A). Within viremic individuals, PD-1 expression on total CD8 T cells was high irrespective of the CD4 count, whereas PD-1 expression on Treg cells and total CD4 T cells was higher in individuals with low CD4 counts (<500 CD4/μL of blood).

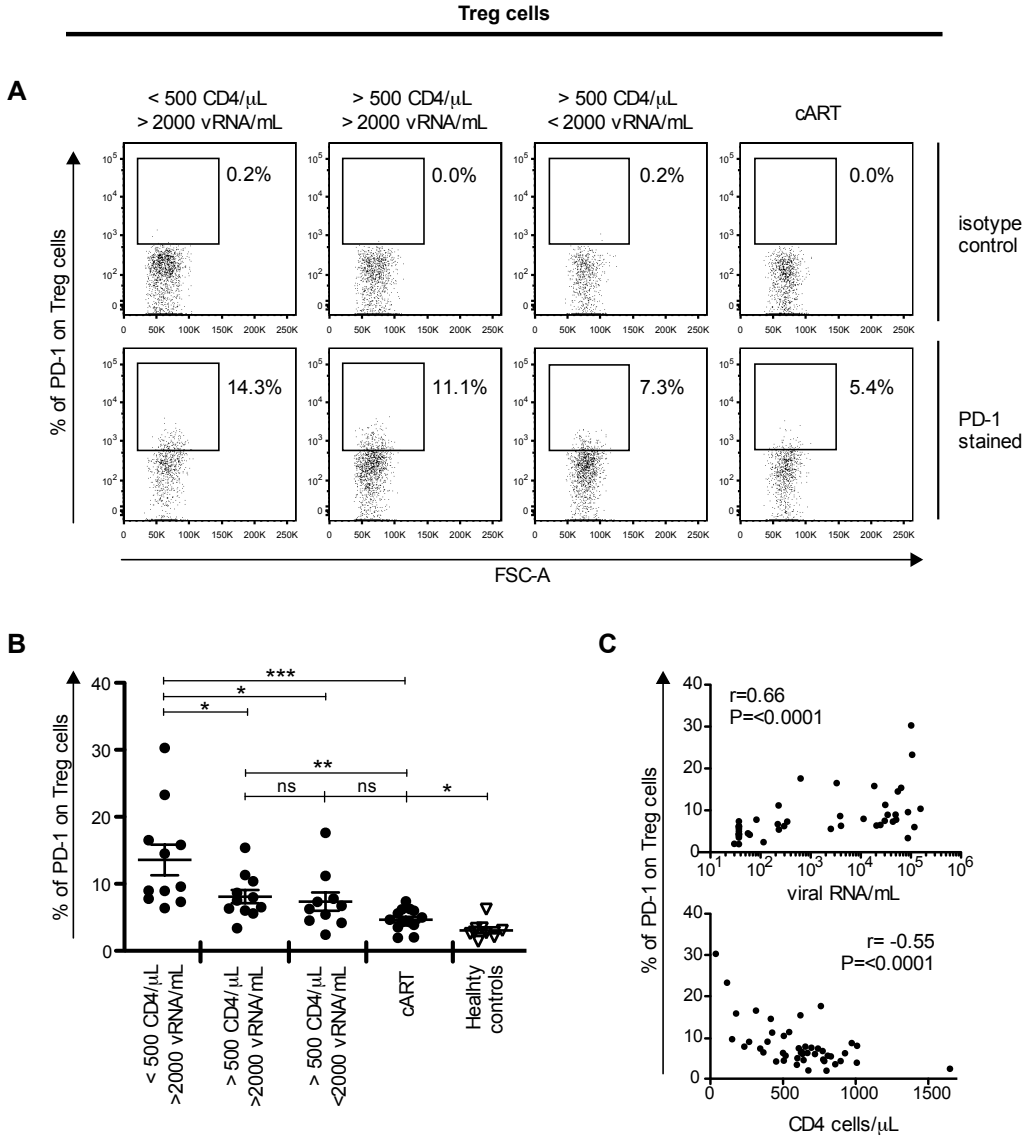

Figure R3. PD-1 expression on Treg cells correlates with markers of disease progression. (A) Representative flow cytometry dot plots showing PD-1 gating on Treg cells (including effector and resting Treg). One HIV-infected individual from each study group is displayed. Numbers indicate the percentage of PD-1+ Treg cells in PD-1 stained samples (down) compared with isotype control antibodies (up). (B) PD-1 expression on Treg cells from different HIV-infected study groups (black circles) and healthy controls (empty triangles) are shown as indicated. Each dot represents the result from one individual.

From Figure R3: The mean ± SEM is shown. Significant differences were determined by a Mann-Whitney U test and indicated by asterisks (*p <0.05; **p <0.01; ***p <0.001; ns: non significant). (C) Correlation of PD-1 expression on Treg cells from HIV-infected individuals with viral loads (up) and CD4 counts (down), respectively. Spearman's rank correlation coefficients (r) and p values (P) are indicated.

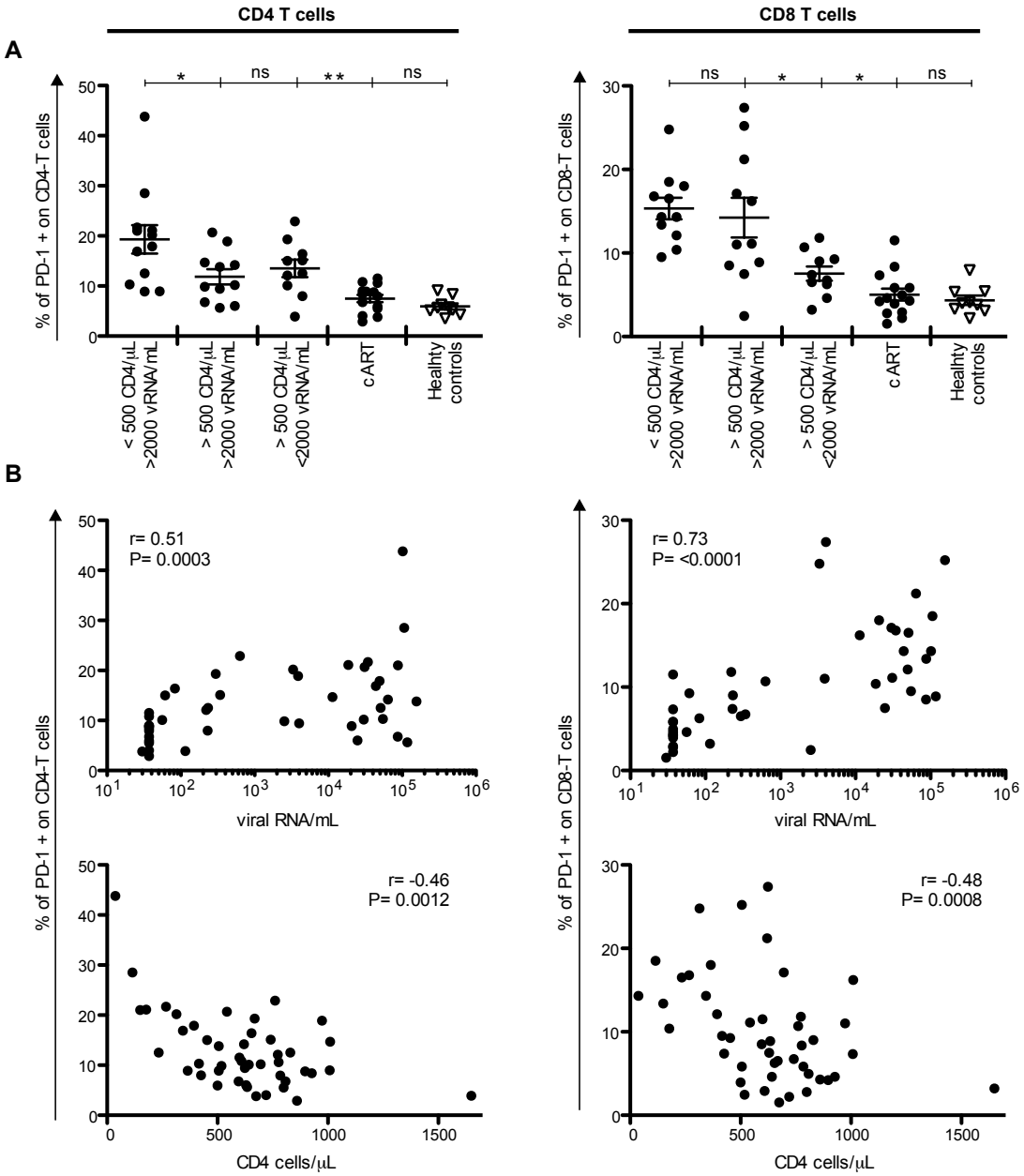

Figure R4. PD-1 expression on CD4- and CD8- T cells. (A) Percentages of PD-1-expressing CD4- and CD8- T cells from HIV-infected individuals (black circles) and healthy controls (empty triangles) are shown. The mean ± SEM is shown. Significant differences were determined by a Mann-Whitney U test and indicated by asterisks (*p <0.05; **p <0.01; ns: non significant). (B) Correlations of PD-1 expression on CD4- and CD8- T cells with viral loads and CD4 T cell counts are shown, respectively. Each dot represents the result from one individual. Spearman's rank correlation coefficients (r) and p values (P) are given for each correlation.

Consistently, PD-1 on Treg cells correlated positively with viral load and negatively with CD4 T cell counts (Figure R3-C). This is concordant with previous observations made for CD4- and CD8- T cells from HIV-infected individuals (Day et al. 2006; Zhang et al. 2007; Porichis et al. 2011) and reproduced here with individuals of our study groups (Figure R4-B). To further examine the relation between PD-1 expression on Treg cells and antigen exposure we followed 5 patients before and after antiretroviral treatment interruptions. Samples were collected from the same patient at 4 time-points: (1) before starting treatment, (2) during treatment, (3) upon interruption of treatment, and (4) after restarting treatment (characteristics of HIV-infected individuals in Table M3, group A). As can be seen in Figure R5 the percentage of PD-1-expressing Treg cells followed viremia and cART reduced PD-1 expression on Treg cells.

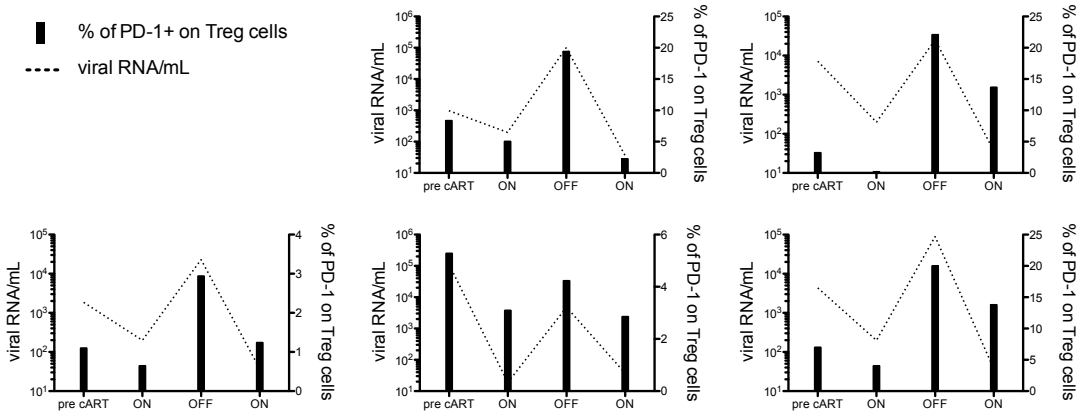

Figure R5. PD-1 expression on Treg cells follows HIV viremia. PD-1 expression on Treg cells (black bars) from 5 HIV-infected individuals followed longitudinally before and after antiretroviral treatment interruptions (pre-cART, on, off, on, respectively). Each graph represents one individual. The dashed line represents the plasma viral load.

PD-1 is a negative regulator of the proliferative capacity in effector T cells. To characterize the relationship between PD-1 expression and the proliferative capacity of Treg cells, PBMC from individuals of the different HIV study groups were labelled with CFSE, stimulated with HIV gag peptides and analysed for proliferation by CFSE dilution via flow cytometry. As shown in Figure R6-A, the proliferative capacity of Treg cells was strikingly impaired in non-treated individuals. It correlated positively with CD4 T cell counts and negatively with viral loads (Figure R6-B and C) and PD-1 expression on Treg cells prior to stimulation (Figure R6-D). These observations parallel those reported for effector T cells (Day et al. 2006) and suggest a negative role of PD-1 for Treg cell proliferation.

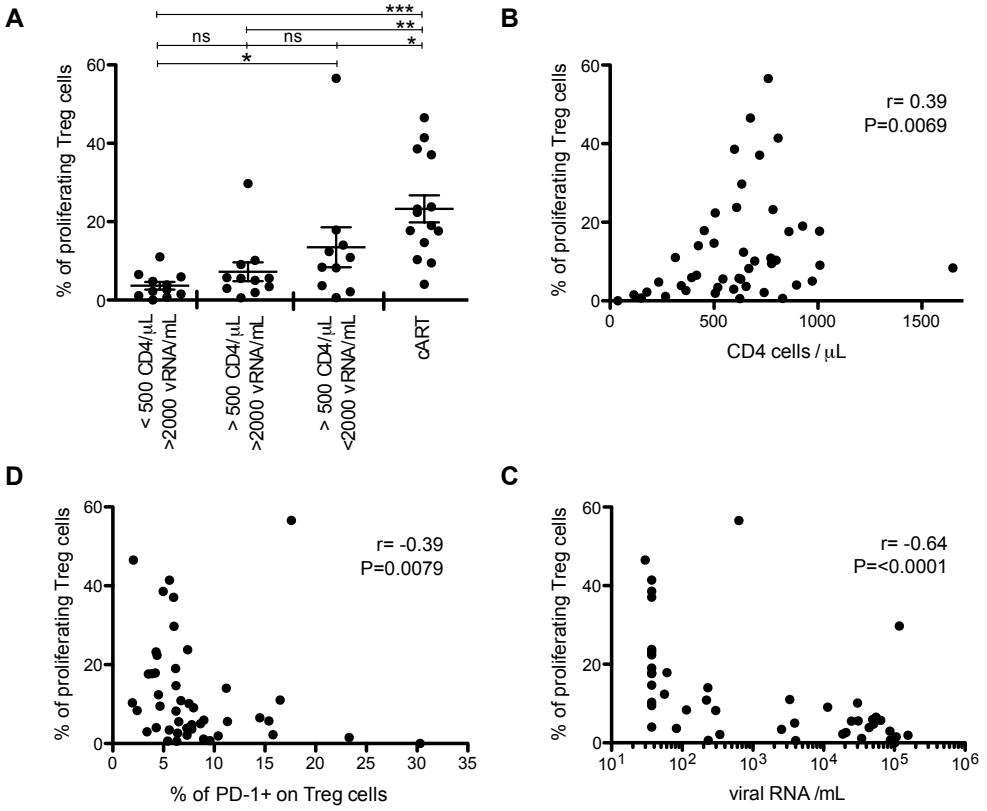

Figure R6. Treg cells from viremic individuals show impaired proliferative capacity that correlates with PD-1 expression. Each dot represents the result from one HIV-infected individual. The percentage of proliferating Treg cells after 6-day gag peptide stimulation of PBMC (A) from different HIV-infected study groups is given as indicated. The mean ± SEM is shown. Significant differences were determined by a Mann-Whitney U test and indicated by asterisks (*p <0.05; **p <0.01; ***p <0.001; ns: non significant). Panels B to D show correlations between the percentage of proliferating Treg cells and CD4 T cell counts (B), HIV viral load (C), and PD-1 expression on Treg cells before stimulation (D), respectively. Spearman's rank correlation coefficients (r) and p values (P) are indicated.

3. PD-L1 blockade increases the proliferative capacity of Treg cells but not their suppressive capacity *per cell*

To analyse the impact of a PD-L1 blockade on the proliferative capacity of Treg cells from HIV-infected individuals, CFSE-labelled PBMC were cultured in the presence of HIV gag peptides and PD-L1 blocking antibody or an isotype control antibody. Cell proliferation was quantified by CFSE dilution via flow cytometry. A significant gain on the proliferative capacity of Treg cells, as well as that of effector CD4- and CD8- T cells, is shown in Figure R7-B as fold change in proliferation relative to the isotype antibody control stimulations. PD-L1 blockade led to a roughly 2 fold mean increase in the percentage of proliferating Treg cells, comparable to that of effector CD4- and CD8- T

cells. The range of responses was broad. The increase in Treg cell proliferation correlated positively with the viral load of the analysed individuals (Figure R7-C).

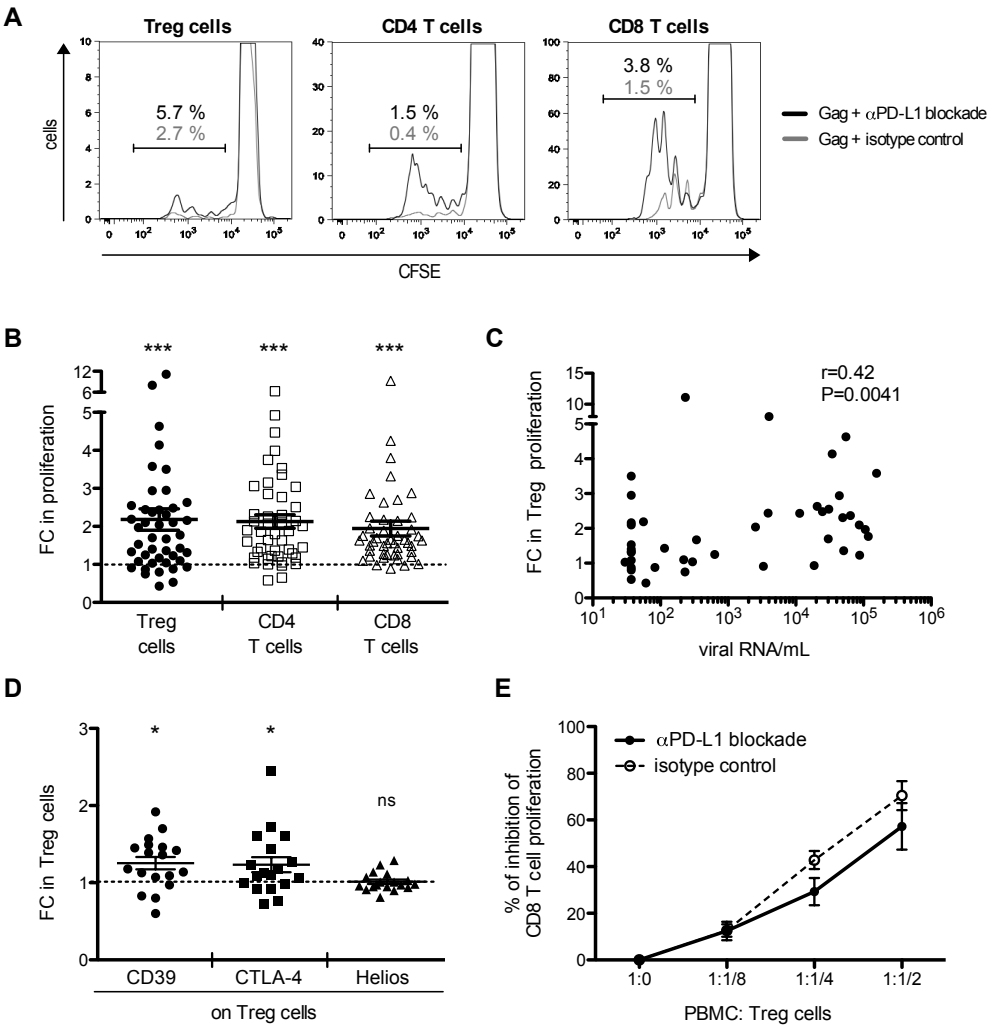

Figure R7. PD-L1 blockade increases Treg cell proliferation but not its suppressive capacity *per cell*. In panels A to D, PBMC from HIV-infected individuals were stimulated with gag peptides for 6 days in the presence of PD-L1 blocking antibody or isotype control antibody. (A) Flow cytometry histograms from an HIV-infected individual with <500 CD4/µL and >2000 viral RNA copies/mL blood showing CFSE dilution on Treg, CD4- and CD8- T cells in presence of PD-L1 blocking antibody (black line) or isotype control antibody (grey line). In panels B to D, each dot represents the result from one individual. FC (fold change) is calculated as the ratio between PD-L1 blockade condition and isotype control condition. The dashed line in panels B and D (FC = 1) indicates no change due to PD-L1 blockade. The mean ± SEM is shown. Significant differences between PD-L1 blockade conditions and isotype controls were determined by a Wilcoxon matched pairs test and indicated by asterisks (*p <0.05; ***p <0.001; ns: non significant). (B) Fold change in the proliferation of Treg (black circles), CD4 (empty squares) and CD8 (empty triangles) cell populations were determined by CFSE assay. (C) Correlation between the fold change in proliferating Treg cells and HIV viral load. (D) Fold change in the frequencies of CD39-, CTLA4- or Helios- expressing Treg cells.

From Figure R7: (E) PBMC from HIV-infected individuals were stimulated with gag peptides in the presence of a PD-L1 blocking antibody or an isotype control antibody, after 6 days Treg cells were isolated and co-cultured with CFSE-labelled-PBMC at different PBMC-to-Treg cell ratios. The percentage of inhibition of CD8 proliferation after anti-CD3/anti-CD28 T cell stimulation is shown as a function of different PBMC-to-Treg cell ratios. Black circles connected with a solid line correspond to suppression assays performed with Treg cells from PD-L1 blockade conditions whereas empty dots connected with a dashed line correspond to suppression assays performed with Treg cells from isotype control conditions. Mean and SEM from 4 independent experiments are indicated.

To further analyse the functional consequences that a PD-L1 blockade may have on Treg cell function after an antigenic stimulation, we analysed the increase of Treg cells expressing effector molecules such as CD39 and CTLA4 as well as their suppressive capacity. First, upon PD-L1 blockade, we observed a slight but significant increase in the frequency of CD39- and CTLA4- expressing Treg cells relative to the control condition. These increases were also significant relative to Helios (p=0.027)(Figure R7-D). The latter is a transcription factor suggested to identify thymic Treg cells, and used as a control. As expected, the frequency of Helios-expressing Treg cells did not increase upon PD-L1 blockade. Second, to test the capacity of expanded Treg cells to suppress CD8 T cell proliferation, Treg cells were isolated from PBMC after a 6-day-culture in the presence of a PD-L1 blocking antibody or an isotype control antibody. Then isolated Treg cells were co-cultured with CFSE-labelled PBMC in the presence of anti-CD3/anti-CD28 and interleukin-2. After 3 days, proliferation of CD8 T cells was quantified by analysing CFSE profiles by flow cytometry. A dose-dependent inhibition of CD8 T cell proliferation was observed that was not significantly different from that of isolated Treg cells expanded under control conditions (Figure R7-E). Thus the presented data suggest that the relief of the PD-1/PD-L1 interaction during expansion does not alter the suppressive capacity of Treg cells on a *per cell* basis.

4. The proliferative capacity of Treg cells and CD8 T cells from HIV-infected individuals is differentially restored by PD-L1 blockade and depends on the plasma viremia of the host

To analyse whether the PD-L1 blockade-mediated restoration of the proliferative capacity of Treg, CD4- and CD8- T cells as shown in Figure R7-B was dependent on the HIV infection stage of the host, the respective data points were grouped according to CD4 T cell count, viral load and antiretroviral treatment (patient grouping as of Table M2). The PD-L1 blockade significantly increased the proliferation of Treg cells from patients with high viremia irrespective of their CD4 T cell count (Figure R8-A). Treg cells from patients that controlled viremia (either spontaneously or by cART) showed no significant increase in proliferation compared with the control. In contrast, PD-L1

blockade significantly increased CD8 T cell proliferation for all 4 patient groups with respect to the control (Figure R8-A).

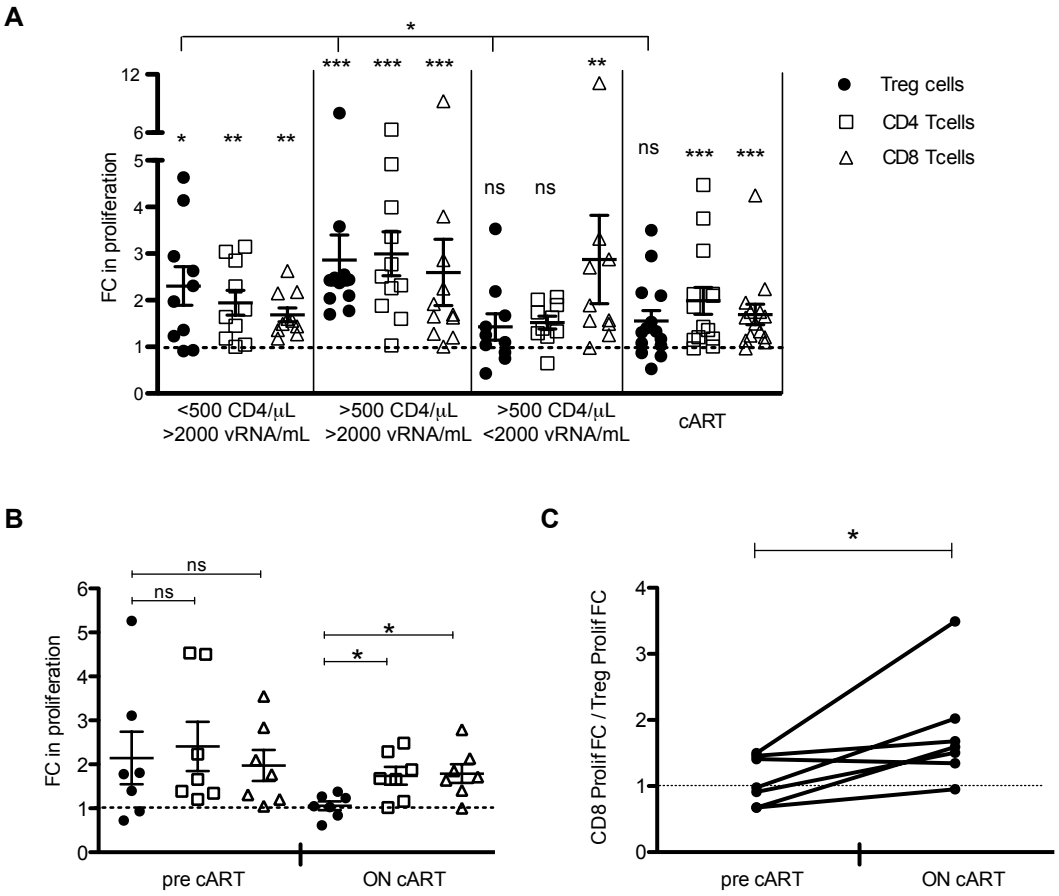

Figure R8. PD-L1 blockade differentially increases Treg and CD8 T cell proliferative capacity depending on host viremia. PBMC from HIV-infected individuals were stimulated with gag peptides for 6 days in the presence of a PD-L1 blocking antibody or an isotype control antibody. Proliferation was determined by CFSE dilution (A) and alternatively by Ki67 staining (B and C). FC (fold change) in proliferation is calculated as the ratio between PD-L1 blockade condition and isotype control condition. Each symbol represents the result from one individual. In panels A and B, the dashed line (FC = 1) indicates no change due to PD-L1 blockade. The mean ± SEM is shown. (A) Given are the FC in proliferation of Treg (black circles), CD4 (empty squares) and CD8 (empty triangles) T cell populations of different HIV-infected study groups as indicated. Significant differences between PD-L1 blockade conditions and isotype controls were determined by a Wilcoxon matched pairs test and indicated by asterisks (*p <0.05; ***p <0.001; ns: non significant). Significant differences in FC of proliferation of Treg cells among the 4 HIV study groups were determined using Kruskal-Wallis test. (B) Fold changes in proliferation of Treg, CD4- and CD8- T cell populations upon PD-L1 blockade, were measured longitudinally in 7 individuals before and after >2 years of antiretroviral treatment (pre cART and ON cART, respectively). Significant differences in FC of proliferation between Treg, CD4- and CD8- T cells were determined by a Wilcoxon matched pairs test (*p <0.05; ns: non significant). (C) Ratio between the FC in proliferation of CD8 T cells and FC in proliferation of Treg cells upon PD-L1 blockade. The dashed line (FC = 1) indicates the same FC in proliferation for CD8 T and Treg cells upon PD-L1 blockade. Significant differences between the ratios before and after antiretroviral treatment were determined by Wilcoxon matched pairs test (*p <0.05).

To confirm that PD-L1 blockade differentially impacts Treg cells and CD8 T cells depending on the plasma viremia of the host, fold changes in proliferation upon PD-L1 blockade were measured longitudinally in 7 individuals before and after antiretroviral treatment (pre cART and ON cART, respectively). In samples before cART, PD-L1 blockade increased Treg cell proliferative capacity by approximately 2 fold, which is comparable to that of effector CD4- and CD8- T cells. However, in samples from the same patients after a cART period, PD-L1 blockade had no significant effect on Treg cell proliferative capacity. In contrast, on effector T cells proliferative capacity was increased 2 fold upon PD-L1 blockade comparable to that of pre cART samples (Figure R8-B). As shown in Figure R8-C, PD-L1 blockade preferentially increased the proliferative capacity of effector T cells over regulatory T cells in samples from individuals under cART. Altogether the presented data indicate that the net gain of T cell effector function after PD-L1 blockade may critically depend on plasma viremia.

5. PD-L1 blockade enhances HIV reactivation, which is related to an increased percentage of Treg cells

To analyse the consequences of the PD-L1 blockade for *ex vivo* HIV reactivation, supernatants of the above-described PBMC cultures were collected and tested for viral production by HIV p24 antigen determination. HIV production was detectable in most PBMC cultures from the viremic patient groups (16 from 19 samples) (Figure R9-A). The blockade of PD-L1 consistently increased viral production relative to the isotype antibody control stimulations. This increase in HIV production correlated with the increase in CD4 T cell proliferation and the fraction of Treg cells in the lymphocyte population (Figure R9-B and R9-C). Furthermore, the increase in HIV production correlated with the decrease in CD4 T cell to Treg cell ratio as well as CD8 T cell to Treg cell ratio (Figures R9-D and E). Together this suggests that the inhibitory function of the Treg cells rather than their capacity of being an HIV target cell may play a role in virus expansion under these conditions.

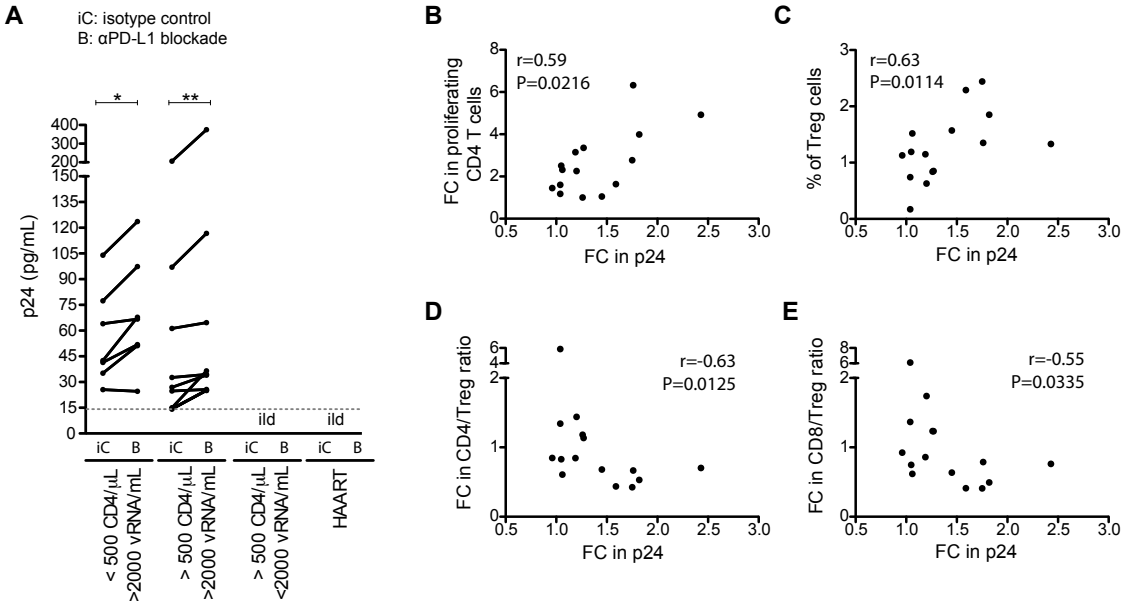

Figure R9. Virus reactivation upon PD-L1 blockade is related to increased percentage of Treg cells. (A) PBMC were stimulated with gag peptides in the presence of PD-L1 blocking antibody or isotype control antibody. After 4 days in culture, supernatants were harvest to quantify the p24 HIV core antigen by ELISA. The dashed line indicates the ELISA cut-off. Significant differences were determined by a Wilcoxon matched pairs test (*p <0.05; **p <0.01). Iid: inferior to the limit of detection. Panels B to E show correlations between Fold change (FC) in p24 and FC in percentage of proliferating CD4 T cells (B), percentage of Treg cells (C), FC in the CD4 T cell to Treg (CD4/Treg) percentage ratio (D), and FC in the CD8 T cell to Treg (CD8/Treg) percentage ratio (E); respectively. Spearman's rank correlation coefficients (r) and p values (P) are indicated.

The results from this thesis have been submitted as a journal article that is under revision:

PD-L1 Blockade Differentially Impacts Regulatory T Cells from HIV-Infected Individuals Depending on Plasma Viremia

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DISCUSSION

In this thesis I studied PD-1 and PD-L1 expression on regulatory T cells (Treg cells) from human immunodeficiency virus (HIV)-infected individuals, and explored the consequences of *ex vivo* PD-L1 blockade on this cell subset. The presented findings provide new insights into the biology of Treg cells in HIV infection and elucidate previously unknown PD-L1 blockade effects on the HIV immune response and virus reactivation.

1. PD-1 and PD-L1 on Treg cells in HIV infection

Our results expand previous work on PD-1-expressing Treg cells from HIV-infected individuals. We described an increase in the percentage of PD-1-expressing Treg cells in HIV-infected individuals compared with healthy controls, in line with previous reports that used classical Treg cell gating (Rueda et al. 2013; Lim et al. 2009; Cho et al. 2014). We confirmed this observation using a different gating strategy that allowed us to better identify *bona fide* Treg cells (Simonetta & Bourgeois 2013). In addition, using this gating strategy we observed that the difference in PD-1 expression on Treg cells between HIV-infected patients and healthy controls was due to PD-1 expression on eTreg cells. We found that very little PD-1 was expressed on rTreg cells, which is consistent with previous reports that described PD-1 upregulation in activated T cells (Agata et al. 1996; Raimondi et al. 2006). Next, we analysed PD-1-expressing Treg cells in patients categorized into 4 groups according to CD4 T cell counts, viral load and antiretroviral treatment. Relative to healthy controls, the frequency of PD-1-expressing Treg cells was significantly increased in all 4 groups of HIV-infected individuals. It correlated with markers of disease progression: positively with virus load and negatively with CD4 T cell counts. Consistently, PD-1 expression on Treg cells dropped under antiretroviral treatment. With this, PD-1 on Treg cells followed the same trend as PD-1 on total CD4- and CD8- T cells as previously described in several reports (Day et al. 2006; Zhang et al. 2007; Porichis et al. 2011).

The observed PD-1 upregulation on regulatory T cells is intriguing. A coordinated up-regulation of a co-inhibitory receptor on both effector T cells and regulatory T cells seems counterintuitive. However, it is consistent with the model of dynamic co-evolution of memory and regulatory T cells at sites of infection (Akbar et al. 2007; Vukmanovic-Stejic et al. 2008) and data from a subsequent study on Treg cells in hepatitis C virus (HCV) infection (Franceschini et al. 2009; Barnaba & Schinzari 2013). According to this model, virus expansion triggers an effector T cell response, but effector T cells cause collateral tissue damage. To control this damage, effector T cells are suppressed by PD-1 and also by expanding Treg cells. To limit exaggerated suppression and achieve homeostasis, Treg cell expansion is also controlled by PD-1 on

Treg cells. The data from our cross-sectional study do not enable the analysis of the temporal appearance of the PD-1-expressing T cell subsets. However, the observed distinct expression of PD-1 on Treg and CD8 T cells could reflect differences in the activation and expansion of these subsets during HIV disease progression, being concordant with this model of an antigen-driven coordinated response.

The observed correlation of PD-1 on Treg cells with patient's viral load is consistent with the idea that persistent antigen exposure is a main trigger of PD-1 expression (Agata et al. 1996; Day et al. 2006; Trautmann et al. 2006; Zhang et al. 2007). However, exposure of PBMC to HIV under non-stimulating conditions did neither induce PD-1 on effector nor on resting Treg cells significantly, thus suggesting that additional activation signals or direct infection are required. Interestingly, however, exposure to HIV was sufficient to massively up-regulate PD-L1 on both eTreg and rTreg cells. This might explain why we found an increase in the percentage of PD-L1-expressing resting and effector Treg cells in HIV-infected individuals compared with healthy controls, and why we did not observe differences in PD-L1 expression between effector and resting Treg cells. The observed massive upregulation of PD-L1 upon HIV exposure was in agreement with previous observations were exposure to either competent or AT-2-inactivated virus led to upregulation of PD-L1 in several cell subsets such as monocytes, DC, CCR5+ T cells and neutrophils (Meier et al. 2008; Boasso et al. 2008; Rodríguez-García et al. 2011; Planès et al. 2014; Bowers et al. 2014).

As PD-L1 can mediate suppression (Baecher-Allan et al. 2003; Francisco et al. 2009; Gotot et al. 2012), an interesting question that arises from the data presented in this thesis is whether rTreg cells could exert PD-L1-mediated suppressive capacity without TCR activation and how relevant this could be in the *in vivo* context. Although we found a low percentage of PD-L1-expressing Treg cells in PBMC from HIV-infected individuals, one could expect much higher PD-L1 expression in other anatomical sites such as lymph nodes where viral loads are higher (Pantaleo et al. 1991). A mechanism of suppression exerted by rTreg cells could be relevant because rTreg cells are preferentially preserved during HIV, while eTreg cells are depleted very early during infection (Simonetta et al. 2012). In addition to PD-L1 mediated suppression, PD-L1 on Treg cells could induce the generation of more Treg cells in the periphery through PD-1/PD-L1 interaction as previously described (Aramaki et al. 2004; Krupnick et al. 2005; Wang et al. 2008; Francisco et al. 2009; Periasamy et al. 2011; Amarnath et al. 2011; Trinath et al. 2012). Therefore, PD-L1 induction mediated by HIV might increase the overall suppressive capacity of the Treg cell population by increasing Treg cell generation and their suppressive capacity, further contributing to effector T cell impairment.

The presented data collectively suggests that HIV infection leads to expression of PD-1 and PD-L1 on Treg cells. This is consistent with the notion that PD-1 and PD-L1 can be upregulated by the virus itself and by immune activation, which is a hallmark of HIV-infection (Agata et al. 1996; Muthumani et al. 2008; Keir et al. 2008; Kinter et al. 2008; Meier et al. 2008; Boasso et al. 2008; Rodríguez-García et al. 2011; Planès et al. 2014; Bowers et al. 2014). These data are also in line with the observation of higher PD-1-expressing T cells, B cells and NK cells (Day et al. 2006; Trautmann et al. 2006; Souza et al. 2007; Zhang et al. 2007; Nicholas et al. 2013; Norris et al. 2012) as well as higher PD-L1-expressing dendritic cells, macrophages, B cells, T cells and neutrophils in HIV-infected individuals compared with healthy controls (Trabattoni et al. 2003; Sachdeva et al. 2010; Rosignoli et al. 2007; Bowers et al. 2014). In conclusion, the up-regulation of PD-1 and PD-L1 in Treg cells suggests that this cell subset is also likely to be influenced by immunotherapy targeting the PD-1/PD-L1 pathway. How this compartment will be influenced will depend on the yet to be defined role of the PD-1/PD-L1 pathway in regulatory T cells in HIV-infected individuals, as well as PD-1 and PD-L1 expression at different anatomical sites during HIV disease progression.

2. Are Treg cells impaired during HIV infection?

PD-1 has been described as an exhaustion marker for effector T cell. In HIV infection the percentage of PD-1-expressing CD4- and CD8- T cells is higher than in healthy controls. PD-1 is mainly upregulated on HIV- specific CD4- and CD8- T cells, which fail to clear the virus. It is associated with an impaired proliferative capacity and reduced cytokine production (Day et al. 2006; Trautmann et al. 2006; Zhang et al. 2007; Souza et al. 2007; Porichis et al. 2011). Likewise, PD-1 has been associated with impaired regulatory T cells in HCV infection (Franceschini et al. 2009). In particular, PD-L1 blockade restored Treg cell proliferative capacity and increased STAT5 phosphorylation, demonstrating that PD-1 inhibits Treg cell proliferation by inhibiting the IL-2 signalling cascade (Franceschini et al. 2009).

In line with this previous study of HCV infection, the data presented in this thesis suggest that Treg cell proliferative potential might be compromised in HIV infection by PD-1 expression as well. In this context, PD-1 may stand as a negative regulator of effector T cells, but also of regulatory T cells in HIV infection. We observed that (1) *ex vivo* Treg cell proliferative capacity was strikingly impaired in non-treated patients in response to gag peptides, (2) Treg cell proliferative capacity inversely correlated with PD-1 expression on Treg cells (prior to stimulation) and (3) PD-L1 blockade on PBMC increased Treg cell proliferation. In view of these data and previous literature

(Franceschini et al. 2009), we attribute the impaired Treg cell proliferative capacity partly to PD-1 expression on Treg cells. However, further work will be needed to validate whether PD-1 directly inhibits Treg cells from HIV-infected individuals, as well as to define whether the impaired *ex vivo* Treg cell proliferation is physiologically significant *in vivo*.

Other studies have instead explored *ex vivo* Treg cell proliferative capacity in HIV-infected individuals by directly analysing Ki67 expression without reestimulation (Xing et al. 2010; Bi et al. 2009). These studies showed that Ki67 expression in Treg cells and conventional CD4 T cells correlate with immune activation and disease progression (negatively with CD4 counts and positively with plasma viral load). In particular, Treg cells and conventional CD4 T cells from individuals with low CD4 counts presented the highest levels of Ki67, which decreased under antiretroviral treatment. While these studies analysed *ex vivo* Ki67 in the whole Treg cell subset, we analysed the proliferative capacity of Treg cells in response to HIV gag peptide stimulation. In our experimental approach, Treg cells that proliferated might be a mixture of HIV-specific Treg cells and also Treg cells that proliferated in a bystander manner as a result of HIV-specific CD4- and CD8- T cell proliferation. In this context, Treg cells that proliferate in a bystander manner might be less frequent in viremic individuals, in whom HIV-specific CD4- and CD8- T cell responses are also diminished.

Besides Treg cell proliferative capacity, there is still no consensus on whether Treg cell suppressive capacity is preserved during HIV infection. Conflicting data have been reported probably as a result of different methodological approaches (i.e. kind of stimulation, groups of patients and Treg cell phenotype used). Some studies showed evidences supporting that Treg cells maintain their suppressive capacity in chronic HIV infection (Moreno-Fernandez et al. 2009; Angin et al. 2012; Phetsouphanh et al. 2015). In particular, Treg cells from healthy controls and HIV-infected individuals (including elite controllers and chronic progressors) were able to suppress T cell proliferation in a similar way when stimulated with CD3/CD2/CD28 coated beads (Angin et al. 2012). However, other studies that used CD3 or HIV-specific stimulation reported that Treg cell suppressive capacity is compromised during HIV-infection. For instance, it has been shown that Treg cells from high viremic individuals display a lower suppressive capacity compared to those from low viremic individuals (Kinter et al. 2004; Tsunemi et al. 2005), and that Treg cells from treated-individuals that do not recover CD4 counts present reduced suppressive capacity (Mendez-Lagares et al. 2012). In this context, our data on impaired Treg proliferation also supports that Treg function is compromised in HIV infection.

3. How could PD-L1 blockade impact Treg cells from HIV-infected individuals?

Inhibiting PD-1 signalling has a potential therapeutic value for treating cancers and persistent viral infections (Okazaki et al. 2013; Harvey 2014; Pauken & Wherry 2015; Nguyen & Ohashi 2015; Velu et al. 2015; Kamphorst et al. 2015). In cancer, the first PD-1/PD-L1 inhibitors have been approved to treat melanoma (Sheridan 2014) and the approval for other types of cancer is expected in the near future. In persistent infections, the PD-1/PD-L1 pathway blockade showed encouraging therapeutic effects in animal models (Velu et al. 2009; Shetty et al. 2012; Fuller et al. 2013; Palmer et al. 2013) and in a clinical trial with HCV-infected patients. In this clinical trial, one single dose of anti-PD-1 antibody led to a reduction in viral loads in 10% of individuals, and remarkably in one patient, HCV remained undetectable one year later (Gardiner et al. 2013). In the case of HIV infection, several studies showed that *in vitro* blockade of PD-L1 restored T cell effector responses and immunoglobulin production (Day et al. 2006; Trautmann et al. 2006; Souza et al. 2007; Porichis et al. 2011; Cubas et al. 2013). After this promising results in pre-clinical studies, a phase I clinical trial has started to evaluate the safety of immunotherapy based on PD-L1 blocking antibodies in HIV-infected patients under antiretroviral treatment (www. clinicaltrials.gov; identifier: NCT02028403).

In this thesis, we observed that PD-L1 blockade impacts not only effector T cells but also Treg cells from HIV-infected individuals. We showed that PD-L1 blockade affects the proliferative capacity of Treg cells. The observed increase in Treg cell proliferation upon PD-L1 blockade is consistent with a previous report on Treg cells from HCV-infected individuals (Franceschini et al. 2009), and also with previous observations in the friend virus infection model. In this mouse model for chronic infection, *in vivo* PD-L1 blockade resulted in an increased number of activated Treg cells in the spleen (Joedicke et al. 2014). In addition, it has been reported that the blockade of other coinhibitory receptors such as Tim-3 also increases Treg cell proliferation (Moorman et al. 2012), further supporting the notion that some inhibitory receptors regulate effector and regulatory T cells in a similar manner.

We showed that PD-L1 blockade has an effect on Treg cells proliferative capacity, but this effect differed among groups of patients. *Ex vivo* PD-L1 blockade significantly increased the proliferation of Treg cells from viremic patients but had no significant effect on the proliferation of Treg cells from patients in whom viremia is controlled (either spontaneously or by combination antiretroviral therapy: cART). In these latter patients, PD-1 expression was low and the Treg cell proliferative capacity was

remarkably high without signs of impairment. This observation was corroborated by experiments on longitudinal samples that allowed us to eliminate the bias caused by different host and virus genetics. We observed a significant increase in Treg cell proliferation upon PD-L1 blockade only in pre-cART samples and not in cART samples, as well as a reduction in PD-1 expression on Treg cells upon antiretroviral treatment.

In our study, a high HIV viremia was associated with PD-1 expression, an impaired Treg cell proliferative capacity and a greater impact of PD-L1 blockade. However, differences in the fold change of Treg cell proliferation might be explained by other factors in addition to PD-1 expression on Treg cells. One good candidate for this is IL-2, which is produced upon T cell stimulation but may have been limiting. IL-2 is essential for Treg cells and may have been scarce particularly in viremic individuals due to PD-1-mediated exhaustion of CD4 T cells, the main IL-2 producers. Consistent with this view are previous observations showing that exogenous IL-2 can overcome PD-1/PD-L1-mediated inhibition of proliferation (Carter et al. 2002; Bennett et al. 2003). Besides IL-2, alternative mechanisms might contribute to the differences in the fold change of proliferating Treg cells. Among these alternative mechanisms there are CD80 and PD-L1. CD80 is another known ligand for PD-L1 (Butte et al. 2007), so the effects of the PD-L1 blockade might have been mediated through CD80. Regarding PD-L1, we found that the percentage of PD-L1- and PD-1- expressing Treg cell correlated positively. On the basis of this correlation and previous evidence on PD-L1 inhibitory signalling into the PD-L1-expressing cell (Hirano et al. 2005; Azuma et al. 2008; Kuipers et al. 2006), one might also hypothesize that the PD-L1 blockade effects could have been partly mediated via PD-L1 expressed on Treg cells.

We can discard that differences in the fold change of proliferating Treg cells upon PD-L1 blockade were caused by different PD-L1 availability among the HIV-study groups. We did not find significant differences in PD-L1 expression on PBMC between the studied groups (including monocytes that are the main PD-L1-expressing cells in PBMC) (Annex 2). These results are in good agreement with previous data (Porichis et al. 2011), but opposite to other reports. These other studies found a significant increase in PD-L1 on B cells, monocytes and neutrophils in high viremic individuals compared with low viremic or treated individuals (Trabattoni et al. 2003; Sachdeva et al. 2010; Bowers et al. 2014). Although the mechanisms underlying the increased Treg cell proliferation remain to be defined, our data suggest that upon PD-L1 blockade impaired Treg cell proliferative capacity from viremic individuals can be improved. This indicates that on viremic individuals, PD-L1 blockade could increase the suppressive capacity of Treg cells at a population level.

It has been previously shown that PD-L1 blockade increases the suppressive capacity of Treg cells from HCV-infected individuals (Franceschini et al. 2009). The authors of this study suggest that the increased Treg cell suppressive capacity might have been the cause of increased Treg cell numbers. In line with this hypothesis, we found that PD-L1 blockade increases the proliferative capacity but not the suppressive capacity of Treg cells expanded in the presence of PD-L1 blockade. However, the data that result from *in vitro* Treg suppressive assays might not reflect the Treg suppressive capacity *in vivo*. While we focused on the capacity of Treg cells to suppress CD8 T cell proliferation, other effector functions might also be affected and play a role *in vivo*. For instance, it has been reported that the suppression of certain effector cytokines and cytotoxic granules can occur without concomitant suppression of proliferation (Mempel et al. 2006; Schmidt et al. 2011; Sojka & Fowell 2011; Schmidt et al. 2012).

As PD-L1 blockade might also impact other aspects of Treg cell biology, the translation of our *in vitro* results to *in vivo* conditions should be done with caution. For example, Treg cells can use PD-L1 to suppress PD-1-expressing cells (Gotot et al. 2012). Thus, binding of PD-L1 antibodies to Treg cells might reduce their suppressive capacity. In addition, it is well known that the PD-1/PD-L1 pathway is important for Treg cell induction in the periphery (Aramaki et al. 2004; Krupnick et al. 2005; Wang et al. 2008; Francisco et al. 2009; Periasamy et al. 2011; Amarnath et al. 2011; Trinath et al. 2012), so the binding of PD-L1 antibodies may also diminish Treg cell induction. In conclusion, we observed that Treg cell proliferative capacity is modified by *in vitro* PD-L1 blockade; nonetheless other aspects of Treg cell biology might also be influenced. Therefore, the *in vivo* net effect on the Treg cell subset will depend on the weight of the different PD-1/PD-L1 roles in Treg cell biology as well as PD-1 expression and PD-L1 availability at distinct anatomical sites. At this point, only the ongoing clinical trial can potentially provide a clearer picture on how PD-L1 blockade impacts Treg cells in patients.

4. PD-L1 blockade on Treg cells: Implications for virus control

The success of several immunotherapies in cancer and persistent infections seems to be related to an increase in effector T cells over Treg cells. In cancer, Treg cells are present at tumour sites and a high effector to Treg cell ratio has been associated with a favourable prognosis (Curiel et al. 2004; Sato et al. 2005; Kamphorst et al. 2015). A recent study described that an increase in the CD8 to Treg cell ratio is related to tumour regression in mice (Twyman-SaintVictor et al. 2015). This reshaping of the effector to regulatory T cell balance was achieved by a therapy that included radiation, CTLA4 blockade and PD-L1 blockade. The efficacy of CTLA4 blockade in melanoma

resulted from enhancement of effector function and depletion of antibody bounded-Treg cells by macrophages (Simpson et al. 2013; Peggs et al. 2009; Roychoudhuri et al. 2015). In line with this, the blockade of PD-L1 also resulted in increased effector to Treg cell ratio as a result of strongly increased effector CD8 T cell responses within the tumour (Twyman-SaintVictor et al. 2015).

Similarly, Penaloza-MacMaster and co-autors showed in mice infected with LCMV (lymphochoriomeningitis virus) that PD-L1 blockade together with Treg depletion results in a significant reduction of the viral load compared with PD-L1 blockade alone. This suggests that Treg cells contribute to exhaustion and that an increase in CD8 to Treg cells relates to virus reduction (Penaloza-MacMaster et al. 2014). In this context, our findings on a differential impact of PD-L1 blockade on effector and regulatory T cells might have relevant implications for virus control. We found that *in vitro* PD-L1 blockade increased effector CD8 T cell proliferation in all HIV-study groups, but only resulted in increased proliferation in Treg cells from viremic patients. This suggests that the net gain of T cell effector function after PD-L1 blockade may critically depend on the plasma viremia of the host. We thus hypothesize that in individuals that control viremia, PD-L1 blockade could restore effector T cells while leaving Treg subset unchanged resulting in an effector to Treg cell ratio in favour of viral control**.** On the contrary, in patients with high viremia, PD-L1 blockade could restore effector T cell responses but also Treg cells having an overall detrimental impact on viral control.

The PD-L1 blockade of PBMC from viremic HIV-infected individuals under stimulating conditions led to increased reactivation of HIV *ex vivo*. Virus reactivation was related to CD4 T cell proliferation. The increased CD4 T cell proliferation implies increased CD4 T cell activation and increased number of HIV target cells, which might well be the cause of the enhanced virus production. However, reactivation of HIV was also related to an increase in the percentage of Treg cells and a decrease in CD4 T cell to Treg cell ratio as well as CD8 T cell to Treg cell ratio. This suggests that a relative increase in Treg cells over CD8 T cells is a factor promoting virus expansion during HIV infection. In line with this interpretation, an increase in the effector to Treg cell ratio has been related to HIV control. A recent study reported that a therapeutic DC-based HIV vaccine increases effector T cells relative to regulatory T cells. This increase in effector to Treg cell ratio correlated negatively with viral rebound following antiretroviral treatment interruption (Brezar et al. 2015). In view of our data and published reports, we hypothesize that an increase in Treg cell proliferation upon PD-L1 blockade, and consequently Treg cell numbers, might be detrimental for HIV control.

Our observation on increased HIV reactivation upon PD-L1 blockade is in line with previous work that described PD-1hi CD4 T cells as a major reservoir of latent virus (DaFonseca et al. 2010). In this study, the blockade of PD-1/PD-L1 pathway enhanced spontaneous release of HIV virions by CD4 T cells. Although we could not detect virus production in culture supernatants from patients under cART, it is believed that PD-L1 blockade could reactivate latent virus in this group of patients. Evaluating whether or not PD-L1 blockade can reduce the latent reservoir in patients under cART is one of the objectives of the first PD-L1 blockade phase I clinical trial in HIV-infected patients. If proven, this might be of great importance in the search for an HIV cure (Battistini & Sgarbanti 2014) (www.clinicaltrials.gov identifier NCT02028403).

In summary, this thesis shows a previously unknown effect of PD-L1 blockade on regulatory T cells from HIV-infected individuals. This indicates that PD-L1 blockade therapy will not only impact effector T cells but also regulatory T cells. Indeed, *ex vivo* PD-L1 blockade on PBMC from HIV-infected individuals differentially increases the proliferative capacity of regulatory- and effector- T cells depending on the subject's plasma viremia. On the basis of the data reported in this thesis, it can be hypothesized that PD-L1 blockade will skew the effector to regulatory T cell ratio in favour of effector cells only in patients in whom viremia is controlled. In patients with uncontrolled viremia, PD-L1 blockade will not favour effector- T cells over regulatory- T cells, and might also boost virus reactivation. This has direct consequences for patient selection to enter clinical trials targeting the PD-1/PD-L1 signalling pathway. Our findings support the rationale to combine PD-L1 blockade with antiretroviral treatment to restore effector responses in HIV-infected individuals. Only this approach may maximize the net gain of effector T cell function and the subsequent better immunological control over HIV.

CONCLUSIONS

The main conclusions of the present work are the following:

HIV infection leads to an increase in PD-1+ and PD-L1+ regulatory T cells.

- The percentage of PD-1- and PD-L1- expressing Treg cells is increased in HIV-infected individuals compared to healthy control individuals.

- *in vitro* HIV exposure induces PD-L1 in both eTreg and rTreg cells, whereas other activation signals might be required for PD-1 induction in Treg cells.

PD-1 expression in Treg cells seems to follow similar rules than PD-1 expression of effector T cells.

- As for effector CD4- and CD8- T cells, PD-1 expression on Treg cells correlates with disease progression (positively with viremia and negatively with CD4 T cell counts) and it is reduced upon antiretroviral treatment.

- Treg cells from viremic HIV-infected individuals show impaired *ex vivo* proliferative capacity in response to HIV gag peptides in comparison to HIV-infected individuals under treatment. The proliferative capacity negatively correlates with PD-1 expression on Treg cells.

PD-L1 blockade differentially impacts regulatory T cells from HIV-infected individuals depending on the plasma viremia of the host.

- *ex vivo* PD-L1 blockade increases the proliferative capacity of Treg cells from viremic HIV-infected individuals, but not their suppressive capacity *per cell*.

- *ex vivo* PD-L1 blockade has no significant effect on the proliferative capacity of Treg cells from individuals in whom viremia is controlled (either spontaneously or by antiretroviral treatment).

- In contrast*, ex vivo* PD-L1 blockade significantly increases the proliferative capacity of effector CD8 T cells from all HIV-study groups.

- *ex vivo* PD-L1 blockade in PBMC from viremic HIV-infected individuals enhances HIV reactivation, which is related to an increased percentage of Treg cells.

Based on our *ex-vivo* results, PD-L1 blockade should only be given to HIV-infected individuals with controlled viremia because these should experience an optimal gain of effector T cells over Treg cells.

ANNEXES

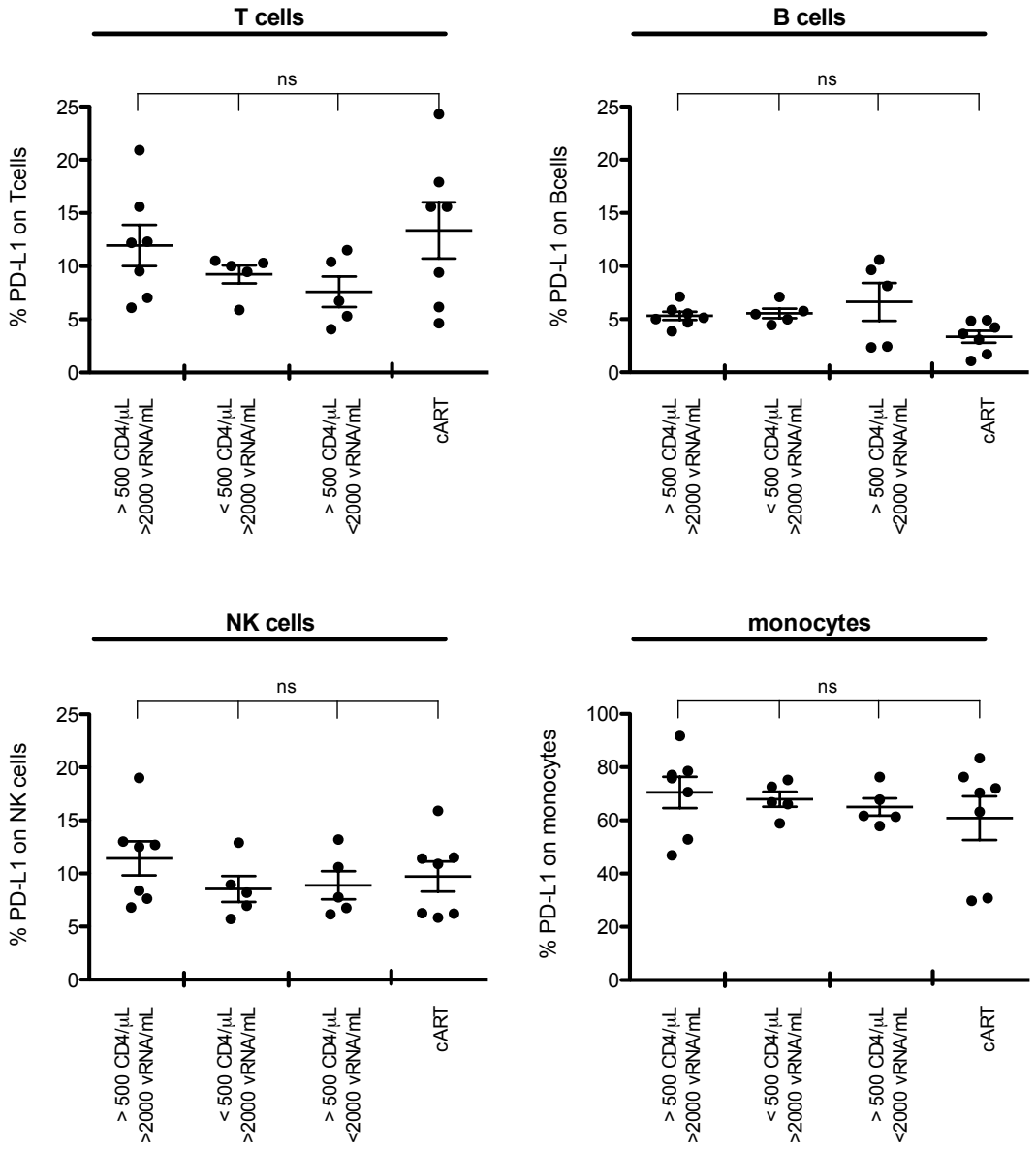
ANNEX 1

List of abbreviations

AIDS	acquired immunodeficiency syndrome
APC	antigen presenting cell(s)
ART	antiretroviral therapy
Blimp-1	B lymphocyte-induced maturation protein 1
BSA	bovine serum albumin
BTLA	B and T lymphocyte attenuator
cAMP	cyclic adenosine monophosphate
cART	combination antiretroviral therapy
CCR5	C-C chemokine receptor type 5
CFSE	carboxyfluorescein succinimidyl ester
CTL	cytotoxic T cell(s)
CTLA4	cytotoxic T lymphocyte antigen 4
DC	dendritic cell(s)
DMSO	dimethyl sulfoxide
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
Eomes	eomesodermin
eTreg cells	effector regulatory T cells
FACS	fluorescence activated cell sorting
FBS	fetal bovine serum
FC	fold change
FOXP3	forkhead box P3
GALT	gut associated lymphoid tissue
GM-CSF	granuolcyte macrophage colony-stimulating factor
KLRG1	killer cell lectin-like receptor subfamily G member 1
HCV	hepatitis C virus
HBV	hepatitis B virus
hi	high
HIV	human immunodeficiency virus
IDO	indoleamine 2,3-dioxygenase
IFN	interferon
Ig	immunoglobulin
IL	interleukin
Int	intermediate

IPEX	immunodysregulation polyendocrinopathy and enteropathy X-linked
IQR	interquartile range
KLRG1	killer-cell lectin like receptor G1
Lag-3	lymphocyte activation gene 3
LCMV	lymphocytic choriomeningitis virus
Lo	low
LPS	lipopolysaccharides
MOI	multiplicity of infection
NFAT	nuclear factor of activated T cells
NK cells	natural killer cells
PBMC	peripheral blood mononuclear cell(s)
PBS	phosphate-buffered saline
PD-1	programmed cell death 1
PD-L1	programmed death ligand 1
PD-L2	programmed death ligand 2
PHA	phytohemagglutinin
pTreg	peripheral regulatory T cells
RGMb	repulsive guidance molecule family member b
rh IL-2	recombinant human interleukin 2
RPMI	Roswell Park Memorial Institute Medium
rTreg cells	resting regulatory T cells
SEM	standard error of the mean
SIV	simian immunodeficiency virus
STAT 5	signal transducer and activator of transcription 5
TCR	T cell receptor
Tfh	follicular helper T cell(s)
TGFβ	transforming growth factor β
Th	helper T cell(s)
TIGIT	T cell immunoglobulin and ITIM domain
Tim3	T cell immunoglobulin mucin 3
TNFα	tumour necrosis factor α
Treg cells	regulatory T cells
tTreg	thymus regulatory T cells
vRNA	viral ribonucleic acid

ANNEX 2



Annex 2 Figure. No differences in PD-L1 expression on PBMC were observed between the HIV-study groups. PD-L1 expression on T cells, B cells, NK cells and monocytes from different HIV-infected study groups are shown as indicated. Each dot represents the result from one individual. Significant differences in PD-L1 expression among the 4 HIV study groups were determined using Kruskal-Wallis test.

ANNEX 3

Other contributions during this thesis

Proliferative capacity is an important quality parameter of T cell responses. Traditionally only static parameters of proliferation have been measured for instance the average number of divisions that cells have undergone or the percentage of proliferating cells. However, these static parameters give no information on the dynamics of proliferation such as rates at which lymphocytes divide and die. During my PhD I have participated in a project that aims to define dynamic proliferation parameters of T cells by using CFSE assays combined with mathematical analysis. This project is in collaboration with Tom Banks (Centre for Research in Scientific Computation, Raleigh, USA) and Gennady Bocharov (Institute of Numerical Mathematics, Russian Academy of Science, Moscow, Russia). I provided the experimental data that have been used to develop various mathematical models to estimate dynamic proliferation parameters from the flow cytometry output of a CFSE assay. This contribution is reflected in the following publications in which I have participated:

Banks H.T., Kapraun D.F., **Peligero C**., Argilaguet J., Meyerhans A. (2015) Evaluating the importance of mitotic asymmetry in Cyton-based models for CFSE-flow cytometry data. International Journal of Pure and Applied Mathematics. 100(1):131-156. Currently available as a technical report of the Center for Research in Scientific Computation (CRSC-TR15-02) at http://www.ncsu.edu/crsc/reports/reports15.html

Banks H.T., Kapraun D.F., Link K.G., Thompson W.C., **Peligero C**., Argilaguet J., Meyerhans A. (2014) Analysis of variability in estimates of cell proliferation parameters for cyton-based models using CFSE-based flow cytometry data. J.Inverse and Ill-posed Problems. DOI: 10.1515/jiip-2013-0065

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Un altre cop vols agitar les aigües
del llac.
Està bé, però pensa
que no serveix de res tirar una sola pedra,
que has d'estar aquí des de la matinada
fins a la posta, des que neix la nit
fins al llevant
−tindràs la companyia
de les estrelles, podràs veure l'ocellassa
de la nit negra covant l'ou de la llum
del dia nou−,
assajant sempre cercles,
per si al cap de molts anys, tota una vida, et sembla
−i mai potser no n'estaràs segur−
que has assolit el cercle convincent.

Joan Vinyoli, *Cercles*
Antologia poètica
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