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Co-Inhibitory Molecule Programmed Death-1 and its Ligands: A New Alternative Therapy for Human Immunodeficiency Virus Infection?

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ABSTRACT

Chronic viral infections are characterized by the up-regulation of a set of immunomodulatory receptors. The over-expression of co-inhibitory molecules on T cells leads to a dysfunctional T cell response with an "exhausted" phenotype. Programmed Death-1 (PD-1) is a molecule that exerts an inhibitory signal on the T cell receptor when it binds to the PD-L1 or PD-L2 ligands present on antigen-presenting cells. Also, the expression of these molecules has been associated to the loss of T cell functions as well as clinical markers of the progression of HIV infection. The study of these molecules has gained attention due to reports indicating that blockade of PD-1 pathway could partially reconstitute T cell functions. In fact, this mechanism has been proposed as an alternative treatment for some chronic viral infections such as HIV infection. This review is focused on those mechanisms that might be favouring the over-expression of PD-1 and its ligands during HIV infection and on the possible new approaches that, by reducing its expression, might represent new strategies for the treatment of HIV infection. Knowing the exact mechanism leading to PD-1, PD-L1 and PDL2 expression in physiologic and pathological conditions is essential for the development of successful treatments. Novel molecular mechanisms inhibiting PD-1 activation might have potential therapeutic use not only in HIV infection but also in other diseases.

Keywords: HIV, T-Cell Dysfunction, Programmed-Death 1 (PD-1), Programmed-Death Ligand (PD-L1), Human Leukocyte Antigen (HLA), Myeloid-Derived Suppressor Cells (MDSC)

1. INTRODUCTION

Despite extensive research on the Human Immunodeficiency Virus (HIV) for over 30 years, eradication of HIV-1 infection and treatment of AIDS remain a long-term challenge UNAIDS, 2010. The virus has developed strategies to spread and persist on infected tissues. On this basis, understanding the alterations to the immune system caused by the virus is one of the major goals for creating an effective treatment or vaccine. CD4+ and CD8+ T cells play an active role in the control of viral infections by producing soluble factors such as cytokines and chemokines and trough the cytotoxic activity against infected cells mediated by molecules such as granzyme and perforin (Freel *et al*., 2011; Swain *et al*., 2012). The T cell function against viruses and other microorganisms relies on the cellular activation status. According to the classic model, T cell activation results from the interaction of two signals (Baxter and Hodgkin, 2002): The first one ensures specificity by Antigen Presenting Cells (APC), which presents viral peptides processed and bonded to class Human Leukocyte Antigen (HLA) molecules to specific T Cell Receptor (TCR). However, this first signal does not trigger complete activation of T cells in absence of a second signal. Co-stimulatory molecules belonging to the B7/CD28 family are able to provide this second signal. Adequate stimulation of T cells through both

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signals leads to clonal expansion and promotes effector functions against pathogens. Both processes are accompanied by cell surface phenotype changes characteristic of cellular activation. In absence of a second signal T lymphocytes become anergic and their functions are limited (Wells, 2009). Co-stimulatory signals are classified as positive or negative depending on their effect on the TCR signalling. In this context, the co-stimulation process involves surface proteins that reduce or amplify the signal given by the TCR complex modulating T cell responses. One remarkable property of some members of this family is their capacity for providing co-inhibitory or co-stimulatory signals, depending on the binding site of the protein and on the type of receptor or ligand participating in the interaction (Alegre *et al*., 2001; Carreno and Collins, 2002). Co-stimulatory signals are fundamental for physiological processes such as maintenance of immune tolerance (Bour-Jordan *et al*., 2011), T-cell development (Keir *et al*., 2005), pathogeninduced T cell activation (Paterson *et al*., 2009) and immune response limitation once the antigen has been eliminated, preventing host damage (Iglesias-Chiesa *et al*., 2008). In some situations, these molecules also contribute to pathological conditions such as the generation of autoimmunity and inflammation implicated in human rheumatoid arthritis (Raptopoulou *et al*., 2010), atherosclerosis (Gotsman *et al*., 2008), systemic lupus erythematosus (Hu *et al*., 2004), multiple sclerosis (Wiendl *et al*., 2003), parasitic infections (Bhadra *et al*., 2012) and certain cancers (Lee *et al*., 2010). In addition, an over expression of co-inhibitory proteins has been consistently documented in chronic viral infections (Jin *et al*., 2011). As an example, HIV infection leads to a major expression of PD-1 and its ligands PD-L1 and PD-L2 (Yamamoto *et al*., 2011), with important implications on the immune response (Yamamoto *et al*., 2011). The over expression of PD-1 on CD4+ and CD8+ specific T cells during HIV infection is associated with a decrease in cytokine production, a gradual loss of CD8+ T cell cytotoxic function and impaired cell proliferation, rendering T cells unable to control viral infection (Day *et al*., 2006; Petrovas *et al*., 2006; Trautmann *et al*., 2006). In recent years, consensus has been reached on the relevant role of PD-1 as a negative regulator of T cell response during HIV infection; blockade of the intracellular pathway regulated by this molecule leads to restoration of certain T cell functions, representing novel approaches for the treatment of HIV infection (Porichis and Kaufmann, 2012) and for several other pathologies. There are few publications about mechanisms leading to the expression of PD-1 and its ligands; knowledge of these mechanisms in physiologic and pathological conditions is essential for the development of successful treatments that, together with the antiretroviral treatment, would improve the immune response. This review is focused on the mechanisms that induce expression of PD-1 and its ligands in homeostatic processes and in pathological conditions. Novel molecular mechanisms inhibiting PD-1 activation with potential therapeutic use in different diseases are also discussed here.

1.1. Molecular Characteristics of PD-1 and its Ligands

The inmunoreceptor Programmed Death-1 (PD-1; PDCD1; CD279) is a 50-55 kDa type 1 transmembrane glycoprotein of 288 amino acids (aa) and is a member of the immunoglobulin (Ig) super family. The PD-1 gene (Pdcd1) has an extension of 2106 cDNA nucleotides, is located in the human chromosome 2q37.3 (Shinohara *et al*., 1994) and is encoded by 5 exons (Finger *et al*., 1997; Ni *et al*., 2007). PD-1 has a transmembrane region, an extra cellular region and a cytoplasmic region. The extracellular part of PD-1 is composed of approximately 147aa forming a single extracellular immunoglobulin with a variable-like domain (IgV-like) responsible for PD-1 binding with their specific ligands: PD-L1 (B7-H1; CD274) and PD-L2 (B7-DC; CD273) (Lazar-Molnar *et al*., 2008; Zhang *et al*., 2004). The transmembrane domain of PD-1 is composed of twoloops of 27aa: The first is located in the middle of the protein and plays a role in anchoring the cell membrane; the second is located at the N terminus and is presumed to be a signal peptide (Zhang *et al*., 2004). PD-1 cytoplasmic domain is composed of 94aa, responsible for triggering a signal through the activation of two tyrosine motifs: An ITIM (immune-receptor tyrosine motif) and an ITSM (immune-receptor tyrosine based switch motif) (Finger *et al*., 1997; Ishida *et al*., 1992). PD-1 has four potential post-transcriptional glycosylation sites in positions 49, 58 and 74, 116 (Finger *et al*., 1997). PD-1 is considerably different from other B7/CD28 family members (e.g., CTLA-4 and ICOS), as exemplified by the lack of a proline-rich ligand recognition loop and the absence of a cysteine residue responsible for a disulfide bond formation (Zhang *et al*., 2004). PD-1 is monomeric in solution and on cell surface, contrasting with other co-inhibitory molecules such as CTLA-4 and ICOS, which are presented as disulfide-linked homodimers (Mages *et al*., 2000; Schwartz *et al*., 2001; Zhang *et al*., 2004). Highresolution crystallographic analysis revealed that PD-1/PD-L2 complex is different from CTLA-4/B7

inhibitory complexes in both, overall organization and particular molecular interactions, responsible for binding and specificity (Lazar-Molnar *et al*., 2008).

The ligands of PD-1 are also well described, the type 1 transmembrane glycoprotein PD-L1 belongs to the immunoglobulin super family and is composed of 229 aa (Dong *et al*., 1999). The PD-L1 gene (Cd274) is located in the chromosome 9p24.2 and is composed of seven exons (Ni *et al*., 2007). This protein has an extracellular region of 122 aa, formed by two immunoglobulin-like domains: An IgC2- like domain and an IgVlike domain, with a potential transmembrane region of 21 hydrophobic aa and a small intracellular tail of 31 aa. PD-L1 has four potential glycosylation sites located in position 35, 192, 200 and 219 of the aa sequence with four disulfide bonds at position 40, 114, 155 and 209 (Dong *et al*., 1999). There are some registered isoforms of PDL1, all of them produced by alternative splicing (He *et al*., 2005). PD-L2, the second known PD-1 ligand, is a type 1 glycoprotein of 273 aa belonging to the immunoglobulin super family; the topographic protein sequence is constituted by an extracellular immunoglobulin with an Ig-like C2 type and a Ig-like V type domains, that contain a transmembrane region of 21 aa and an intracellular tail of 32 aa (Lazar-Molnar *et al*., 2008). PD-L2 has some potential glycosylation sites at the 36, 64, 157, 163 and 189 positions, with four potential disulfide bounds at positions 42, 102, 143 and 192, with protein isoforms (Lazar-Molnar *et al*., 2008; Ni *et al*., 2007). The PD-L2 gene (*Pdcd1Ig2*) is located in the chromosome 9 comprising six exons close to PD-L1 gene by only 42kb (Lazar-Molnar *et al*., 2008).

1.2. Expression of PD-1 and its Ligands

Upon activation, PD-1 is expressed on CD4+ and CD8+ T lymphocytes (Bennett *et al*., 2003; Vibhakar *et al*., 1997), on B cells (Agata *et al*., 1996), on stimulated monocytes/macrophages (Huang *et al*., 2009) and on NKT cells (Moll *et al*., 2009). It can also be expressed on non-lymphoid organs such as retina (Chen *et al*., 2009). PD-L1 has a wider range of expression including dendritic cells (Wolfle *et al*., 2011), macrophages (Wagner *et al*., 2010), neutrophils (Bankey *et al*., 2010) and activated T and B-lymphocytes (Francisco *et al*., 2010; Rosignoli *et al*., 2009). PD-L1 can also be expressed on pancreatic islet cells (Rajasalu *et al*., 2010), keratinocytes (Youngnak-Piboonratanakit *et al*., 2004), vascular endothelial cells (Mazanet and Hughes, 2002), astrocytes (Lipp *et al*., 2007), cardiac endothelial cells (Grabie *et al*., 2007), placenta (Petroff and Perchellet, 2010) and kidney (Menke *et al*., 2007). The diversity of cells expressing PD-L1 suggests that it could have a role not only in APC but also in cells closely related to the immune system. PD-L2 is expressed in low levels on antigenpresenting cells (Tseng *et al*., 2001), bone marrow-derived mast cells (Nakae *et al*., 2006) and on activated human T cells (Ishida *et al*., 2002; Messal *et al*., 2011). Despite the well-defined expression of PD-1 and its ligands, little is known about the mechanisms regulating its expression. It is known that PD-1 can be induced with T-cell activation and by anti- and pro-inflammatory cytokines such as INFα, IL-10 and gamma chain cytokines IL2, IL-7, IL15, IL21 (Kinter *et al*., 2008). Additionally, epigenetic mechanisms are involved in the control of PD-1 (Zhang *et al*., 2011). It was recently shown that DNA methylation down regulates PD-1 expression resulting from viral-induced TCR activation; as the virus is cleared and TCR stimulation decreases, the PDCD1 gene is remethylated and the expression of PD-1 is down regulated (Youngblood *et al*., 2011). Sustained demethylation of PCDC gene was observed in T cells during chronic virus infections and, consequently, increased PD-1 expression was also observed. PD-1 and PD-L1 are also up regulated on APC and CD8+ T cells after stimulation with TLR ligands (Berthon *et al*., 2010; Meier *et al*., 2008; Wong *et al*., 2009). PD-L2 is mainly regulated by cytokines such as IL-10, IL4 and INF-γ (Rodriguez-Garcia *et al*., 2011; Stanciu *et al*., 2006). Apparently, IFN-γ is also one of the main regulators of PD-L1 expression in a wide range of cell types (Berthon *et al*., 2010; Chen *et al*., 2012; Gong *et al*., 2009; Liang *et al*., 2009). Further investigation on the mechanisms of PD-1, PD-L1 and PD-L2 expression and regulation is required to understand the role of these molecules in pathological processes.

1.3. PD-1 Activation by its Ligands

The effect of PD-1 binding to its ligands has been well characterized in T cells and B cells during chronic viral infections. PD-1 activation has been associated with T cell dysfunction due to inhibition of the TCR signalling and to altered expression of proteins and genes. As mentioned before, there are two main ligands for PD-1, being PD-L2 the one with more affinity for PD-1 than PD-L1 (Youngnak *et al*., 2003). After binding with its ligands, PD-1 activation transmits an inhibitory signal to the TCR or BCR signal and down-modulates TRC complex (Karwacz *et al*., 2012; Latchman *et al*., 2001; Okazaki *et al*., 2001; Pentcheva-Hoang *et al*., 2007). PD-1 ligation results in phosphorylation of the tyrosines located in its cytoplasmic domain and

recruitment of the SHP- 1 and SHP-2 to the C-terminal tyrosine in the ITSM (Chemnitz *et al*., 2004). SHP-2 then dephosphorylates TCRassociated CD-3 ξ, ZAP70 Kinase Ch (PKCh) and Erk (Sheppard *et al*., 2004), resulting in downstream direct inhibition of PI3K signalling (Parry *et al*., 2005; Riley, 2009). Triggering of PD-1 pathway leads to alterations on cell cycle progression (Latchman *et al*., 2001), on cytokine production (Cho *et al*., 2009; Latchman *et al*., 2001) and cells become susceptible to death by apoptotic mechanisms (Petrovas *et al*., 2006). Specifically, blocking PI3K after PD-1 activation affects IL-2 production and glucose metabolism, compromising cell survival (Parry *et al*., 2005). PD-1 pathway also prevents production of transcriptional factors associated with T cell effector functions such as GATA-3, T-bet and Eomes (Nurieva *et al*., 2006). PD-1 activation induces a particular pattern of proteins and activation of genes. Studies on CD4+ T cells comparing the transcriptional profile during PD-1 inhibition *versus* other co-inhibitory transcriptional profiles such as CTLA-4 showed that PD-1 has a major influence in T cell activation (Parry *et al*., 2005; Riley, 2009). The number of transcripts derived from the interaction of CD3 and CD28 is reduced in 67 and 90% by CTLA-4 and PD-1 respectively (Parry *et al*., 2005; Youngblood *et al*., 2012). CTLA-4 induces the expression of the anti-apoptotic gene Bcl-xL, while PD-1 does not. This might be the reason why PD-1 pathway renders T cells susceptible to apoptotic stimulus (Parry *et al*., 2005).

1.4. PD-1 and its Ligands During Chronic Viral Infections Such as HIV Infection

T cell exhaustion was initially observed in mice with LCMV infection (Zajac *et al*., 1998). It is characteristic of an antigen-persistence environment caused by chronic infection with microorganisms such as, Epstein Barr Virus (EBV), Hepatitis B or C Virus (HBV or HCV), mycobacterium tuberculosis or HIV (Jin *et al*., 2011). This phenotype of T cells is often called "exhausted" and is characterized by a progressive loss of T cell functions and a deteriorated immunological control of pathogens (Freeman *et al*., 2006). The loss of functions is hierarchical, being the cytotoxic function, the proliferative capacity and IL-2 production lost at first, followed by the loss of TNF-α production and subsequently by the loss of IFN-γ production (Wherry and Ahmed, 2004). Several extrinsic and intrinsic mechanisms are involved in the negative regulation of immune responses during an environment of antigen persistence (Virgin *et al*., 2009). Extrinsic mechanisms, referred as factors that extracellularly promote immune impairment trough cell-cell or cell-protein interaction, include: (1) dysfunctional or suppressive Antigen-Presenting Cells (APCs) (Jin *et al*., 2011; Rodrigue-Gervais *et al*., 2010), (2) increase of regulatory cell populations just as regulatory T cells (Tregs) (Dolganiuc *et al*., 2008; Manches *et al*., 2008) and Myeloid-Derived Suppressor Cells (MDSC) (Chou *et al*., 2012). Intrinsic mechanisms are referred as intracellular mechanisms that promote cell dysfunction, like the up-regulation of inhibitory receptors (Blackburn *et al*., 2009; Fourcade *et al*., 2010) and transcriptional repressor proteins (Shin *et al*., 2009) due to an increased duration and magnitude of antigen stimulation, or by an environment enriched with immunosuppressive cytokines (Coussens and Werb, 2002; Virgin *et al*., 2009). Thus, it has been suggested that the over-expression of co-inhibitory proteins is related to the dysfunction of T cells during chronic infections (Boni *et al*., 2007; Day *et al*., 2006; Freeman *et al*., 2006; Petrovas *et al*., 2006; Radziewicz *et al*., 2007; Trautmann *et al*., 2006; Urbani *et al*., 2006). Based on studies made in animal models and in humans, it is currently known that several chronic infections such as HCV and HBV up-regulate the expression of inhibitory proteins just as PD-1 and PD-L1 on virus-specific T cells (Trautmann *et al*., 2006; Urbani *et al*., 2006).

The effect of this protein up-regulation on T cell exhaustion has been extensively studied on HIV infection, becoming a potential hallmark of AIDS progression (Day *et al*., 2006; Holm *et al*., 2008). PD-1 overexpression on CD8+ and CD4+ T cells was first described by Petrovas *et al*. (2006), who showed that PD-1 favours the unresponsiveness of T cells against HIV. Since then, studies have focused on the patterns of expression and the relation of this protein with the functional status of T cells and on the screening of cells that express PD-1 and its ligands in several cohorts of HIV individuals (Barber *et al*., 2006; Day *et al*., 2006; Petrovas *et al*., 2006; Porichis and Kaufmann, 2011; Trautmann *et al*., 2006). PD-1 expression is dynamically regulated during T cell differentiation; from PD-1- naïve T cells to PD-1low memory T cells and PD-1hi effector Tcells (Rosignoli *et al*., 2009). PD-L1 is highly expressed on terminally differentiated effector T cells from viremic and aviremic individuals, while it is expressed in low levels by their naïve CD4+ T cells (Rosignoli *et al*., 2009). PD-1 expression is higher in anatomic compartments with elevated viral replication such as lymph nodes, gut and peripheral lymphoid

tissues, than in peripheral blood cells (D'Souza *et al*., 2007; He *et al*., 2004; Velu *et al*., 2009). However, it has not been defined if the higher expression of PD-1 in these anatomical compartments is also related to a more "exhausted" phenotype. The influence of the over-expression of these molecules on T cell functions has been clarified by using specific antibodies to block the interaction of PD-1 with its ligands (Finnefrock *et al*., 2009; Freeman *et al*., 2006). Blockade of the PD-1 pathway shows improvement in some T cell functions, enhances proliferation of HIV-specific CD4+ and CD8+ T cells (Porichis *et al*., 2011) and increases the secretion of some cytokines (Muthumani *et al*., 2011). Additionally, PD-1 expressed on monocytes might also be contributing to IL-10 production, which in turn favous T cell dysfunction (Said *et al*., 2010). The effect of PD- 1 on cellular cytotoxicity remains to be explored. One of the most relevant studies regarding blockade of PD-1 pathway is the one of Velu group in a model of SIV infection. This study showed an improvement of cellular and humoral responses not only in blood but also in gut (Velu *et al*., 2009). Therefore, there is no doubt that the PD-1 pathway contributes significantly to the T cell dysfunction observed during HIV infection however, due to the increasing reports showing not only the increase of PD-1 expression on immune cells but also of its ligand PDL1, it is important to evaluate the role of its ligand in the context of HIV infection. Also, it might also be important to know other effects of the PD-1 activation pathway in order to revert T cell dysfunction. As an example, it is now known that PD-1 up-regulates a unique set of genes on exhausted T cells which includes BATF. Over-expression of BATF results in an exhaustion-like T cell phenotype, while silencing of BATF gene is able to restore the function of impaired T cells (Quigley *et al*., 2010).

1.5. Factors Contributing to PD-1 and PD-L1 Over-Expression during HIV Infection

Although PD-1 expression has been described on cells of HIV+ individuals, little is known about the factors inducing PD-1 expression on HIV infection. Data indicate that the virus is the main factor inducing the expression of PD-1 and its ligands. HIV-specific CD8+ T cells express higher levels of PD-1 than HCV-, HBVor CMV- specific CD8+ T cells (Day *et al*., 2006; Trautmann *et al*., 2006), being the cells with immunodominant epitopes the ones that express higher levels of PD-1 (Conrad *et al*., 2011). This might imply that HIV provides additional factors for the up-regulation of PD-1 and possibly for its ligands. The expression of PD-1 on CD4+ and CD8+ T cells has a positive correlation with HIV plasma viral load and a negative correlation with peripheral blood CD4+ T cell count (Zhang *et al*., 2007). PD-1 and PD-L1 are highly expressed on virus specific effector memory CD4+ T cells from acute and chronic infected subjects, while all T cell subsets of elite controllers (a minority group that naturally controls HIV replication) and uninfected subjects express PD-1 and PD-L1 in low levels (Porichis *et al*., 2011; Rosignoli *et al*., 2009), corroborating the idea that the virus directly influence the expression of such molecules. Correlation of PD-1 expression with viral load has been confirmed by studies describing that the reduced HIV-replication observed as a consequence of long-term combined Antiretroviral Therapy (cART), apparently decreases PD-1 expression on specific CD4+ T cells, leading to an increase in the number of functional T cells. Nevertheless, the fact that PD-L1 expression remains elevated on CD8+ T cells from viremic and aviremic subjects receiving cART (Rosignoli *et al*., 2007), suggests a different mechanism of regulation for PD-L1 expression on these subjects. Furthermore, it has been proposed that viral load affects PD-1 expression in a context favouring chronic activation, a predominant feature of HIV infection (Estes *et al*., 2008; Sauce *et al*., 2007; Venkatachari *et al*., 2008). The cell chronic activation characteristic of HIV infection results from the interaction of multiple factors like persistent antigenic load, cytokine environment and bacterial translocated products from gut or colorectal tissue in blood (Jin *et al*., 2011). The close relation between activation markers and PD-1 over-expression suggests that chronic activation might also influence PD-1 expression during HIV infection (Hokey *et al*., 2008; Holm *et al*., 2008; Sauce *et al*., 2007; Tendeiro *et al*., 2012). On the contrary, other reports have shown that PD-1 is responsible of some characteristics of the hiper-immune activation (Holm *et al*., 2008; Nakanjako *et al*., 2011; Porichis *et al*., 2011). Blockade of the PD-1 pathway during SIV infection reduces the expression of activation markers in T cells and, importantly, reduces the translocation of bacterial products in the colorectal tissue of rhesus macaques (Shetty *et al*., 2012).

Although viral presence seems to be close related to PD-1 expression (Porichis *et al*., 2011), there is no evidence that HIV-infection *per se* promotes PD-1 expression. The impact of HIV infection in PD-1 and PD-L1 has not been fully evaluated. In 2008 the group of Muthumani *et al*. (2008) reported that the viral protein

Nef induces the expression of PD-1 in CD4+ T cells, representing a viral mechanism for inducing the expression of this protein. In contrast, the group of Venkatachari *et al*. (2008) reported in the same year that HIV infectiondown-regulates the expression of PD-1 in CD4+ T cells, inhibiting apoptotic cell death. Differences in the expression of PD-1 depending on the type of virus, being the subtype D the one inducing higher expression of PD-1 in T cells than the subtype A, were subsequently reported (Bousheri *et al*., 2009). The effect of HIV infection *per se* on PD-1 ligand expression is less clear; most studies have analysed the expression of these ligands in antigen presenting cells. It has been found that PD-L1 and PD-L2 can be induced in monocytes/macrophages after exposure to competent and inactivated HIV (Rodriguez-Garcia *et al*., 2011). PDL1 could be preferentially up-regulated in macrophages in an anti-inflammatory environment and PD-L2 might be predominantly up-regulated in a pro-inflammatory environment (Rodriguez-Garcia *et al*., 2011). HIV infection could also induce the expression of PD-L1 in DC (Muthumani *et al*., 2011). Additionally, PD-L1 over-expression has also been observed in T cell subsets of HIV infected individuals (Rosignoli *et al*., 2009), the mechanism by which this over-expression occurs remains poorly studied but, the over-expression of PD-1 ligands on T cells opens the possibility of a new T cell-T cell interaction that might be contributing to PD-1 up-regulation. Recently it has been shown that cytotoxic CD8+ T cells obtain the PD-L1 molecule form mature DC by a new antigenspecific mechanism called trogocytosis. Functional studies showed that the CD8+ T cells that acquire PD-L1 from mature DC are able to induce apoptosis of CD8+PD-1+ T cells (Gary *et al*., 2011), opening the possibility for mechanisms based on cellular interactions for PD-L1expresion.

1.6. Inhibition of PD-1 Pathway as Alternative Therapy to Control HIV

Understanding the mechanisms responsible o the T cell dysfunction observed during chronic viral infections could provide novel therapeutic targets for the treatment of persisting infections. Accumulating evidence on the effect of PD-1 on T cells has increased the knowledge about the mechanisms of T cell exhaustion characteristic of chronic infections, placing PD-1 pathway as a novel strategy for HIV therapy (Porichis and Kaufmann, 2012). Several strategies are being developed for supporting the immune system not only in chronic viral infections but in other diseases affecting the immune system, some of them are: (1) blockade of PD-1/PD-L1 interaction with specific antibodies or trough other molecular approaches (Berger *et al*., 2008; Brahmer *et al*., 2010; Petrovas *et al*., 2006); (2) use of PD-1 blockade as an adjuvant in ART treatment or in vaccines trials (Finnefrock *et al*., 2009; Ha *et al*., 2008); (3) blockade of several inhibitory receptors expressed on T-specific exhausted cells (Blackburn *et al*., 2009; Jin *et al*., 2010; Jones *et al*., 2008); and (4) use of iRNA for PD-L1 in APC (Muthumani *et al*., 2011).

The use of antibodies to prevent interaction of PD-1 with its ligands represents a novel strategy for T cell therapy, leading to restoration of T cell functions in a fraction of CD4+ and CD8+ T cells, improvement of cellular proliferation (Trautmann *et al*., 2006) and a modest increase on cytokine production (Barber *et al*., 2006; Petrovas *et al*., 2006). However it has been suggested that inhibiting the PD-1/PD-L1 pathway may lead to sustained TCR signalling leading to hyper-activated T cells, or may generate inflammatory responses. Blockade of PD-1 pathway in tumour-bearing mice increases the cytotoxic responses against tumoral cells without remarkable autoimmune responses (He *et al*., 2004). The clinical relevance of blocking this pathway is supported by pharmacokinetic studies in patients with hematologic malignancies (Berger *et al*., 2008) or with solid tumours (Brahmer *et al*., 2010). The effects of blocking PD-1 pathway in presence of chronic viral infections have not been assessed and the SIV infection model might be suitable for initiation of such studies. Additionally, the use of PD-1 blockade together with cART or vaccination may potentiate HIV-therapy as the presence of antigen would be reduced and the immune response would be stimulated (Finnefrock *et al*., 2009). It has been shown that blockade of the PD-1 pathway in combination with vaccination synergistically enhance specific T cell response during LCMV infection and has an important impact on viral control (Ha *et al*., 2008). Additional studies are required to assess the synergic effect of PD-1 blockade after cART initiation. It will be also interesting to know the effect of this blockade in T cells of other anatomic compartments in which antiretroviral treatment is poorly accessible, as well as to determine optimal timing for cART initiation and PD-1 blockade. The use of siRNA to avoid PD-L1 expression on APC has also been described as an alternative way to block PD-1 pathway and rever T cell dysfunction (Borkner *et al*., 2010; Iwamura *et al*., 2012). Blocking PD-1 pathway, either through anti-PDL1 antibody or SiRNA seems to be a good strategy to restore T cell functionality. However, PD-L1 also supplies a co-stimulatory signal by interacting with B7-1 and

considerations about silencing completely this molecule should also be taken. Further studies will elucidate possible alterations in this co-stimulatory signal caused by PD-L1 blockade.

Exhausted T cells express several co-inhibitory receptors showing that PD-1 is not the only molecule affecting T cell function. It is said that the more exhausted the cell is, the more co-inhibitory molecules are expressed, this molecules co-expression correlates with a faster progression to AIDS (Kassu *et al*., 2010). Phenotype and gene expression profile studies showed the co-expression of several inhibitory receptors onexhausted CD4+ and CD8+ T cells, like T cell immunoglobulin mucin 3 (ITIM-3), Cytotoxic T-Lymphocyte Associated protein 4 (CTLA-4), natural killer cell receptor (2B4), Lymphocyte Activation Gene 3 (LAG-3), CD160, Leukocyte Immunoglobulin-Like Receptor superfamily B member 3 (LILRB3) and 4 (LILRB4), that act synergistically with PD-1 (Porichis *et al*., 2011; Yamamoto *et al*., 2011; Jones *et al*., 2008). Thus, blocking more than one of these co-inhibitory receptors should improve not only the proliferative capacity of T cells (Yamamoto *et al*., 2011) but also cytokine production and cytotoxic functions, strategy that has been less evaluated.

1.7. Final Remarks

In the last years, the PD-1 pathway has become a novel target for restoring the progressive T cell dysfunction observed on viral chronic infections. Combined antiretroviral therapy can achieve undetectable viral loads, but it is unable to eliminate HIV reservoirs or fully restore the immune system. Recent reports have shown that blocking PD-1 pathway can partially restore T cell functions. Because PD-1 has distinct roles in both physiological and pathological conditions, a better understanding of the molecular behaviour and biological functions of PD-1 and its ligands may contribute to the development of effective alternative or co-adjuvant therapies for HIV. As an example, there is a lack of knowledge on the cellular and molecular mechanisms affecting the cytotoxic function of CD8+ T cells after activation of PD-1 intracellular pathways, an essential function for the elimination of infected cells. To our knowledge, only the group of Trautmann *et al*. (2006) has been able to show that blockade of PD-1/PD-L1 interaction slightly improves CD8+ T cell degranulation. Additional studies exploring the expression of PD-1 and its ligands and their role in modulating the cytotoxic function of CD8+ T cells are required. The mechanism by which PD-1 activation modulates cytokine and other anti-viral factors production is also unknown. The fact that a dysfunctional cell not only expresses one inhibitory molecule but also a set of diverse inhibitory molecules emphasises the relevance of defining the T cell functions affected by the different phenotypic profiles. Exploring the clinical relevance of these events is essential for the knowledge of new targets and development of therapeutic models. PD-1 expression on HIV infection has been widely studied, but little is known about the expression and function of PD-1 ligands. The fact that a soluble form of PD-L1 has been found in plasma from patients with cancer (Chen *et al*., 2012) represents a new mechanism of activating PD-1 pathway that may favours T cell dysfunction and mediate apoptotic mechanisms. Further studies are needed to elucidate PD-L1 and PD-L2 roles in HIV infection.

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