SAPIENZA
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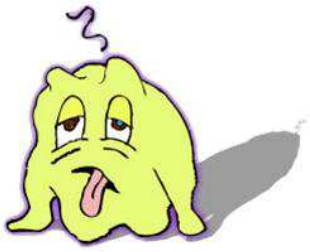
"T cell exhaustion and microRNAs"



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Abstract

Functional exhaustion of antigen-specific T cells is a feature of many chronic viral infections and the role of miRNAs in this process are not well understood.

A typical exhausted T cell is characterized by dysfunctional responses such as low proliferative potential and decreased ability of cytokine production such IL-2, TNF-α and IFN-γ. These defects have been associated with upregulation of inhibitory receptors such as programmed death-1 (PD-1), a member of the B7:CD28 family, capable to induce T cell exhaustion following the interaction with its own ligands (PD-L1 expressed on all cells and/or PD-L2 particularly expressed on dendritic cells). In this project we studied the early phases of PD-1-dependent T cell exhaustion in healthy individuals and we correlated this phenomenon with the microRNAs profile in these cells.

PD-1 positive and PD-1 negative PBL populations from six healthy donors were FACS-sorted and the functional features of $CD4^+$ and $CD8^+$ T cells/ expressing or not PD-1 were analyzed for the intracellular cytokine production in response to polyclonal stimuli.

The exhaustion is a gradual process in which IL-2 production is the first function to be lost: our data demonstrated that two samples analyzed were characterized by PBLs/PD-1+ in terminal exhaustion because they produced less IL-2 and IFN-γ after antiCD3/CD28 stimulation than PD-1- T cells; the other four samples analyzed instead were characterized by T lymphocytes in early exhaustion, because PD-1+ T cells produced only less IL-2 when stimulated with antiCD3-CD28. This result demonstrates that PD-1 expression correlates with different degree of exhaustion.

To validate both PD-1 and TIM-3 as exhaustion markers, we stimulated T cell clones with mitogenic stimuli *in vitro* and we analyzed the expression level of both PD-1 and TIM-3, as well as the cytokine production upon the appropriate stimuli. In these conditions, T cell clones upregulated both PD-1 and TIM-3 in relation with impaired cytokine production. Interestingly, upon repeated rounds of stimulation *in vitro*, T cell clones rescued the capacity of performing their functions in relation with a

dramatic downregulation of both PD-1 and TIM-3, suggesting that the exhaustion phenomenon is reversible. This was confirmed by experiments showing rescued cytokine production by PD-1+ T cell clones that had been stimulated in the presence of a blocking anti-PD-L1 antibody.

To identify candidate microRNAs in early phases of the exhaustion process, we analyzed the microRNAs expression profile by Taqman Low density array on PD-1+ T cells producing low amounts of IL-2, but normal IFN-γ. The analyses showed that only 23 microRNAs are deregulated in PD-1+ PBLs compared to PD-1- PBLs and that miR-21 resulted the most upregulated. The upregulation of miR-21 were also validated by RT-PCR in functionally-exhausted CD4+ T cell clones coexpressing PD-1 and TIM-3. This dimostrated that miR-21 could be a candidate microRNA in the early phases of T cell exhaustion.

Introduction

1. Immune system

From birth our organism comes in contact with external stimuli and a great variety of extraneous agent, such as bacteria, parasites and viruses; the immune system is a defense system that during its evolution has developed mechanisms to efficiently protect us from these continuous insults.
The complex array of cells in the immune system are able to recognize differences that distinguish one pathogen from another, through a specific manner attaining an appropriate response during the effector phase to eradicate the foreign organism.
The subsequent exposure to the same pathogen induce a memory response, in which the immune reaction is more rapid and efficient, serving to eliminate the microorganism and prevent disease.
The immune system performs this through two fundamental arms of the immune response, innata and adaptative immunity that work collectively to provide a high degree of protection for living species.
Innate immunity evolved earlier and is mediated by cells, such as macrophages, neutrophils and dendritic cells (DC) barriers, such as skin, and a variety of antimicrobic compounds that provide the first line of defence against infection.
The later evolved adaptative immunity is mediated by T and B cells and enhances pathogen eradication by adding antigen specificity and memory onto pre-existent innate immunity.
Forthermore, the immune system discriminates between foreign and the body's own cells and proteins establishing and manteining unresponseveness to its compound self.

1.2 T lymphocytes and their roles in immune system

1.2.1 CD4+ T cells

CD4+ T cells, also called T helper cells, play central role in mediating adaptative immunity, sustaining the activation and survival of other cells in the immune system by helping B cells make antibodies, also regulating macrophage function, enhancing their phagocytic and anti-viral function and orchestrating immune responses against a great variety of pathogenic microorganisms (1).
T helper cells are also involved in immunological memory: if their functions and number are lost or decreased respectively, the individual becomes susceptible to infections. In 1986 Coffman and Mosmann revealed that cytokine profile can be used to distinguish CD4+ effector T cells, called helper-T-cell-1 and -2 (Th1 and Th2), which possess different physiological functions (2,3).
Since then, new types of effector T cells have been documentated and CD4+ T cells, upon TCR activation can be functionally distinguished into 4 major T helper subsets. This is on the basis the milieu of cytokines present at the time of TCR engagement and also through the expression of different patterns of cell surface molecules and by cytokines profile produced by the cells (figure 1).

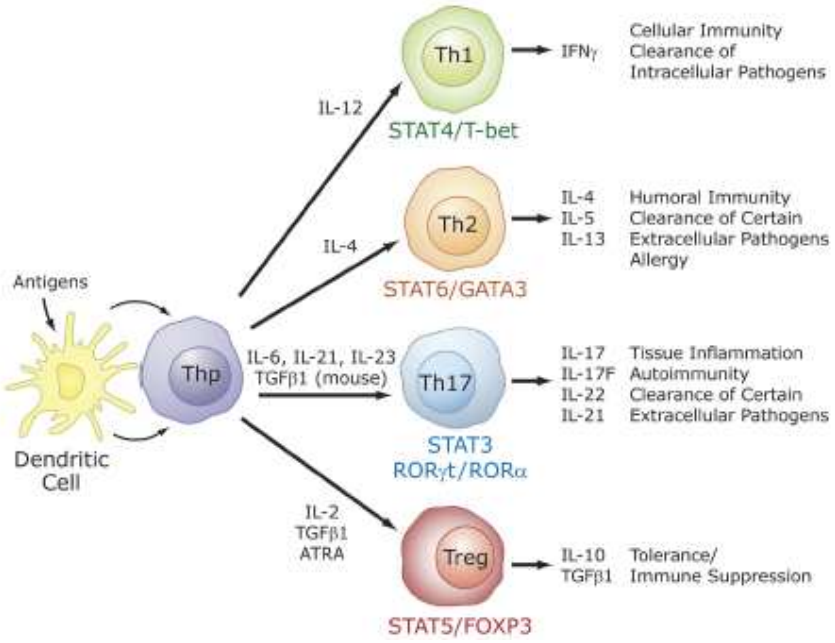


Figure 1. Differentiation of uncommitted (naïve) CD4+ T helper cells (Thp) into different effector CD4+ T cell lineages and their functions.

Th1 cells produce pro-inflammatory cytokines such as IFN-γ, TNF-α and IL-2 to stimulate innate and T cell immune responses and to protect the host from intracellular pathogens.
Th2 cells are producers of IL-4, IL-5, IL-9, IL-10 and IL-13 and their response is often direct against extracellular pathogens such as helminthes and nematodes (4) .

Th17 cells are characterized by IL-17. They have potent inflammatory functions and play an important role in the induction and propagation of autoimmunity and are implicated in a number of immunopathologies such as rheumatoid arthritis and colitis (5).
T-regulatory cells, (Treg) are phenotypically defined by the receptor CD25 and the transcription factor FoxP3. They play a suppressive role through IL-10 and TGF-β production and are involved in maintaining self-tolerance and immune homeostasis.
Disruption of Treg function contributes to a plethora of autoimmune and inflammatory pathologies (6,7).

1.2.2 CD8+ T cells

CD8+ T cells are the most important component of the adaptive immune response against intracellular infections. They are sometimes referred to as cytotoxic T lymphocytes (CTLs) because their principal function is the ability to eliminate viruses by killing infected cells through lytic granule production such as granzymes that are directed to the target cells with perforin and drive the cell to apoptosis. In addition to cytotoxic functions, CD8+ T cells secrete potent antiviral cytokines such as IFN-γ and TNF-α (8) .
The importance of CD8+ T cells is evident in chronic viral infections and various studies have documented roles for CD8 CTLs in controlling persistent infections in human (9,10).
In chronic infection, the continuous presence of functional CD8+ T cells is essential to keep the virus in check, and loss of their response can lead to recurrence of virus.

1.2.3 Activation of CD4+ and CD8+ T cells

Naïve CD4+ and CD8+ T cells must be activated to perform their immunological functions.
In the lymph nodes (DLNs), the DCs recognize antigens and present them to CD8 and CD4+ T cells in the context of MHC class I and class II respectively; naïve T cells can come in contact with their cognate antigen through DLNs and can initiate their commitment to effector T cell lineage.
According to the model proposed by Kevin Lafferty two signals are necessary to activate T cells: the first signal (also called "stimulatory signal") that confers specificity to the immune response is the antigen presented in the context of MHC and is recognized by the T cell receptor (TCR) (11).
The second signal (also called "antigen-indipendent costimulatory signal") is mediated by costimulatory molecules expressed on APCs to receptors expressed on T cells. Both stimulatory and costimulatory signals are important: in absence of costimulation the cells become unresponsive or anergic to subsequent antigenic challenge (12,13).
Finally cytokines such as IL-12 and IL-4 produced by activated T cells of the innate and also the adaptive immune system allow proper activation of naïve T cells and also promote differentiation into fully functional effector T cells.
The initial activation of naïve T cells is followed by the clonal expansion process in which T cells initiate massive expansion and acquire an activate phenotype. They then exit by lymphoid organ and extravase into peripheral tissue to rapidly control infection elaborating effector functions (i.e. cytokine production and killing of infected cells (14).
After the clonal expansion of both CD4+ and CD8+ T cells undergo to a process of cell death, also called contraction phase, during which the vast majority (90%) of stimulated effector cells are eliminated. The small population that survives is the memory pool, characterized by long term survival in absence of antigen and a faster

and more efficient secondary immune response upon subsequent exposure antigenic (15).

1.2.4 T cell memory

1.2.4.1 Memory T cell subsets

Subsets of memory T cells were identified in peripheral blood mononuclear cells (PBMCs) of human and for both CD4+ and CD8+ T cells two major subgroups were characterized on the basis of the cell-surface markers expression, such as the lymphoid organ homing receptor CCR7: Central memory (T_{CM} cells) and Effector memory (T_{EM} cells).
T_{CM} cells are present in lymph nodes, spleen and blood and are capable of expanding rapidly in response to antigen but are less-efficient in producing cytokines. They express CD44, CD127, CD62L and are CCR7+ (16).
T_{EM} cells are present in periphery, rapidly producing IFN-γ and TNF-α and expressing a low level of CD62L and are CCR7- but also retain a high level of CD44 and CD127 (17).
The effector memory T cells rapidly respond to antigen and become effector more quikly to eradicate the pathogen than to central memory T cells.

1.3 CD4+ and CD8+ T cells in viral infections

1.3.1 T cell responses during acute and chronic infections

In viral infection both pathogen and host compete for dominance: following the acute phase of infection, when the antigen and inflammation are reduced or when eliminated memory CD8+ and CD4+ T cells acquire the characteristics of long-lived memory cells that allow them to confer protective immunity (18). In fact these cells have capacity to persist long periods in the absence of antigen via homeostatic proliferation driven by IL-7 and IL-15and to respond rapidly to an subsequent re-exposure to the pathogen via coproduction of multiple cytokines, chemokines, cytotoxicity, vigorous proliferation associated with an increase in cell numbers and migration of effector T cell to sites of infections.
In contrast, during chronic viral infection when both antigen and inflammation persist the effector functions and key characteristics of long-lived memory T cells can differ dramatically.
In fact, they gradually lose their multi-functionality and the reduced expression of IL-7 and IL-15 receptors on virus-specific CD4+ and CD8+ T cells and the relative incapacity to effectively respond to these cytokines, leads to defective antigen-independent homeostatic self-renewal. This results in virus-specific T cells that become antigen-dependent are lost if the antigen is removed. This defect in of T cell responses during chronic viral infection is referred as T cell exhaustion.

1.4 T cell exhaustion: an overview

T cell exhaustion is a common feature of many chronic viral infections. It was initially identified and described in 1998 during persistent LMCV infection in mice as dysfunctional T cell responses (virus-specific, tetramer-positive CD8+ T cell) and physical or clonal delection of non-responding T cells following infections (19,20).

Since then, T cell exhaustion was characterized and studied in other infections, both in mice and in humans, including adenovirus, polioma virus, Fried leukaemia virus, mouse hepatitis virus, human hepatitis B and C virus (HBV and HCV), human immunodeficiency virus (HIV) as well as bacterial and parasitic infections (21-31).
Impaired T-cell responses have also been observed and documented in patients with cancer and also for CD4+ T cells following several infections (32).
In each case, despite that the phenomenon differs for specific pathogens, general phenotypic details and functional features of exhausted T cells are becoming clearer and have been widely discussed by Virgin et al., 2009 (figure 2).
It is now well known that a typical exhausted cell differs from effector and memory T cells. During exhaustion T cells gradually lose the usual array of effector activities due to persistent antigenic stimulation, and show phenotypic and functional defects, such as the inability to proliferate and to produce cytokines in stepwise manner. This defect is a principal reason for the inability of the host to eliminate the persisting pathogens (33,34).

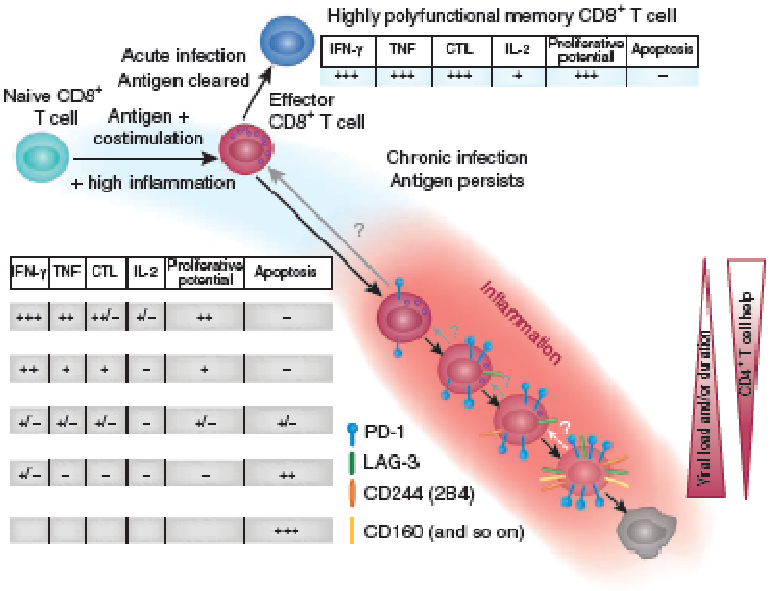


Figure 2. Stepwise and hierarchical T cell exhaustion during chronic infection. During chronic infection and in the persistence presence of antigen the effector CD8+ T cells gradually lose functional properties and potential proliferative. This phenotype is correlated with the upregulation of inhibitory receptors. T cell exhaustion is aggravated by increased viral load and is inversely correlated with the count of CD4+ T cells. Finally exhausted T cell can be deleted.

A feature of functional exhaustion is the progressive lost of T-cell function, both in mice than in humans. Generally IL-2 production, robust proliferative capacity and cytotoxic activity are the first functions to be lost, whereas, other properties, such as the ability to produce TNF are lost later at more intermediate stages of exhaustion.
At severe stages of dysfunction IFN-γ production is partially or completely lost.
Exhausted CD8+ T cells, despite their functional ineptness, display high levels of phenotypic markers typically associated with cellular activation such as CD43,

CD69 and also the inhibitory receptor but low levels of CD62L (L-selectina), CC-chemokine receptor 7 (CCR7) and CD127, which are associated with effector activity cell population (35).
Finally exhausted T cells are physically deleted if the high antigenic load persists, which may be due to expression of pro-apoptotic factors such as BCL-2 which are upregulated by exhausted T cells both in mice as in humans during chronic infection.
Now it is known that the levels and the strength of antigenic stimulation are critical determinants of the process and that more severe exhaustion is correlated with a higher viral load. Decreasing viral load, as during resolution of acute infection generally can help the exhausted T cells regain functionality though completely exhausted T cells that may be refractary to reactivation (36,37).
In addition to antigenic load, longer duration of infection or loss of CD4+ T cells leads to increasingly severe exhaustion and cases of exhaustion are documented also for virus-specific CD4+ T cells though little is known about their dysfunction (38).
CD4+ T cells are required for optimal CD8+ T cell responses and, as helper cells, their requirement becomes more stringent under condition of antigen-persistence because they to sustain CTLs; in fact during exhaustion CD4+ T cells gradually succumb or undergo deletion and this is associated with more severe defective memory CD8+ T cells.
CD4+ T cells produce IL-21 that seem to have an important role in fostering CD8+ T cell responses, limiting the rapid generation of terminally differentiated effector-like T cells and also attenuating T cell senescence during acute and chronic infections, both in mice as in humans.
In fact, the absence of IL-21 or its receptors, does not affect primary CD8+ T-cell responses induced following LCMV infection, but the virus-specific cells subsequently lose their capacity to produce cytokines and to control infection as well as CD4-deficient mice suggesting that IL-2 is a vital helper factor.
In CD4-deficient mice infected with LCMV clone 13, the administration of IL-21 reduced the viral load, increased the responsiveness of virus-specific CD8+ T cells and also suppressed deletion of epitope-specific population (39).

Human IL-21 also encourages functional CD8+ T cell responses: during HIV infection the amount of IL-21 in the blood correlates with CD4+ T cell count and patients with a high level of IL-21 have enhanced frequencies of virus-specific CD8+ T cells (40-42).
Other immunoregulatory cytokines can both positively and negatively influence immune responses and therefore T cells exhaustion. For example, many chronic infections such as HIV, HCV and also HBV and LCMV are associated with high expression of IL-10 and their homologues (43,44).
IL-10 plays multiple roles and has been shown to decrease pro-inflammatory cytokine production, prevent the function of antigen-presenting cells, cut down T cell responses and also affect B cells.
The main source of IL-10 are dendritic cells and CD4+ T cells, like regulatory T cells, B cells and CD8+ T cells.
Analyses of polymorfisms in IL-10 and within the IL-10 promoter have been associated with outcome of infections and also disease progression. In fact promoter sequences with low IL-10 production are mainly prevalent in individuals with asymptomatic HBV infection and those associated with higher production, are a risk factor for the development of chronic HCV infection (45,46).
This is one reason why the expression of this cytokine, IL-10, is linked to T cell exhaustion during persistent viral infections, and its blockade increases viral control and enhances T cell responses (47).
LCMV system has helped explain the existing relationship between IL-10 and T cell dysfunction: mice undergoing chronic infection are associated with T cell exhaustion and with elevated levels of IL-10, both protein and messenger levels (48,49).
Transforming growth factor-β (TGF-β) has also been involved in T cell impairment limiting the size of pathogen-specific T cell responses and the propensity of CD8+ T cells to undergo apoptosis (50).
In fact, during T cell exhaustion the CD8-virus specific responses are reduced and these dysfunctional T cells finally succumb to deletion. TGF-β in acute infections limits CD8+ T cells responses, most likely inducing the expression of the pro-

apoptotic protein Bim and by downregulation of the expression of the anti-apoptotic gene *Bcl-2*.
In persistent LCMV infection the high levels of TGF-β, that prevent the ability of T cells to receive TGF-β signals, improves the CD8+ T cells functionality and prevents the severe exhaustion and also deletion of some CD8+ T cell-specificities.
Other cytokines such as IL-27, which regulate the immune responses during certain parasitic infection and autoimmune disease by reducing T-cell activities and IL-35, that is produced by regulatory T cells and is linked with immunosuppression, can also influence T-cell exhaustion.
Tregs have also been linked to chronic infections and to exhaustion. Tregs are CD4+ T cells that mature in the thymus and represent 5-10% of the CD4+ T cell population and are characterized by the constitutive expression of CD25 (51,52).
Tregs have suppressive activity; they suppress the activation and/or expansion of multiple types of immunocompetent cells.
It was first shown that Tregs suppress the activation and expansion of CD4+ T cells, and *in vitro* studies have also shown that they abolish the cytokine production of CD4+ and CD8+ T cells even in the absence of antigen presenting cells (APC) and moreover inhibit the function of effector and memory T cells.
Foxp3+CD4+ Tregs can negatively regulate effective immunity and are more often associated with ineffective immune responses during chronic infections, such as Friend leukemia virus, HCV or HBV and during persisting parasitic and bacterial infections and also in cancer.
Tregs may act through cell-to-cell contact, inhibition of APC maturation and CD8+ T cell effector function, production of regulatory cytokine and therefore limiting effective viral control and then prolonging antigen-expression and subsequent T cell exhaustion.
Tregs can also increase immunity of the early stage of acute infections by modulating effector T cell recruitment to infected sites.
Pathways such IL-10, TGF-β and inhibitory receptors have been linked to mechanism of suppression of Tregs and in addiction, there are other CD4+ Treg

populations that produce IL-35 which can induce or else effector T cells to become Treg cells (53,54).
In addiction, has been recently observed that also transcriptional pathways are implicated in CD8+ T cell exhaustion. This role has been attributed to Blimp-1 that controls the terminal differentiation of T cells (55).
Blimp-1, (B lymphocyte-induced maturation protein-1) was initially known as a decision maker in the fate of B cells through regulation of genes promoting B cell terminal differentiation into plasma cells but not into memory B cells. However, it may provide similar functions for CD8+ T cells, by promoting the terminal differentiation of most into short-lived cytotoxic T lymphocytes (CTLs) rather than long-lived central memory (CM) T cells.
Has been demonstrated in fact, that during acute infection Blimp-1 is associated with terminal differentiation of effector CD8+ T cell, but smaller amounts of this factors are found both in precursors and mature memory CD8+ T cells (56,57).
In addiction studies in which genetic ablation of Blimp-1 reverses the pattern of memory differentiation have suggested that small or moderate amounts of Blimp-1 promote the formation of memory T cells, intermediate amounts favor the terminal differentiation of functional effector T cells, but very high amounts of Blimp-1 foster the exhaustion. (58).
Blimp-1 suppress IL-2 transcription and capacity proliferative probably via alterations in the expression of the pro-proliferative factor inhibitor of DNA binding 3 (ID3) (59).
As for Blimp-1 also for T-bet has been attributed a role for exhaustion though the precise function of these transcription factors might be dependent on context such as acute and chronic infection.
Now is clear that T cell exhaustion is an active controlled process due to many factors such as viral load, disposability of CD4+ T cells that sustain CD8+ T-cell responses, level of immunoregulatory cytokines, as well as lymphoid architecture and cellular and tissue tropism (60).

1.4.1 Inhibitory receptors and T cell exhaustion: PD-1

During acute infections many inhibitory receptors are only transiently expressed on functional effector T cells, as a result of activation of cells to limit the severity of the responses and are then down-regulated when the pathogen is cleared. Conversely, the establishment of the exhausted state (and also during chronic infections) is associated with constitutive expression of a wide variety of these inhibitory receptors, that regulate the functional and proliferative potential of the responding cells (61).
The first to be involved in exhaustion is programmed death 1 (PD-1; also called CD279) receptor.
PD-1 is a 288-aminoacid type I transmembrane protein in the CD28 family of receptors discovered in 1992 as a gene upregulated in T cell hybridoma undergoing cell death (62).
Its negative regulative function was discovered 7 years later by the autoimmune-prone phenotype of $Pdcd1^{-/-}$ (63).
In human PD-1 is expressed on CD4+ T cells, CD8+ T cells, natural killer T cells, B cells, DCs and activated monocytes and its expression is induced by T cell receptor (TCR) or B cell receptor signaling and is augmented by stimulation with tumor necrosis factor. PD-1 expression remains high in persistent antigenic stimulation and is highly detectable on dysfunctional, exhausted T cells in chronic viral infections.
The two PD-1 ligands differ in their expression patterns and also in their affinities for PD-1, with expression of PD-L2 being much more restricted than PD-L1 expression. PD-L2 (also called B7-DC and CD273) is inducible on dendritic cells (DCs), macrophages and cultured bone marrow–derived mast cells and share thre-fold high affinity for PD-1. In contrast, PD-L1 (also called B7-H1 and CD274) is expressed constitutively on murine T cells, B cells, DCs, macrophages, mesenchymal stem cells and cultured bone marrow–derived mast cells; PD-L1 expression is further upregulated after activation. Constitutive expression of PD-L1 is lower in humans than in mice. PD-L1 is also expressed on a wide variety of non-

hematopoietic cell types, including vascular endothelial cells, epithelial cells, muscle cells, hepatocytes, pancreatic islet cells and astrocytes in the brain, as well as at sites of immune privilege, including the placenta and eye. In humans but not in mice, PD-L2 is also expressed on vascular endothelial cells. (64,65).

The PD-1 receptor is a cell surface monomer consisting of a single immunoglobulin variable-like domain and a cytoplasmic domain containing two tyrosine-based signaling motifs (called ITIM and ITISM motifs).

PD-1 transduces an inhibitory signal when simultaneously engaged with the TCR or B cell receptor but does not transduce a signal when crosslinked alone. Phosphorylation of the second tyrosine residue, located in an immunoreceptor tyrosine-based switch motif, recruits the phosphatases SHP-2 and, to a lesser extent, SHP-1 to the PD-1 cytoplasmic domain. Recruitment of the phosphatases leads to the dephosphorylation of effector molecules activated by TCR and the B cell receptor signaling (such as Syk and phosphatidylinositol-3-OH kinase) leading to eventual inhibition of T-cell activation. (66-68).

The functions of PD-L1 and PD-L2 in T cell activation are only beginning to be understood. Some *in vitro* studies suggest that PD-L1 and PD-L2 can inhibit T cell proliferation and cytokine production, whereas others indicate that PD-1 ligands enhance T cell activation.

1.4.2 PD-1/PD-L1 pathway

Recent studies indicate that the PD-1/PD-L1 pathway has important roles in regulating self-tolerance but also has key roles in regulating anti-microbial immune defense. This pathway controls immune responses to microorganisms that cause acute or chronic infection. A variety of microorganisms that cause chronic infections appear to have exploited the PD-1/PD-L pathway to attenuate anti-microbial immunity and to facilitate persistent infection.
In fact PD-1 signaling may result in decreased T-cell proliferation, survival, protein synthesis with greater effect on cytokine production (such IFN-γ, TNF-α and IL-2 production) (figure 3) (69).

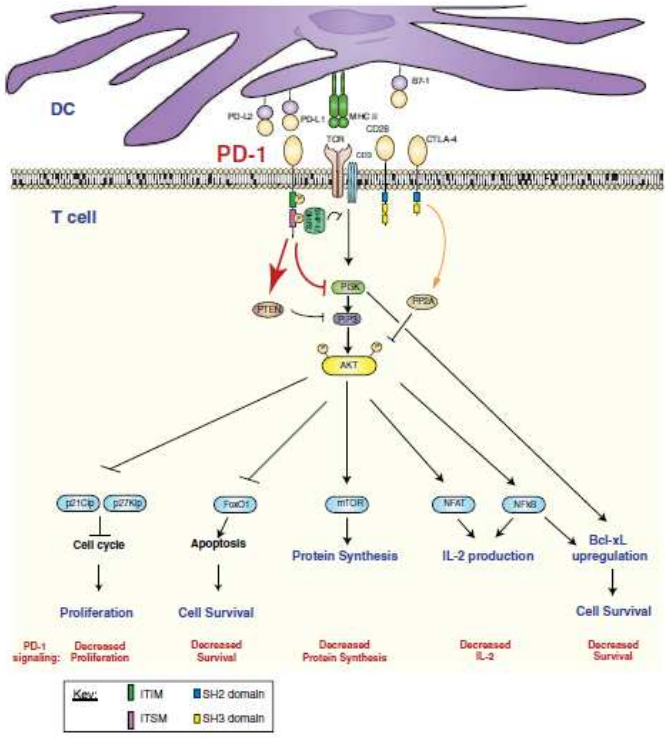


Figure 3. PD-1 signaling may result in decreased T-cell proliferation, survival, protein synthesis, and IL-2 production.
Ligation of TCR and PD-1 leads to tyrosine phosphorylation (P) of the ITIM and ITSM of PD-1. Binding of the ITSM by SHP-1 or SHP-2 results in the dephosphorylation of proximal signaling molecules and augmentation of PTEN expression. This effectively attenuates the activation of the PI3K and Akt pathways. PD-1 signaling may result in decreased T-cell proliferation, survival, protein synthesis, and IL-2 production.

The involvement of this pathway in chronic viral infection-associated T-cell exhaustion was first demonstrated in a murine lymphocytic choriomeningitis virus (LCMV) model, following micro-array analysis of gene expression profiles of virus-specific CD8+ T cells during chronic LCMV infection with clone 13 by Barber et al., 2006 (70).
In chronically infected mice, PD-1 is highly upregulated on the functional exhausted T cell but only transiently expressed on the effector T cell during acute infection and is absent on functionally memory populations.
After the initial studies in mice, other studies have shown that PD-1 is implicated in T cell exhaustion in humans, such as HIV (figure 4) where the PD-1 pathway limits the functionality of antigen-specific T cells during other persisting viral infections, such as HBV and HCV, and also in non viral infections as well as in cancer (71,72).

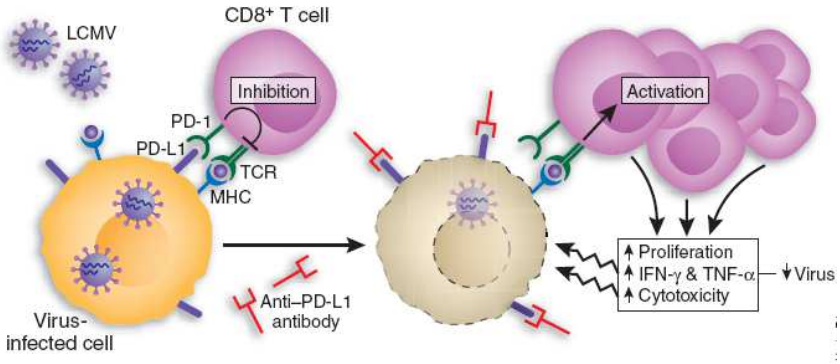


Figure 4. Mechanism of suppression. Therapy with PD-L1-specific antibody restores impaired CD8+ T-cell functions in LCMV infections.

Several groups have demonstrated that blockade of the PD-1/PD-L1 pathway leads to increased T-cell proliferation and TNF-α, IFN-γ and granzyme production, indicating improved T effector cell function of HIV-specific CD8+ T cells.
Remarkably, anti-PD-L1 blockade also restored CD4+ T-cell proliferative responses to HIV antigen. Similarly, anti-PD-L1 enhanced proliferation of HCV-specific CD8+Tcells, but CD8+ effector functions were not examined.
These observations have been extended to infections with simian immunodeficiency virus (SIV) where blockade PD-1 showed an improvement in frequencies, function of virus-specific- CD8+ T cell responses, survival and also enhancement of B cell responses (73).
Taken together, these findings suggest that the PD-1/PD-L1 pathway might be operating to deter anti-viral T-cell responses during chronic HIV and HCV infection, and suggest that blockade of this pathway might provide a new therapeutic approach to revive dysfunctional T cells.
It appears that PD-1 also might serve as a useful marker on virus-specific CD8+ T cells to indicate the degree of T-cell exhaustion and disease severity.
The level and percentage of PD-1 expression on HIV-specific CD8+ T cells correlates with viral load, declining CD4 counts, and decreased capacity of CD8+ T cells to proliferate on HIV antigen *in vitro*. PD-1 expression on HIV specific CD8+ T cells decreased upon effective highly active anti-retroviral therapy, suggesting that high viral load stimulates PD-1 expression and functional exhaustion.
Similarly, PD-1 might be a marker for disease progression in HCV infection since during acute HCV infection, PD-1 is upregulated on HCV-specific CD8+ T cells, declines in patients with resolving infection, but remains high in patients who develop chronic infection (74).
The therapeutic vaccination during chronic viral infection, in combination with PD-L1 blockade, synergistically boosts epitope-specific CD8 + T cell responses and promotes viral control. These experiments demonstrate that blocking this major inhibitory pathway during chronic infection improves the efficacy of therapeutic

vaccination and may prove useful for the treatment of persistent infections, in general, as well as to improve immune responses to tumors (75).
Several groups in fact have demonstrated that the PD-1/PD-L1 pathway is involved in T cell exhaustion in cancer: in a study of Ahmadzadeh and Mumprecht in 2009 it showed that PD-1 expression is found on tumor-infiltrating CD8+ T cells and on antigen-specific CD8+ T cells in multiple solid tumor and in hosts with nonsolid tumors, respectively (76,77).
In many tumors these PD-1+ T cells are functionally exhausted: they expressed PD-L1 at high levels and this high expression is strongly associated with poor prognosis (78).
In addiction, they have showed that interference with PD-1/PD-L1 signaling, either through antibody blockade or PD-1 deficiency, improves the clinical outcome and restores functional T cell responses in several cancers (79).
However, targeting the PD-1/PD-L1 pathway does not always result in reversal of T cell exhaustion as recent studies showed that exhausted T cells are a heterogeneous population on the basis of the different potential for recovering functions after blockade PD-1 pathway (80).
Exhausted CD8 T cells expressing intermediate levels of PD-1 (called PD-1 ^{Int} cells) can be potently re-invigorated by blockade PD-1 pathway, but those with high levels of PD-1 (PD-1 ^{Hi} cells) cannot: an example of these situations has been described in humans during chronic HCV infection where PD-1^{Lo} HCV-specific CD8 T cells from the blood responded to PD-1 pathway blockade while PD-1^{Hi} HCV-specific CD8 T cells from the livers of the same individuals did not (81).
In addiction PD-1 expression is not always associated with exhausted phenotype: in a study on patients with a self-limiting HBV infection during the acute phase, the PD-1 blockade restored IFN-γ production despite the elevated levels of PD-1 with desease progression, indicating that other molecules are likely involved in T cell exhaustion (82-84).

1.4.3 TIM-3 and T cell exhaustion

Another inhibitory receptor upregulated in dysfunctional T cells is TIM-3.
TIM-3 is a member of the T cell immunoglobulin mucin, (also called TIM), family of receptors; the murine Tim-family genes were positionally cloned in 2001 from the locus associated with susceptibility to airway hyper-responsiveness (AHR), a mouse model of human asthma (85).
Tim-family proteins have been repeatedly associated with various autoimmune and allergic diseases in both humans and mice (86).
Recently, the Tim-family receptors have also emerged as important regulators of effector CD4+ T cell functions, regulating Th1- and Th2-mediated immunity.
The Tim family genes consist of eight members in mice (referred as mTIM-1/8) and three in humans (called TIM-1, TIM-3 and TIM-4) which are located on two genetic regions associated with allergic and autoimmune disease (chromosome 11 and 5q33 in mice and in humans, respectively) (87).
TIM molecules are type 1 membrane proteins with a common structural organization: an N-terminal IgV domain, a mucin domain, a transmembrane domain, and a cytoplasmic tail (figure 5) (88-91).

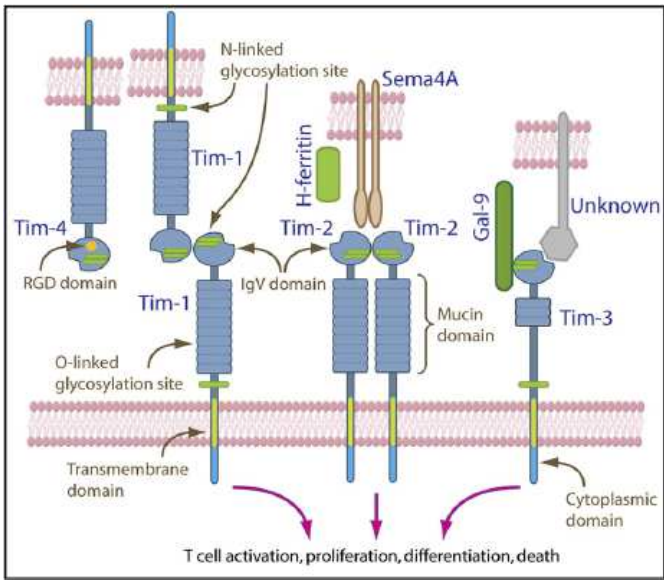


Figure 5. Mouse Tim Family Proteins, Their Ligands and functions.
Tim protein might deliver multiple differential signals in regulating T cell responses such as activation, proliferation, differentiation, and death, depending on the receptor-ligand interaction.

Tim proteins were initially thought to be expressed on the surface of differentiated effector T cells to directly regulate their activity. Now it is known that TIMs are also expressed and regulate the functions of antigen-presenting cells (92).
Tim-1 is preferentially expressed on Th2 cells and functions as a potent costimulatory molecule for T cell activation, whereas Tim-4 is expressed on antigen-presenting cells (APCs) and represents the only natural ligand reported for Tim-1 (93).

The Tim-1-Tim-4 pathway appears to positively regulate CD4+ T cell activity, as indicated by the fact that an agonistic monoclonal anti-Tim-1 and Tim-4.Ig fusion protein stimulate both T cell proliferation and cytokine production.
In contrast, signaling through Tim-2 has been suggested to impede Th2-mediated immunity, because blockade of Tim-2 by Tim-2.Ig treatment enhances Th2-prototypic cytokine secretion and ameliorates Th1- mediated experimental autoimmune encephalomyelitis (EAE) (94).
TIM-3 was originally identified as a molecule expressed on T helper (Th-1) in mice In human TIM-3 is preferentially expressed on Th-1 cells and is also expressed constitutively on dendritic cells and macrophages (95).
Galectin-9, a ligand for Tim-3, binds to Tim-3 via carbohydrates present on the IgV domain and this interaction inhibits TH-1 responses and induces peripheral tolerance (96-98).
Galectin-9 is expressed in immune cells, such as T and B cells, macrophages, endothelial cells and fibroblasts. The production of gal-9 is stimulated by IFN-g, the major cytokine produced by Th-1 cells, and thus may be part of a negative feedback loop leading to the death of TIM-3positive Th-1 cells.
As for PD-1 has been demonstrated an inhibitory role in human T cells also for TIM-3; for example reduction of TIM-3 expression in T cells through blocking antibodies or small interfering RNA induced an increased interferon (IFN)- γ secretion by CD4 + T cells and in patients with autoimmune disease TIM-3 expression is lacking on CD4+ T cells (99,100).
T cell clones of patients with multiple sclerosis (MS) isolated from the cerebrospinal fluid express a lower level of TIM-3 and secrete a higher amount of IFN-γ compared to clones isolated from healthy control subjects.
A recent study in patient with progressive HIV showed that TIM-3 is expressed at high levels on both CD4+ and CD8+ T cells and TIM-3 upregulation on HIV specific CD8+ T cells has been correlated with exhausted phenotype. These CD8+ T cells expressing high levels of TIM-3 showed impaired functions, such as cell survival, proliferation, as well as decreased cytokine production (101).

The frequency of TIM-3 positive cells positively correlated with viral load and inversely with CD4+ T cell number.
In a study in patients with HIV infection sorting of TIM-3 positive and TIM-3 negative populations showed that HIV-specific T cells expressing high level of TIM-3 produced less amount of IFN-γ rather than did TIM-3 negative cell.
This suggests that TIM-3, like PD-1, may be an important marker of exhausted cells during chronic viral infections. As for PD-1, blocking TIM-3 restores T cell proliferation and enhances the INF-γ production (figure 6) (102).

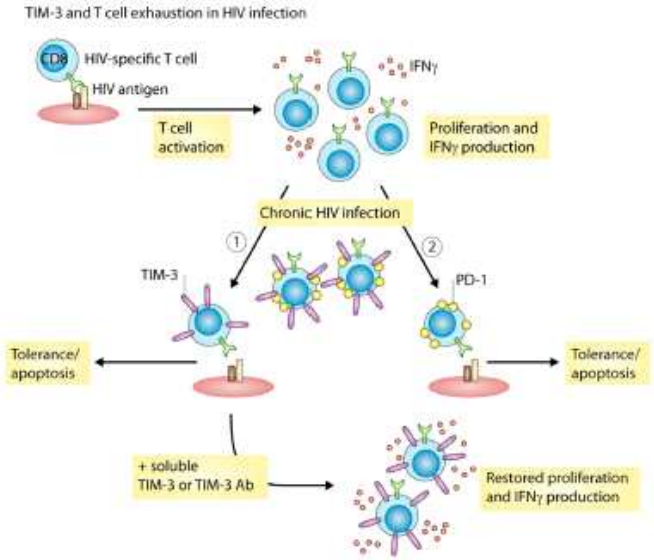


Figure 6. Differential expression of TIM-3 on the surface of T cells regulates susceptibility to viral infection or development of autoimmunity.
In patients with progressive HIV infection, TIM-3 is up-regulated on the surface of CD4+ and CD8+ T cells. Up-regulation of TIM-3 on HIV-specific CD8+ T cells leads to impaired cell survival, proliferation, and cytokine production. Blocking TIM-3 binding to its ligand galectin-9 using soluble TIM-3 or TIM-3 antibody restores functionality of HIV-specific CD8+ T cells from HIV patients. Up-

regulation of PD-1 in HIV patients also leads to T cell dysfunction. Both inhibitory receptors are used as exhaustion markers.

As well as PD-1and TIM-3 other cell surface inhibitory receptors are involved in T cell exhaustion both in human and in animal models: LAG-3 (lymphocyte activation gene 3) is expressed on unresponsive LCMV-specific CD8+ T cells and controls progression of cell cycle; CTLA-4 (cytotoxic T-lymphocyte antigen 4), another member of CD28-family such as CD279, which affects the functional quality of T cell responses during HCV and HIV infections by competition with CD28 for costimulatory ligands; TIM-3 (T cell immunoglobulin domain and mucin domain protein 3), CD244, CD160 and others (103-105).

1.4.4 PD-1/TIM-3 coexpression

As described above during the acquisition of exhausted phenotype, the impaired T cells gradually upregulate inhibitory receptors and more severe exhausted cells coexpress a great number of these receptors during LCMV infection. For example it has been demonstrated that TIM-3 was only transiently expressed after acute infection on CD8+ T cells, but during chronic infection these T cells retained high TIM-3 expression (106). In lymphoid and also in non-lymphoid organs the greater percentage of virus-specific CD8+ T cells coexpressed TIM-3 and PD-1 and this coexpression was associated with decreased proliferation and secretion of effector cytokines (IFN-γ, IL-2 and TNF-α) (107).
Very recently it also was confirmed that in Friend leukemia infection in which the infection results in the generation of virus-specific effector CD8+ T cells terminally exhausted, caused rapid induction of PD-1 and TIM-3 on the effectors T cells (108). This is true also for mice bearing solid tumors where TIM-3 was expressed on CD8+ tumor- infiltrating lymphocytes (TILs): all TIM-3+ TILs also coexpressed PD-1 and even these cells coexpressing both PD-1 and TIM-3 represented the predominant fraction of T cells infiltrating tumors that manifested impaired proliferation and dysfunctional production of cytokines (109).

In mice with advanced acute myelogenous leukemia (AML) this exhausted phenotype of CD8+ T cells characterized by coexpression of TIM-3 and PD-1 was identified in the liver, the first major site of AML metastases. This coexpression increased during desease progression and these T cells were deficient in their ability to produce IFN-γ, TNF-α and IL-2 in response to PD-L1 and Galectina 9 (110).
All these studies demonstrated that the coexpression of TIM-3 on T cells expressing PD-1 leads to a more severe exhausted phenotype.

1.5 Strategies to reverse the exhaustion

Since the T cells exhaustion phenomenon in viral infections is primarily responsible for the inability of the host to fight pathogen agents there has been interest in reversing T cell impairment.
As described above, in chronic LCMV infection in mice and also in humans the blocking of PD-1 pathway leads to the recovery of T cell function and reduced viral load. This shows that some changes in CD8+ T cells during chronic infection are reversible. This is also true for blockade of TIM-3 that can restore proliferation and cytokine production in HIV patients. Unfortunately, these strategies that block antibodies for the ligands of inhibitory receptors show efficacy with T cells that are not completely terminally exhausted (70).
The solution in the case of T cells terminally exhausted to restore or enhance immunity during chronic infection and in cancer could be combining the blockade of inhibitory receptors and/or suppressive cytokines as well as therapeutic vaccination (111,112).
This demonstrates that the efficacy of these strategies depends on several factors, such as the number and expression level of inhibitory receptors that identify exhausted T cells with different potentials for recovering functions.

In a recent study it has been shown that the DNA methylation status of Pdcd1 locus, encoding PD-1, by using human and murine system of acute and chronic viral infection during CD8+ T cell differentiation was inversely correlated with expression of PD-1. The authors show that during acute infection, naive to effector CD8+ T cell differentiation is associated to a transient loss of DNA methylation of the Pdcd1 locus and further differentiation into functional memory cells corresponds to Pdcd1 remethylation. In contrast, in exhausted CD8+ T cells, the Pdcd1 regulatory region is completely demethylated and remains unmethylated even when the virus titer decreases. In addition, to blocking PD-1 signaling, modulating PD-1 expression may also serve to rejuvenate exhausted T cells (113).

1.6 Exhaustion, anergy and senescence

The repeated antigenic stimulation of antigen-specific T cells can lead to exhausted, anergic and also a senescent state that are different processes.
In fact, the activation of T cells is tightly controlled by many positive and negative regulatory processes. This fine-tuning allows productive immunity to pathogens while minimizing the risk of autoimmunity.
One negative regulatory mechanism is clonal anergy, which is a hyporesponsive state that occurs when T cells are stimulated *in vitro* with antigen in the absence of appropriate co-stimulatory signals. These cells become nonresponsive to subsequent stimulation. Both anergy and adaptative tolerance, that is the process of *in vivo* anergy, are states of non-responsiveness distinct from T cell exhaustion; while the unresponsively in anergic T cells is rapidly induced and initiated at the time of first antigenic stimulation, the T cell exhaustion is a progressive process that worsens over time and T cells ongoing to exhaustion probably receive costimulation and undergo massive initial activation (114,115).
In addiction, the gene-expression profiles of anergy and exhaustion seem to be partially different. For example NFAT, the nuclear factor of activated T cells, seems

to play a role in both situations (although to date is not clear how this transcription factor are regulated in exhausted T cell populations) other genes associated with anergy such *Grail*, *Egr2* and *Egr3* seem not to be upregulated in exhausted T cells.
Also senescence and terminal differentiation are important characteristics of the biological system, also for T cell responses. The repeated T cell stimulation can infact lead to a loss of the replicative capacity of antigen-specific T cell populations as a result of unrepaired DNA damage and/or telomere erosion (116-118).
Markers such as KLRG1 in mice and CD57 in human can be used to identify T cells with lower proliferative potential that seem to be senescent or terminally differentiated (119,120).
During T cell responses to infections, some T cells can become terminally differentiated and lose proliferative capacity that correspond with a definition of senescence. Also exhausted T cells show defects in proliferative potential, though severely impaired Tcells expressing markers of senescence at low levels. In a similar manner, the expression of CD57 does not appear to be strongly correlated with PD-1 during HIV infection while there is a connection between PD-1 and telomere length in HIV-infected subject (121).
In many cases CD8+ T cells expressing both KLRG1 or CD57 can still carry out effector functions, unlike exhausted CD8+ T cells and in contrast, repetitively stimulated CD8+ T cells that have some features of terminally differentiated or senescent cells can also undergo some changes associated with T cell exhaustion (122-124).
This data suggest that these processes are distint but the molecular relationships between exhaustion and senescence remain to be completely defined.

1.7 MicroRNAs

MicroRNAs (miRNAs) are a large class of non-coding small RNAs (21-25 nucleotides) that negatively regulate gene expression at the post-transcriptional level

by pairing with specific messenger RNA (mRNA), inducing degradation or preventing the translation in the corresponding protein product (125-127). The first evidence of the existence of microRNAs date back to 1993 when the group of Victor Ambros, through genetic screening of genes underlying defects in the temporal control of post-embryonic development of the nematode *C. elegans,* identified a small 22-nucleotide non-coding RNA, called lin-4. The latter appeared to be partially complementary to seven nucleotides in the conserved region 3' untranslated mRNA of the gene encoding the nuclear protein LIN-14, which sub-expression is essential for the correct temporal progression from first to second stage of larval development (128).

The evidence of complementarity between sequences of lin-4 and lin-14, plus the fact that mutations in lin-4 were able to affect the smooth functioning of development, inspired a large number of molecular and biochemical studies that led to the demonstration that the direct interaction between the two RNA is responsible for controlling expression of the LIN-14 protein (129,130).

As a result a similar mechanism was found that lin-4 could also negatively regulate the gene lin-28 involved in a more advanced stage of larval development of *C. elegans* (131).

These results led to the identification of a completely new mechanism of gene regulation post-transcriptional processes involved in development.

After this discovery, microRNAs have been identified in animals and plants: now it is clear that all eukaryotes use the action of these small RNAs in regulating gene expression (132).

In humans today there have been identified 1048 microRNAs (http://www.mirbase.org/) but research suggests that computing the total number could rise (133,134). This evidence, related to their regulatory nature, confirms the fact, which is now also supported by numerous experimental data, that microRNAs can act not only in development but in most physiological cell processes such as growth, cell division, apoptosis and differentiation.

1.7.1 Biosynthesis of microRNAs

The process leading to the formation of mature microRNAs has been recently clarified (figure 7).

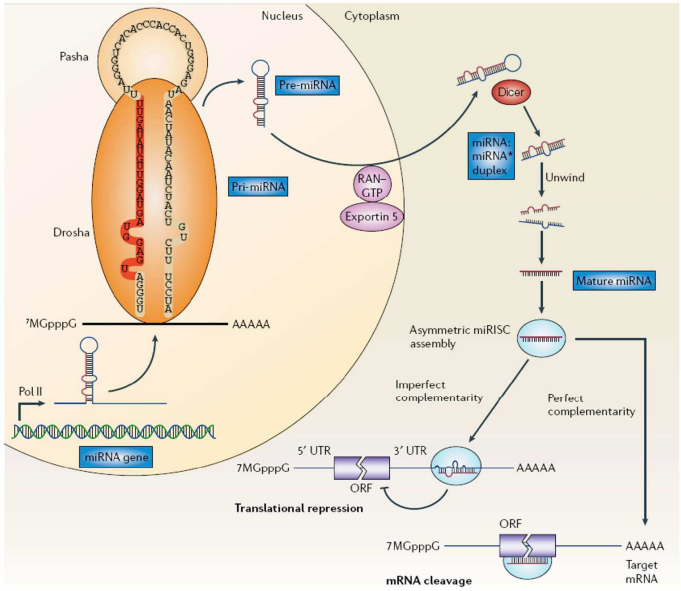


Figure 7. The process of formation of miRNAs.

In the genome the genes for microRNAs can be singly located or in clusters, in intergenic regions or even in the intron sequences of specific genes (135).
The genes for miRNAs are generally transcribed in the nucleus by RNA polymerase II (Pol II) in long precursors, the pri-miRNA. These are processed by endonuclease Drosha (RNase type III) and its cofactor DGCR-8 (Pasha in Drosophila) to form a transcript of about 70 nucleotides called pre-miRNA, with hairpin structure.

Drosha is predominantly localized in the nucleus and contains two domains with sequential activities of RNase III, a binding domain for the double-stranded RNA and a N-terminal region whose functions are still unknown (136).
Although the mechanism by which Drosha is able to discriminate the different precursors of miRNAs is still unknown, there is more evidence that is precisely the structure of the pri-miRNA responsible for this recognition.
In fact, the processing efficiency of Drosha depends on the size of the loop end, the structure of the hairpin and flanking sequences from the recognized site of cut, as it has been observed that mutations that alter the characteristics of these regions significantly induce the inhibition of its activity (137).
After this initial step, the pre-miRNA is exported by Exportin 5, a transporter of the family of RAN-GTPases, from the nucleus to the cytoplasm where another type III RNase, Dicer, catalyzes further pre-miRNA processing leading to the formation of a small (approximately 22 nucleotides) imperfect double-stranded RNA (duplex) (miRNA: miRNA*) that contains both the filament of the mature microRNA (miRNA) and its complementary (miRNA *) (138).
The Dicer protein, about 200 kDa, contains two domains with RNase activities called RIIIa RIIIb, a domain with helicase and ATPase activities, a domain DUF283 with functions still unknown, a C-terminal domain important for the link with the RNA duplex and a PAZ domain (Piwi Argonaute-Zwille).
The latter is especially important in the recognition of the 3' end protruding generated from the cut catalysed by Drosha and could also to be responsible for the correct positioning of the region with RNase activity of Dicer on the cleavage site of pre-microRNAs (139,140).
The last stage of the formation of miRNAs requires the action of RISC (RNA-induced silencing complex), the multiprotein complex that induces RNA silencing. The selection of a specific target messenger RNA and functional efficiency of a microRNA, require that the mature filament of the microRNA generated from the duplex miRNA:miRNA*, is selectively incorporated into the RISC complex (141,142). The complementary strand miRNA * is, however, rapidly degraded.

Although the mature miRNA can reside on both strands of the RNA duplex, it was observed that the preferential incorporation into RISC relative to one another, is mainly due to the instability on the 5 'end: the less stable molecule with its 5 'end is "chosen" as active and mature miRNAs and thus the thermodynamic properties of microRNA precursor to determine the asymmetric assembly of RISC and therefore, the specificity of target mRNA during the post-transcriptional mechanism of inhibition (125).

1.7.2 Mechanism of action and functions of microRNAs

As mentioned previously, the RISC complex is formed by a series of proteins, among which are those of the family of Argonauta (143).
They have two characteristic conserved domains: the PAZ domain, also present in Dicer, and Piwi domain.
The PAZ domain, both in *H. Sapiens* (hAgo2) and in Drosophila, contains a binding site for nucleic acids (OB), while Piwi, given its structural homology with RNase H, would seem to be involved in the process of cutting of the target mRNA (144).
The mechanism by which the miRNA-RISC complex induces the effect of regulatory silencing on messenger RNA is completed by dual mode of action that consists in the degradation of mRNA or in the inhibition of its translation and this depends on the degree of complementarity between the miRNA sequence and specific target. In fact, miRNAs that bind with perfect complementarity to their "target" mRNA will result in the degradation; in this case there is only one annealing site which usually is found in the ORF(open reading frame) or in the coding sequence of the mRNA target. This homologous mode of action to that of RNA interference (RNAi), is commonly found in plants (145).
In contrast, in animals, except for a few exceptions such as that of miR-196, microRNAs induce the inhibition of translation of their messenger target through

imperfect pairing with sequences in the untranslated regions of specific 3' (3'UTR) of their mRNA target (146,147).
In this type of regulation, it was noted that the perfect pairing between first 7-8 nucleotides located on the 5' end of microRNAs and also know as seed sequence, and the 3'UTR of target mRNAs is essential for the proper functioning of the action of microRNAs. Since the initial observation of first microRNA in the model organism *C.elegans* over 1000 microRNAs have been identified in mammalian cells and have been shown to be involved in a range of physiological processes, including development, differentiation and homeostasis.

1.7.3 MicroRNAs in the immune system

The importance of miRNAs in the regulation of the mammalian immune system hematopoiesis has been studied in experiments in which the overexpression of miRNAs in hematopoietic stem cells (HSCs) strongly affected B cell development after transplantation in mice (148).
This observation was further dimostrated by inactivation of components of the machinery of microRNAs that leads to a severely compromised lymphocyte development: in fact, conditional deletion of Dicer , the key enzyme in miRNA biogenesis, in hematopoietic stem cells (HSCs) leads to an inability of these cells to reconstituite the hematopoietic system (149).
This is true also for the development and differentiation of T lymphocytes in mice where deletion of Dicer in immature thymocytes led to a decreased number of total T cells in thymus and pheriphery (150).
Deletion of Ago2, a component of RISC, in hematopoietic cells resulted in compromised development of B and erytroid cells, due to a partial deficiency in microRNAs indicating that miRNAs play key roles for the optimal development of these cells (151).

Studies of expression profiling combined with bioinformatic analyses attempted to identify microRNAs responsible for these phenotypes .
Today it is clear that miRNAs regulate many aspects of the immune system such as both innate and adaptative immune responses and specific miRNAs were attributed the role in immune system (figure 8) (152).

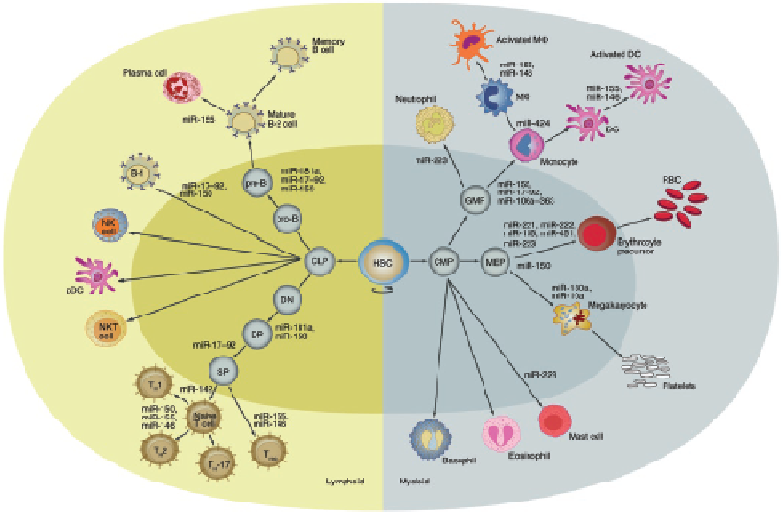


Figure 8. MicroRNAs in hematopoiesis and immune system function.
B-1, B-1 type B cell; B-2, B-2 type 'conventional' B cell; pre-B, pre–B cell; pro-B, pro–B cell; CLP, common lymphoid progenitor; DN, double-negative T cell; DP, double-positive T cell; SP, single-positive T cell; NK, natural killer; pDC, plasmacytoid dendritic cell; NKT, natural killer T; TH1, T helper type 1 cell; TH2, T helper type 2 cell; TH-17, interleukin 17–producing helper T cell; Treg, regulatory T cell; Mφ, macrophage; DC, dendritic cell; GMP, granulocyte-macrophage progenitor; CMP, common myeloid progenitor; MEP, megakaryocyte-erythrocyte progenitor; RBC, red blood cell.

For example miR-150 shows dynamic changes in the expression level during lymphocyte development. This microRNA is expressed at high levels in mature B cells and T cells, but not in their progenitors and its expression is extinguished after differentiation of naïve T cells into effector Th-1 and Th-2 subsets (153,154).
MiR-150 is expressed at high levels also in megakaryocytes and at low level in erythrocytes.
In many laboratories it has been demonstrated the physiological function of miR-150 in hematopoietic development with gain-of- function and loss-of-function mouse models: the overexpression of miR-150 in mouse HSCs leads in defective B cell development during the transition from pro-B cell to pre-B cell. One target of miR-150 is c-Myb that is a transcriptional factor that controls multiple step of lymphocyte development. Studies have confirmed that the gene encoding c-Myb is a miR-150 target and that activated B cells deficient in miR-150 have more c-Myb, whereas B cells from transgenic mouse overexpressing mir-150 have less c-Myb (149). In addiction, in Dicer deficient T cells miR-150 overexpression can restore correct T cell differentiation.
MiR-155 is an another example of a microRNA with specific function in lymphoid differentiation. MiR-155 is processed from a primary transcript, called "BIC" (B cell integration cluster) that was first identified as a frequent site of integration for the avian leucosis virus (155).
In vitro and *in vivo* studies implicated miR-155 in various immune functions, such as innate and adaptative immune responses and also in development of immune cells. Studies have demonstrated that in HSCs this microRNA is expressed at moderate levels whereas mature hematopoietic cells also have a lower miR-155 expression level. Its expression is increased during activation of B and T cells and also in activated monocytes (156, 157). Mir-155 is expressed in many cellular type and seems to have different functions. This microRNA has multiple targets that are regulated in a cell-type-specific manner. In 2007 miR-155 knockout mice were generated by two independent groups and both showed that miR-155 played an essential role in both B and T cell responses: mice knockout for bic/miR-155 gene

were viable but they were unable to develop a proper immune T-, B-, or dendritic-dependent responses (158-160).
MiR-155 is involved in the regulation of T cell lineage fate by promoting T helper type 1 differentiation, possibly by targeting the transcription factor c-Maf.
In addition to B cells and T helper cells, miR-155 is expressed in regulatory T cells and is involved in their development (161,162).
MiR-155 is also involved in innate immunity and in monocytes and macrophages MiR-155 is upregulated after exposure to inflammatory stimuli (163-165).
Another microRNA of interest is miR-146 that was first identified as an immune system regulator that influence the mammalian response to microbial infection.
Many studies have demonstrated distinct patterns of miR-146 expression in various hematopoietic lineages and this has suggested the involvement of this microRNA in the maintenance of lineage identity in lymphocyte subsets: miR-146 is expressed at low level in naïve T cells and is upregulated in T helper type 1 cells but not in T helper type 2 cells suggesting that miR-146 is a Th1 specific miRNA (153, 164).
MiR-146 was reported to be expressed at high levels in human monocytes in response to LPS. In addition, miR-146 is among the most highly expressed miRNAs in regulatory T cells, and overall, the miRNA expression pattern of the regulatory T cell lineage as well as the constitutive expression of CD25 resembles the profile of activated rather than naive T cells.
MiR-181a is another microRNA that plays roles in hematopoietic differentiation and also in T cell differentiation and functions: miR-181a affects B cell lineage selection as well as T cell development and activation (166).
In the bone marrow, progenitor (lineage-negative) cells have low expression of miR-181a and B cells have higher expression of miR-181a. When overexpressed *in vitro* in culture conditions that promote multilineage differentiation, miR-181a induces a two- to threefold increase in CD19+ B cell populations but does not affect T or myeloid cells. In purified thymocytes subsets, miR-181a is dynamically regulated, with expression increasing in populations in double-negative stage 1 through double negative stage 3 and then decreasing during the remainder of T cell development.

Overexpression of miR-181a augments T cell receptor (TCR) signaling strength. Conversely, knockdown of miR-181a results in lower TCR signal strength and in the inhibition of positive and negative selection in an *in vitro* fetal thymic organ culture model. MiR-181a seems to 'tune' TCR signal strength by downregulating expression of several protein tyrosine phosphatases, including SHP-2 and PTPN22 with the result to enhance basal activation of the TCR signaling molecules Lck and Erk. These results indicate that miR-181a has a physiological function during thymic selection by regulating TCR signaling strength in a cell-intrinsic way and that miR-181a may regulate the activation of mature T cells by modulating TCR signal strength. These findings demonstrated that microRNAs play an important role in the development, stability of immune system cells and also in the regulation of their responses.

2. Project aims

The principal aim of our project was to study the early phases of T cell exhaustion in healthy individuals on the basis of the expression level of PD-1, a typical exhaustion marker and valuating microRNAs involved in T cell impairment.
T cells (CD4+ and CD8+) are important for control of infections and their cooperation for killing viruses and pathogens. The persistent antigenic stimulation however drives T cells to an iporesponsive state, termed Exhausted, characterized by lose of effector functions such as cytokine production.
Recently, an increasing number of studies have suggested the importance of inhibitory receptor pathways in functional impairment of T cells.
Particularly, an extraordinary amount of effort has been focused to decipher the role of the PD-1/PD-L1 pathway.
Programmed death-1 (PD-1) is a member of the B7:CD28 family which is mainly expressed on the surface of activated T and B cells. Ligation of PD-1 with the ligand PD-L1 results in dephosphorylation of signaling molecules downstream of the TCR resulting in the dampening of T-cell receptor (TCR) signalling and the eventual inhibition of T-cell activation.
Functional recovery of the exhausted T cells was also shown to be possible with PD-1/PD-L1 blockade, restoring proliferation, cytokine secretion, cytotoxic capability and decreasing viral load.
PD-1 expression remains high in persistent antigenic stimulation and is highly detectable on dysfunctional, exhausted T cells in chronic viral infections and also in cancers. It represents a typical marker of T cell exhaustion.
In addiction to PD-1, many other cell surface inhibitory receptors coregulate T cell exhaustion and more impaired T cells upregulate inhibitory markers.
In a recent study in HIV-1 infection TIM-3 emerged as another receptor that influences the exhausted state.
As with PD-1, blockade of TIM-3 improves the proliferation and responsivity of the exhausted T cells *in vitro*. The coexpression of both PD-1 and TIM-3 in T cells is

associated with a more exhausted phenotype and the blockade of both markers can reverse the functional capacities, such as proliferation, cytokines production and the killing activity.
MiRNAs are a conserved class of non-coding RNAs that negatively regulate gene expression post-transcriptionally. Initially identified in invertebrates, miRNAs are believed to be important during development, and for regulation of cell proliferation, effectors functions and apoptosis in human cells. However, the widespread significance of miRNA regulation in dysfunctional immunity system has yet to be completely demonstrated.
Antisense technologies for inhibition or replacement of miRNA activity will be useful tools for miRNA functionalization, as well as for therapeutic modulation of miRNAs.
Little is know on microRNAs profiling for T cells expressing PD-1 and microRNAs expression profiles of these cells might help understand the early phases of T cell exhaustion and migh have therapeutic utility to eradicate the dysregulated or alterated meccanism that occur in cancer and chronic infections.
In particular, the objectives of our project were:

1) Characterize PD-1/PD-L1 pathway in human T cells.
Human CD4+ and CD8+ T cells of healthy donors will be studied to verify if the PD-1 expression is correlated with dysfunctional capacities analyzing the cytokine production in CD4 + and CD8+ T cells/ PD-1 positive ($PD\text{-}1^{+}$) and PD-1 negative ($PD\text{-}1^{-}$) and evaluating this inhibitory receptor as exhaustion markers.
We analyzed varyious buffycoats of healthy donors (HD) by surface staining of PD-1 and we selected samples in wich PD-1 expression on PBLs was at least 8-9% to recover a sufficient number of cells for subsequent analysis.
PD-1 positive and PD-1 negative PBL population were FACS sorted by peripheral blood population and the functional features of $CD4^{+}$ and $CD8^{+}$ T cells/ PD-1 positive and PD-1 negative were analyzed by intracellular cytokine analysis using flow cytometry technology.

2) To develop an experimental protocol to obtain T cells PD-1^{+}TIM-3^{+} aspecific lines to use them as model of T cells exhausted *in vitro* and to control if the more exhausted phenotype is due to a coexpression of both PD-1 and TIM-3 markers.
We generated CD4+ and CD8+ T cell clones from peripheral blood lymphocytes by limiting dilution and we stimulated these T cell clones *in vitro* and tested the expression of PD-1 and TIM-3 markers.
The functional features of T cell clones expressing low and high level of both PD-1 and TIM-3 were analyzed by intracellular cytokine assay to correlate the cytokine production of levels of exhaustion markers.

3) Comparative analysis was performed of microRNA profiling of PD-1+ and PD-1- PBL in order to study microRNA upregulated and downregulated in PD-1+ T cells that are in early exhaustion.
To study microRNA profiling on sorted PBLs-PD-1+ and PD-1- of Healthy Donors we analyzed the expression of an microRNA panel to detect upregulated and dowregulated microRNAs by TAQMAN LOW DENSITY ARRAY. We selected them for subsequent study and microRNAs resulted upregulated of interest for the immune system in relation on the exhaustion phenomenon.

3. MATERIALS AND METHODS

3.1.1 Healthy donor selection

In this study we recruited healthy donors according to ethical guidelines of the 1975 Declaration of Helsinki.

3.1.2 Reagents and materials

FITC-conjugated anti–PD-1, APC-Cy 7–conjugated anti-CD4 and anti-CD8 were from eBioscence; PE- conjugated anti–TIM-3 were from RNDsystem
PE and FITC-conjugated anti–IL-2, PE and FITC-conjugated anti -IFN-γ and PE-conjugated anti –IL-10 were from eBioscence;
Cytofix/Cytoperm Kit were from BD Biosciences (Pharmingen). Dynabeads CD3/CD28 T Cell Expander (anti-CD3/CD28) was from Dynal Biotech.
PMA, ionomycin and Brefeldin-A was from Sigma-Aldrich.
PHA were from Wellcome, Beckenham, United Kingdom.
Terasaki plates for cloning were from Falcon.
Complete medium was RPMI 1640 medium containing 5% AB serum or 10% fetal calf serum, 2 mM glutamine, 1% nonessential amino acids, 1% sodium pyruvate from Invitrogen and supplementated with recombinant IL-2 (Proleukin) at 50U/ml.

3.1.3 Cell preparation

PBLs from fresh whole blood of Healthy donors were isolated using Ficoll gradient centrifugation on Lymphoprep cushions (Nycomed pharma, AS, Oslo, Norway).

3.2 Surface pre-staining on PBLs of Healthy Donors (HD)

PBLs isolated as described above were pre-stained with FITC-conjugated anti–PD1 mab and then sorted if the PD1expression was at least 8-9%.

3.3 Sorting PBL-PD1^{+}/ PD-1^{-}

PBL-PD1 positive and PBL-PD1 negative were purified from whole PBMCs: briefly, PBMCs were stained with FITC-conjugated anti–PD1 and then processed with FACSAria (BD) to sort PBLs-PD1^{+} and PBLs-PD1^{-} cells. High level of purity ranged from 56-92.2% were found in the positively sorted PD-1^{+} PBLs population, and less than 13% were found in the PD-1^{-}PBLs .

3.4 Intracellular staining on PBL-PD1^{+} and PD-1^{-}

After sorting a portion of cells (50X10^{3} PD1^{+} PBLs and PD1^{-} PBLs) were stimulated with anti-CD3/CD28 and PMA and ionomicina as controls in 96 wells plates for 12 h at 37°C. At the second hour, 5 μg/ml brefeldin-A was added. Cells washed and stained with labeled mAbs to CD4 and CD8; cells were washed, then fixed and permeabilized using Cytofix/Cytoperm solution at 4°C for 20 min, rewashed with Perm Wash Buffer, and stained at intracellular level with labeled mAbs to IL-2 and IFN-γ for 30 min at 4°C. Negative controls were obtained by unstimulated cells. Stained cells were acquired with a FACSCanto flow cytometer and analyzed using FACSDiva software (BD).
Remaining amounts of sorted PD-1^{+} and PD-1^{-} PBLs populations were washed twice in PBS 1X and preserved in Trizol reagent (1ml /5X10^{6} cells) to extract RNA and to analyze microRNAs.

3.5 Generation of T cell clones from peripheral blood lymphocytes

Lymphocytes were separated from the PBMCs cell population by Ficoll-Hypaque density gradient centrifugation, were counted and then cloned. Briefly, T-cell clones were generated by limiting dilution at 0.5 cell/well onto 60-well Terasaki plates (Falcon) in the presence of 1 µg/ml of phytohemoagglutinin (PHA ,Wellcome, Beckenham, United Kingdom), 100 UI/ml of human IL-2, and irradiated (3,000 rads) allogeneic feeder cells (5 X 105 cells/ml) and RPMI supplementated with 10% FCS and culturated at 37°C and 5% CO_2. After 10 to 12 days, cell growth was examined with an inverted microscope. T-cell blasts growth were trasferred in 96 well plates with irradiated allogeneic feeder cells (1 X 10^6 cells/ml)**,** PHA and complete medium supplementated with IL-2; growing cells were expanded in IL-2-containing medium and stimulated with PHA plus APC every 2 weeks.
T clones obtained were tested in order to verify purity; briefly 100X106 T clones cells were stained with mAb anti-CD4 and anti-CD8 and then acquired with a FACSCanto flow cytometer and analyzed using FACSDiva software (BD) to test purity. We obtained a purity > 90 % for all clones.

3.6 In vitro induction of PD-1 and TIM-3 expression and surface staining

T cell clones obtained were stimulated in 24 well plates (1 X 10^6 cells/ml)**,** with PMA/iono (10ug/ml) or irradiated allogenic feeders as APC (1 X 10^6 cells/ml) plus PHA (2µg/ml) and complete medium supplementated with IL-2 (100UI/ml) and cultured at 37°C and 5% CO_2.
Then the PD-1 and TIM-3 expression was monitored before the stimulation (t0) and every 18-48-72 and 96h after the stimulation protocol with PMA/iono and after 10 days after first and second stimulation APC plus PHA: 100X10^3 cells are stained with mab against PD-1 and TIM-3 for 20 min at 3°C. After staining, cells were washed twice and then acquired with a FACSCanto flow cytometer and analyzed using FACSDiva softare (BD). Cells that were unstained were used as a negative control.

3.7 Intracellular staining on CD4+/CD8+ T cell clones

Unstimulated T cell clones (at t0) with low level of marker PD-1 and TIM-3 and stimulated with PMA/iono or PHA-feeders and IL-2 with higher level of both markers (controlled by flow cytometry) were stimulated with PMA /iono, with anti-CD3/CD28 or in 96 wells plates for 12 h at 37°C. At the second hour, 5 μg/ml brefeldin-A was added. Cells were washed, then fixed and permeabilized using Cytofix/Cytoperm solution at 4°C for 20 min, rewashed with Perm Wash Buffer, and stained at intracellular level with labeled mAbs to IL-2 and IFN-γ for 30 min at 4°C. Negative controls were obtained by unstimulated cells. Stained cells were acquired with a FACSCanto flow cytometer and analyzed using FACSDiva software (BD).

3.8 MicroRNAs profiling on total PBLs/PD-1+ and PD-1- in HD: Taqman microRNA array

Total RNA was extracted using the Trizol Reagent (Invitrogen) following manufacturer' s protocol.
1 microliter of 20mg/ml Molecular biology grade Glycogen (Roche) was added to each sample before isopropanol precipitation.
40 ng of total RNA were reversely transcribed using “TaqMan® MicroRNA Reverse Transcription Kit, 200 reactions” (# 4366596, Applied Biosystems) with “Megaplex™ Primer Pools, Human Pools Set v3.0” (# 4444750 Applied Biosystems). Following Reverse Transcription a pre-amplification step was carried out using “TaqMan® PreAmp Master Mix” (#4391128 Applied Biosystems) and Megaplex™ Primer Pools (#4444750 Applied Biosystems). Finally, “TaqMan® Array Human MicroRNA A+B Cards Set v3.0” (#4444913 Applied Biosystems) were loaded and run using “TaqMan® Universal PCR Master Mix, No AmpErase® UNG” (# 4324018, Applied Biosystems). All steps were performed following

manufacturer's protocol. In particular, all quantities and PCR cycling conditions were according to MegaplexTM Pools For microRNA Expression Analysis Protocol (Part Number 4399721 Rev. C, Applied Biosystems).
MiRNA Cards were run on a 7900HT Fast Real-Time PCR System.
Data was analyzed using Applied Biosystem dedicated software (SDS 2.4).
TLDA were run in the 7900 HT Sequence Detection system. The ABI TaqMan SDS v2.4 software was utilized to obtain raw CT values. To review results, the raw CT data (SDS file format) was exported from the Plate Centric View into the ABI TaqMan RQ manager software. Automatic baseline and CT were set for all samples. Delta Ct values were used to compute fold changes.

4. RESULTS

4.1 Screening of healthy individuals

To characterize PD-1/PD-L1 pathway in human T cells and to verify that this marker is a good candidate for the study of the early phase of exhaustion state, we controlled the levels of PD-1 expression on the peripheral blood cells of 90 healthy donors through flow cytometry analyses.
As shown in figure 4.1A most of the tested individuals presented an extremely low level of PD-1 expression and lower PD-1 levels ranged from 0.3-4.2%.
On 20 buffycoats of HD indicated just 6 showed sufficient PD-1 expression. These 6 individuals expressing PD-1 ranged from 8.8-19.8% and were selected to sort total (CD4+ and CD8+) T cells /PD-1^+ and PD-1^-.
As shown in figure 4.1B and figure 4.1C PD-1 expression less than 1% (i.e. 0.8%) would not allow sufficient recovery of $CD4^+$/$CD8^+$ PD-1^+ and PD-1^-. The CD4+ and CD8+ T cell-percentage-expressing PD-1 was only 1.4 and 0.6% respectively: a value that is too low and not sufficient for subsequent sorting experiments.

Indeed, with PD-1 expression of 8.8% , the percentages of T cells (CD4+ and CD8+) PD-1^{+} was good (30.4% and 13.7% respectively) and sufficient to recover an adeguate number of PBLs-PD1^{+} and PD-1^{-} by Facs sorting strategy.
This demonstrates that in healthy individuals there is a very low level of PBLs-PD-1^{+} and that PD-1 expression at least 8-9% is sufficient to recuperate an adeguate number of Total CD4+ and CD8+/ PD1^{+} T cells. In both cases the CD4+ T cells on PBLs expressed a higher level of PD-1.

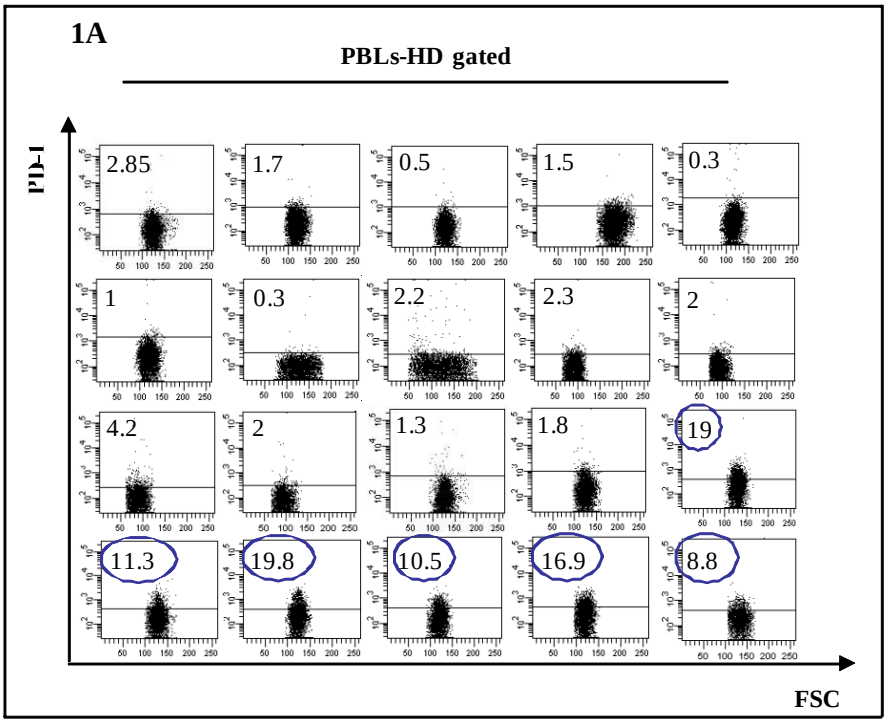


Figure 4.1A. Flow cytometry analyses of PD-1 expression on 20 HD-PBLs.
HD-PBLs stained with mAbs to PD-1. Blue circles indicate buffycoats of HD chosen for sorting experiments. FSC, forward scatter**.**

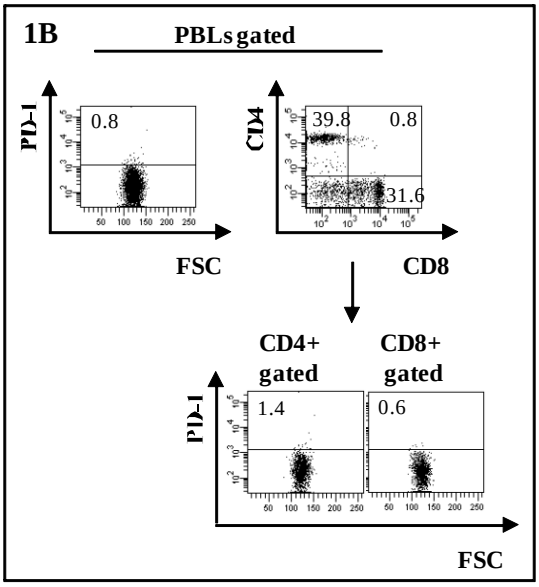


Figure 4.1B. PD-1 expression of 0.8% is not adequate to sort a sufficient number of CD4+ and CD8+ PD-1positive total T cells. HD-PBLs stained as in figure 4.1 A and also with mAbs to CD4 and CD8. The percentage of cells is indicated in each quadrant. FCS, forward scatter.

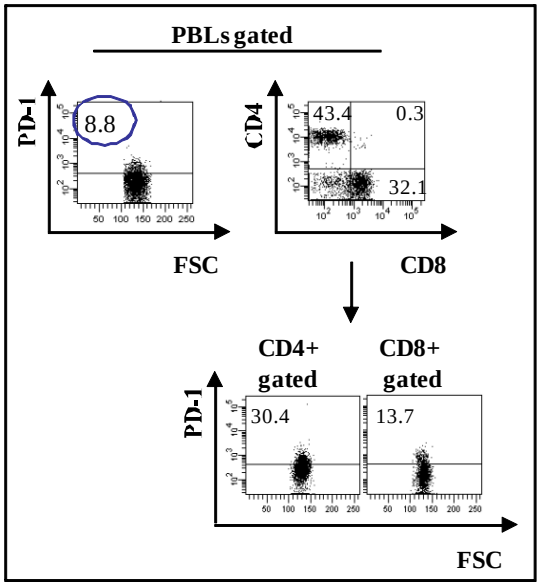


Figure 4.1C. PD-1 expression at least of 8-9% on HD-PBLs is adeguate to sort a sufficient number of CD4+ and CD8+ PD-1positive total T cells. HD-PBLs stained as in 4.1 B. The percentage of cells is indicated in each quadrant. FCS, forward scatter.

4.2 Phenotypic analyses on sorted PBLs and functional analyses on PD-1^+ and $PD1^-$ /$CD4^+$ and $CD8^+$ T cells

In order to study the phenotypic characteristics of CD4+ and CD8+ T cells on the basis of the PD-1 expression we chose 6 buffycoat, in which the mean PD-1 expression on PBLs was 14% (fig 4.1 A) and we proceeded with the Facs sorting of PD-1 positive and PD1 negative PBLs populations after surface staining with mab against PD-1.

As shown in figure 4.2.1 from a sample analyzed in which the PD-1 expression on PBLs was 11.3% we obtained good enrichment and purity of both sorted populations. In fact, flow cytometry analysis showed that 56% of PD-1^+ T cells were found in the positively sorted PD-1^+PBLs population and a total purity (0% $PD1^+$ T cells) for the PD-1^-PBLs population. The exhausted cells are characterized by impaired functional properties such as reduced cytokine production and this defect is associated with upregulation of the PD-1 exhaustion marker. To verify if the existing correlation between high expression of PD-1 and diminishing cytokine production is also true in healthy donors, we tested the Facs sorted PD-1positive and PD-1negative PBLs populations by intracellular staining assay to detect the differences in the secretion of IL-2 and IFN-g by PD-1^+ and PD-1^- /$CD4^+$ and $CD8^+$ T cells. We stimulated CD4+ and CD8+ total T cells with antiCD3/CD28 and PMA/ionomycin as a positive control and unstimulated as a negative control for 12 h at 37°C. Cells were stained with labeled mab against CD4 and CD8 for 20 min and then assessed cytokines secretion by intracellular cytokine staining.

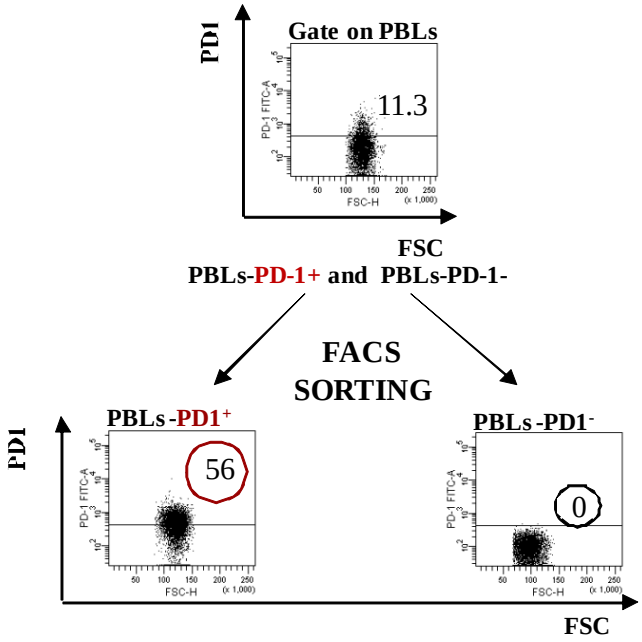


Figure 4.2.1. Strategy of PBLs-PD-1$^+$ and PBLs-PD-1$^-$ sorting.
A representative experiment in which PBLs of a healthy donor sample 2 stained with mab against PD-1 and PD-1 expression was analyzed by flow cytometry. Facs sorting of PD-1+ and PD-1-/PBLs populations were done with FACS ARIA. The purity of sorting was tested by acquiring a portion (50x103) of both facs sorted fractions and also the unstained PBLs as a negative control at the flow cytometer. FCS, forward scatter.

Out of six experiments and only 2 samples analyzed, we observed functional differences of both total CD4+ and CD8+ T cells expressing or not PD-1.
As shown in figure 4.2.2 we observed differences in IL-2 production for CD4 PD-1+ versus PD1- T cells; CD4+PD-1+ T cells (analyzed by PBLs expressing PD-1 at 56%) stimulated with anti-CD3/CD28 produced 2.1% of IL-2 compared at 15.3% of IL-2 produced by CD4+ PD-1- T cells. This difference was also observed for the control stimulus PMA/ionomycin in which CD4+PD-1+ and CD4+PD-1- T cells produced 1.6% and 16.1% of IL-2, respectively. The amount of IFN-γ produced by CD4+PD-1+ T cells was slightly lower compared to that produced by CD4+PD-1- T cells after stimulus with anti-CD3/CD28: 1.1% versus 1.4% and with the control stimulus we did not observed any evident differences.
The percentage of IL-2+INF-γ+ double producing cells between CD4+PD1+ and CD4+PD1- T cells was also different: with anti-CD3/CD28 we observed only 0.9% of CD4+ PD-1+ double producing T cells compared to 2.1% observed in the CD4+ PD-1-, and also with the control stimulus we observed differences (4.1% in CD4+PD1+ T cells versus 6.9% in CD4+PD-1- T cells).
The analysis of cytokines produced by CD8+/PD-1+ and PD-1- showed similar results: as shown in figure 4.2.3 with anti-CD3/CD28 stimulus the CD8+PD-1+ T cells produced only 0.7% of IL-2 compared to 2.8% produced by CD8+PD-1- T cells. This difference was more pronounced with the control stimulus PMA/ionomycin: 0.7% and 9.4% of IL-2 was produced by CD8+/PD-1+ and PD-1- respectively.
The amount of IFN-γ produced by CD8+PD-1+ T cells was much lower compared to that produced by CD4+PD-1- T cells after stimulus with anti-CD3/CD28: 1.7% versus 13.7% while with the control stimulus PMA/ionomycin the CD8+PD-1+ T cells were slightly better producers of IFN-γ compared to CD8+ PD-1- T cells (27.9% versus 27.1% respectively) . The percentage of IL-2+INF-γ+ double producing cells between CD8+PD1+ and CD8+PD1- T cells was also different: with anti-CD3/CD28 we observed only 0.4% of CD8+ PD-1+ double producing T cells compared to 0.7% observed in the CD8+ PD-1-, and also with the control stimulus

we observed that the differences were more pronounced (5% in CD8+PD1+ T cells versus 8.4% in CD8+PD-1- T cells).

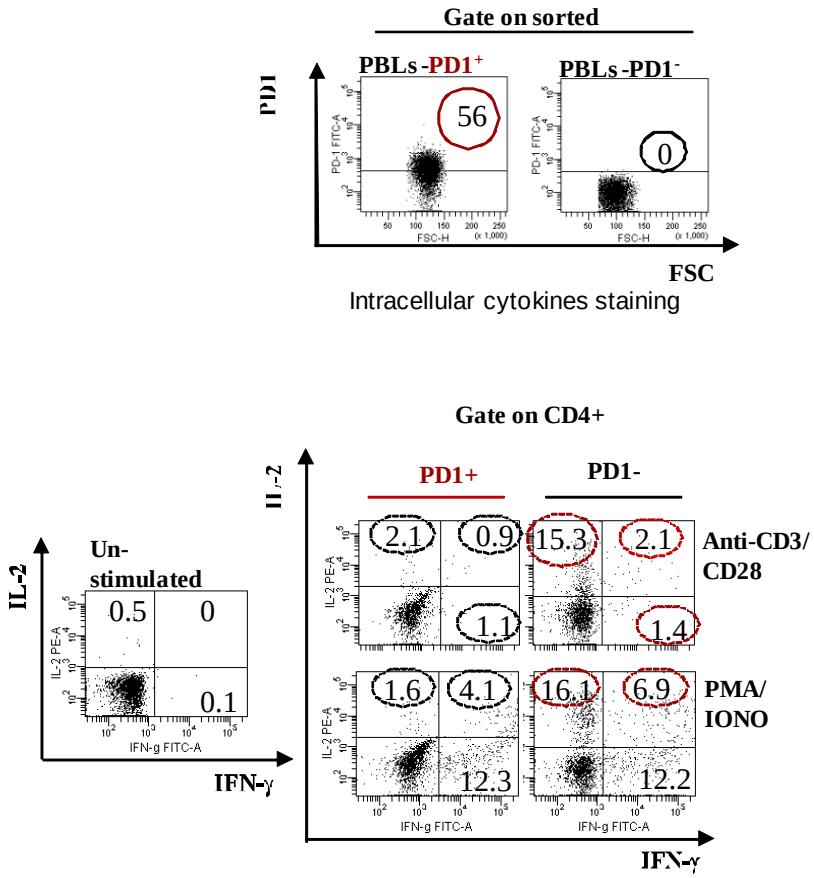


Figure 4.2.2. Enrichment of PBLs-PD-1+ and PD-1- and comparative analysis of cytokine production by PD-1$^+$ and PD-1$^-$ /CD4$^+$ T cells in HD sample 2. A representative experiment in which the FACS sorted population of PBLs-PD-1+ and PD-1- was analyzed by flow cytometry to control the enrichment of both populations demonstrating purity. Sorted PBLs-HD are tested by

intracellular staining assay to analyze the secretion of IL-2 and IFN-γ by CD4+ PD-1+ and CD4+PD-1- T cells. On the left unstimulated T cells are represented as negative controls.

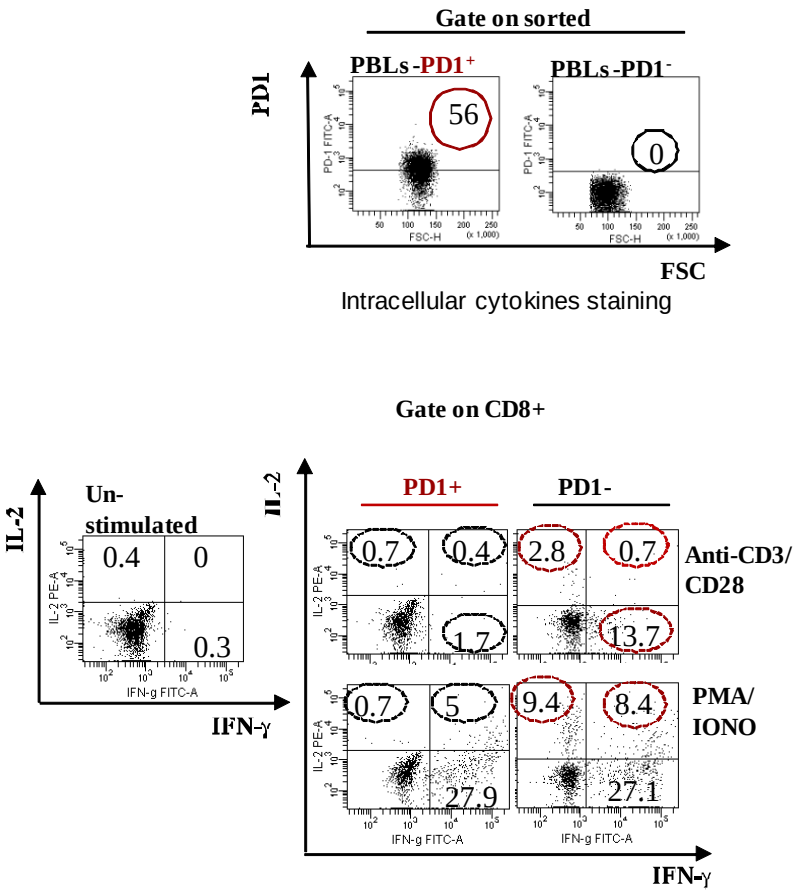


Figure 4.2.3. Comparative analysis of cytokines production in PD-1+ and PD-1- /CD8+ T cells in HD sample 2. PBLs-HD are facs sorted on the basis of PD-1 expression and then the secretion of IL-2 and IFN-γ by CD8+ PD-1+ and CD8+PD-1- T cells was tested by intracellular staining assay. Left are represented T cells unstimulated as negative control.

The cytokine production mean of these two experiments infact demonstrated functional differences between CD4+ and CD8+ T cells expressing or not expressing PD-1. As shown in figures 4.2.4 and 4.2.5 CD4+ and CD8+ T cells PD-1+ produced less IL-2 and IFN-γ with both stimuli compared CD4+ and CD8+ T cells PD-1- though the differences were not statistically significant.

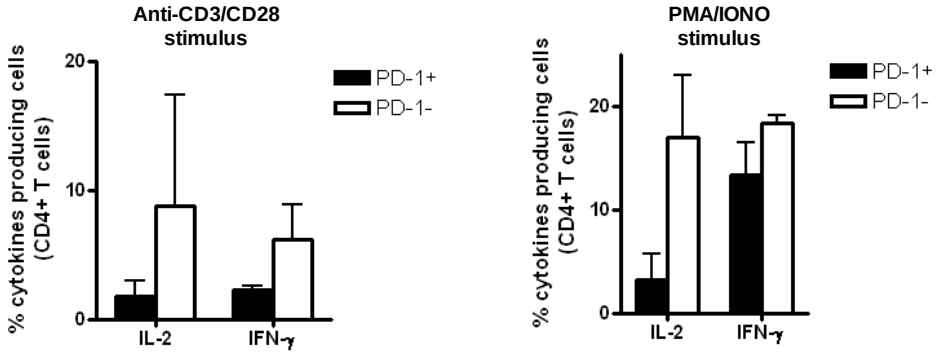


Figure 4.2.4. Cytokine production by CD4+PD-1+ and CD4+PD-1- T cells in two experiments. The figure shows the mean +SD of cytokine production of two experiments. Facs sorted PBLs-HD are stained with labeled ab against surface marker CD4 and then the percentage of CD4+ T cells producing IL-2 and IFN-γ were tested by intracellular staining assay stimulating T cells with anti-CD3/CD28 and PMA/ionomycin. The cytokines produced by PD-1+ CD4+ T cells were compared to those produced by PD-1- CD4+ T cells. The statistical analysis was done by applying the t test. (P>0.05 unpaired t test).

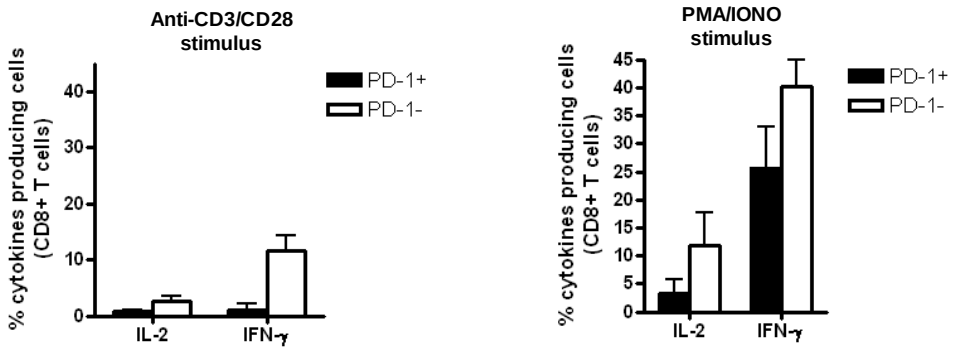


Figure 4.2.5. Cytokine production by CD8+PD-1+ and CD8+PD-1- T cells in two experiments. The figure shows the mean +SD of cytokine production of two experiments. Facs sorted PBLs-HD are stained with labeled ab against surface marker CD8 and then the percentage of CD8+ T cells producing IL-2 and IFN-γ were tested by intracellular staining assay stimulating T cells with anti-CD3/CD28 and PMA/ionomycin. The cytokines produced by PD-1+ CD8+ T cells were compared to those produced by PD-1- CD8+ T cells. The statistical analysis were done by applying the t test. (P>0.05 unpaired t test).

This demonstrated that total T cells (CD4+ and CD8+ T cells) in HD samples 1 and 2 (represented in table 1) expressing PD-1 produced less cytokines (IL-2 and also IFN-γ) compared to total CD4+ and CD8+ T cells/ PD-1 negative.

Healthy Donor samples-analyzed

HD SAMPLES	PD-1 EXPRESSION ON PBLs	PD-1 expression on positively sorted PBLs population	PD-1 expression on negatively sorted PBLs population	Cells number recovered
HD 1	19 %	65.3 %	0 %	$5X10^6$ ON BOTH
HD 2	11.3 %	56 %	0 %	$5.5X10^6$ ON BOTH
HD 3	19.8 %	92.2 %	12.6 %	$3.1X10^6$ ON BOTH
HD 4	10.5 %	83.2 %	0.1 %	$2.85X10^6$ ON BOTH
HD 5	16.9 %	81.2 %	2.8 %	$5.75X10^6$ ON BOTH
HD 6	8.8 %	73 %	0.1 %	$1.156X10^6$ ON BOTH

Table 1. HD samples in which PD1+ and PD-1- /PBLs population have been sorted. Purity of both populations sorted and number of cells recovered.

In another four experiments we obtained different results: analysis on sorted PBLs/PD-1+ and PD-1- in which the enrichment of positively sorted populations was higher (73%) and the purity of negatively sorted population was complete (99.9%), as represented in figure 4.2.6, showed that the CD4+PD-1+ T cells stimulated with anti-CD3/CD28 produced less IL-2 than did CD4+PD-1- T cells:

6.7% of IL-2 were produced by CD4+PD-1+ T cells compared to 14.6% produced by CD4+PD-1- T cells, contrariwise with the control stimulus where the CD4+ PD-1+ T cells produced 44,2% of IL-2 versus 25.4% produced by CD4+PD-1- T cells. The CD4+ PD-1+ T cells produced major amounts of IFN-γ with both stimuli: 6.3% and 74.3% produced by CD4+PD-1+ T cells stimulated with anti-CD3/CD28 and PMA/ionomycin respectively and only 3.5% and 14.2% of IFN-γ was produced by CD4+PD-1- T cells.

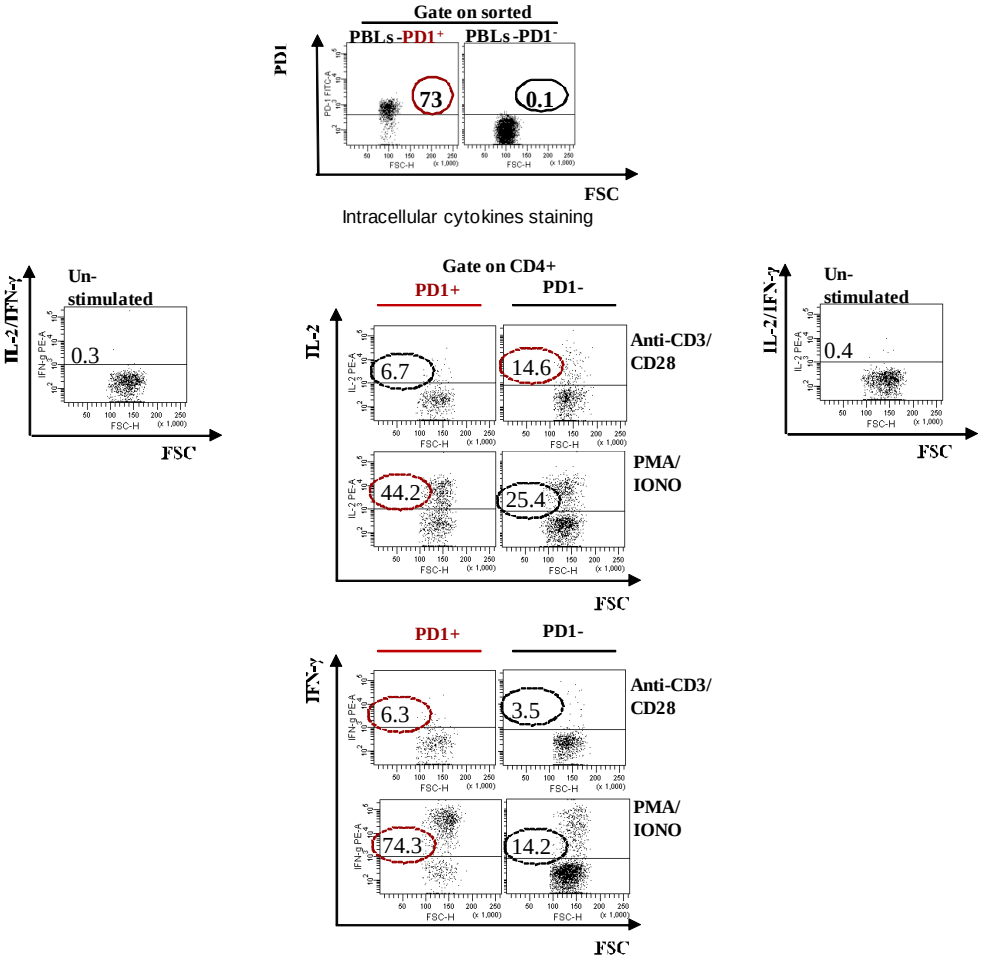


Figure 4.2.6. Comparative analysis of cytokine production by PD-1$^+$ and PD-1$^-$ CD4$^+$ T cells: HD-sample 6. CD4+PD-1+ T cells stimulated with anti-CD3/CD28 produced less IL-2 than did the CD4+PD-1- T cells but CD4+PD-1+ T cells were better producers of IFN-γ. The top left and right represents the CD4+ T cells/PD-1+ and PD-1- unstimulated as negative controls.

Analysis of cytokine production by CD8+ T cells, represented in figure 4.2.7 showed that the CD8+PD-1+ T cells stimulated with anti-CD3/CD28 produced less IL-2 than did CD8+PD-1- T cells: 0.4% versus 0.9%. Alternatively with the control stimulus the CD8+PD-1+ T cells produced slightly more IL-2 compared CD8+PD-1- T cells: 14.8% versus 13.3%.
The CD8+ PD-1+ T cells produced major amounts of IFN-γ with both stimuli: 4.4% and 93.4% of IFN-γ was produced by CD8+PD-1+ T cells when stimulated with anti-CD3/CD28 and PMA/ionomycin respectively and only 2.6% and 13.4% of IFN-γ was produced by CD4+PD-1- T cells.

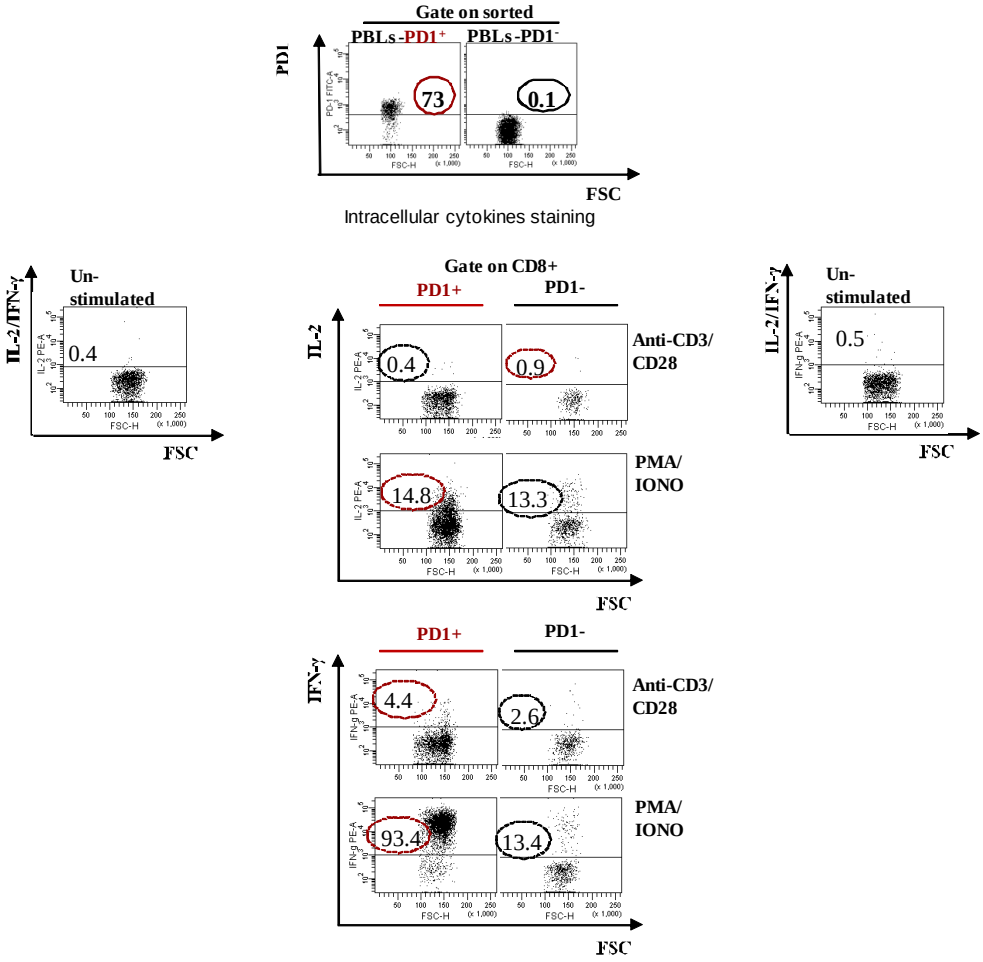


Figure 4.2.7. Comparative analysis of cytokine production by PD-1^{+} and PD-1^{-} CD8^{+} T cells: HD-sample 6. CD8+PD-1+ T cells are bettee producers of IFN-γ than CD8+PD-1- T cells. The top left and right represents the CD8+ T cells/PD-1+ and PD-1- unstimulated as negative controls.

The cytokine production mean on the other four samples (HD samples 3-6) demonstrated a weak differences in the IL-2 production by CD4+ PD-1+ T cells compared to CD4+ PD-1- T cells with anti CD3/CD28 stimulus, conversely with the control stimulus the CD4+PD-1+ T cells were better producers of IL-2 compared to CD4+ PD-1- T cells and these differences were not statistically significant.
CD4+PD-1+ T cells instead produced more IFN-γ with both stimuli compared to CD4+PD-1- T cells and with the control stimulus this difference was statistically significant (figure 4.2.8).

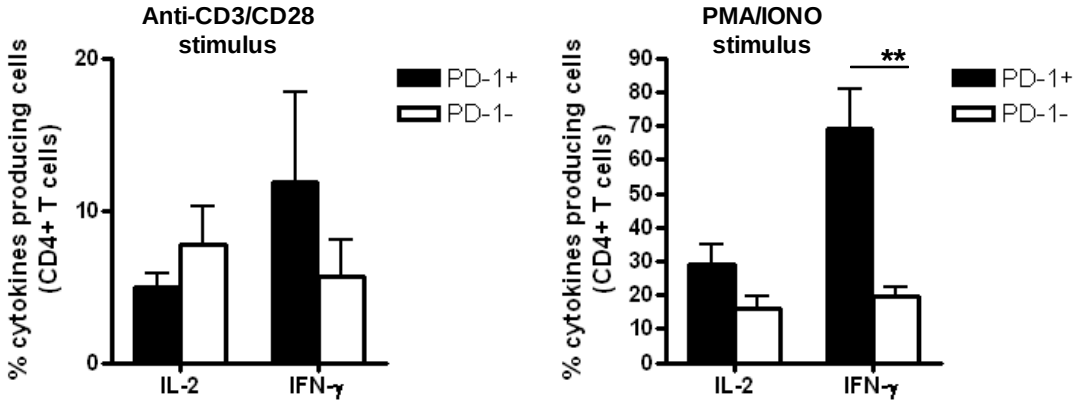


Figure 4.2.8. Means of cytokine production by CD4+PD-1+ and CD4+PD-1- T cells in four experiments. The figure shows the mean +SD of cytokine production of four experiments. Facs sorted PBLs-HD are stained with labeled ab against surface marker CD4 and then the percentage of CD4+ T cells producing IL-2 and IFN-γ were tested by intracellular staining assay stimulating T cells with anti-CD3/CD28 and PMA/ionomycin. The cytokines produced by PD-1+ CD4+ T cells were compared to those produced by PD-1- CD4+ T cells. The statistical analysis was done by applying the t test. (** p=0.0063 unpaired t test).

The analysis of cytokine production means in CD8+ T cells showed that with anti CD3/CD28 stimulus, the CD8+PD-1+ T cells produced less IL-2, in contrast to the control stimulus, the CD8+PD-1+ T cells were better producers of IL-2 compared to CD8+ PD-1- T cells, even though the differences were not statistically significant.
CD8+PD-1+ T cells instead produced more IFN-γ with both stimuli compared to CD8+PD-1- T cells and with the control stimulus this difference was statistically significant (figure 4.2.9).

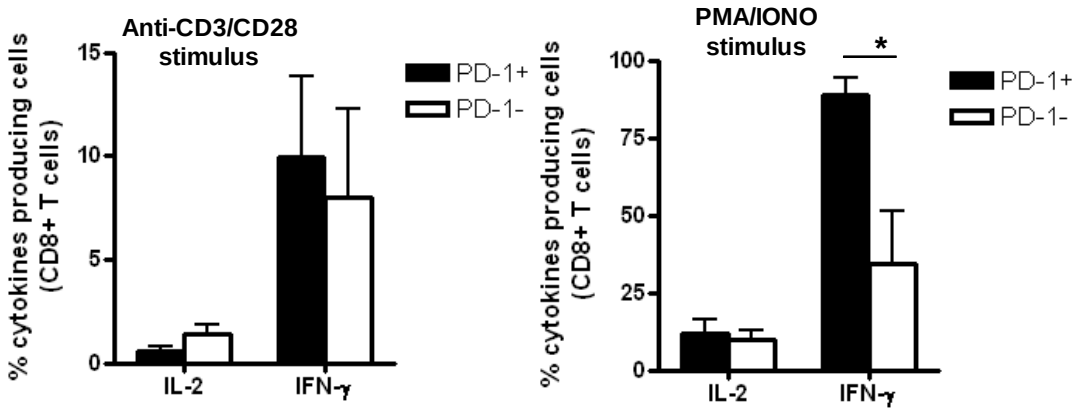


Figure 4.2.9. Means of cytokine production by CD8+PD-1+ and CD8+PD-1- T cells in four experiments. The figure shows the mean+SD of cytokine production of four experiments. Facs sorted PBLs-HD were stained with labeled ab against surface marker CD8 and then the percentage of CD8+ T cells producing IL-2 and IFN-γ were tested by intracellular staining assay stimulating T cells with anti-CD3/CD28 and PMA/ionomycin. The cytokines produced by PD-1+ CD8+ T cells was compared to those produced by PD-1- CD8+ T cells. The statistical analysis was done by applying the t test. (* p=0.0223 unpaired t test).

This demonstrated that in these four healthy donor samples, the total CD4+ and CD8+ PD-1+ T cells showed only impaired IL-2 production with physiologic

stimulus. The analysis on PBLs of six healthy donors on the basis of the total CD4+ and CD8+ T cells producing cytokines has showed that there is a correlation between PD-1 expression and different degrees of exhaustion.

4.3 In vitro induction of PD-1 and TIM-3 expression in CD4+ T cell clones with PMA/ionomycin stimulus

To study T cells exhaustion in an *in vitro* model we generated T cell clones by limiting diluition as described in materials and methods and we stimulated them with transmembrana stimulus PMA/ionomycin. We monitored the level of expression of both PD-1 and TIM-3 markers before the stimulation and also 18-48-72 and 96h after the stimulus in a kinetic study by flow cytometry to test if T cell clones were a good model for T cell exhaustion.
As shown in figure 4.3.1, the analyses of 5 CD4+ T clones showed that PD-1 level exhibited an kinetic expression that is slower. Infact, the PD-1 marker on CD4+ T cell clones, after 96h from the stimulus, does not reach levels of expression of more than 50%.
TIM-3 marker after 18h from the stimulus was expressed on approximately 30% of CD4 T cell clones and already after 48h from the stimulus, it was expressed at high levels (80%), and the difference of expression compared to unstimulated conditions (t0) was statistically significant.
The percentage of CD4+ T cells clones coexpressing PD-1 and TIM-3 gradually increased, reaching levels of expression higher than 25% after 48h (statistically significant) and 72h from the stimulus and increased after 96h from the stimulus.

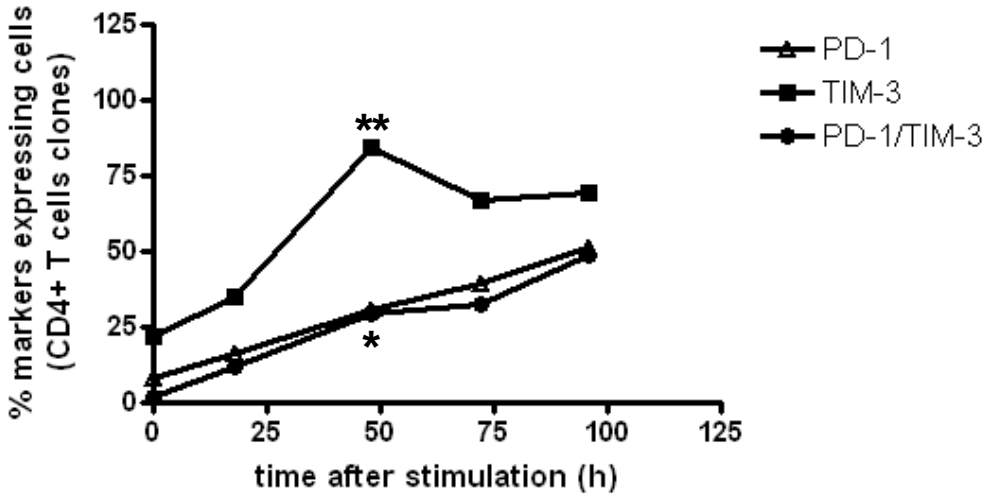


Figure 4.3.1. Mean of expression (%)of PD-1 and TIM-3 on 5 CD4+ T cell clones stimulated with PMA/ionomycin. CD4+T cell clones were stimulated with PMA/ionomycin and after 18-48-72 and 96h from stimulus the percentage of CD4 T cells expressing PD-1, TIM-3 and coexpressing both markers was analyzed by flow cytometry staining CD4 T cells at indicated time points with labeled ab against PD-1 and TIM-3. The mean of 5 independent experiments. The percentage of CD4 T cells expressing PD-1,TIM-3 or both at various times after stimulation was compared to the relative time at t0, that represents the unstimulated condition. (* p=0.0410, ** p=0.0017; unpaired t test).

Figure 4.3.2 represents one experiment on 1 CD4+ T cell clone that actually showed that the TIM-3 marker was more easily upregulated compared to PD-1 as; initially the cells began to express the TIM 3 and then they became double positive.
In fact, at 96 h from the stimulation almost all cells (95%) coexpressed both PD-1 and TIM-3 markers.

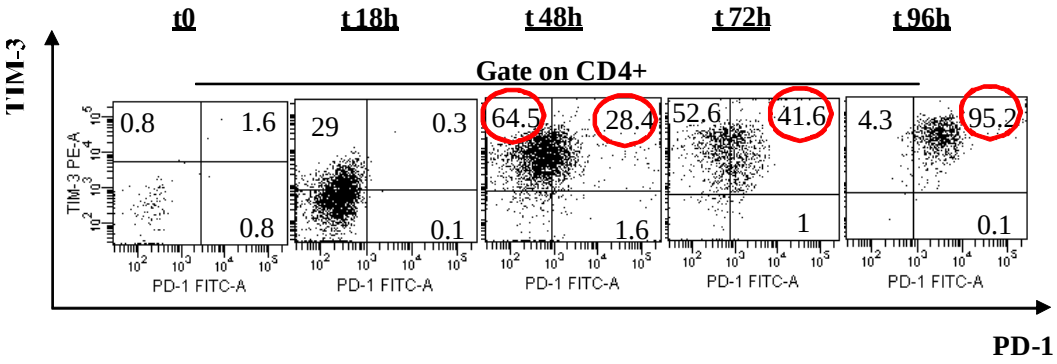


Figure 4.3.2. Kinetics of PD-1 and TIM-3 expression after stimulation with PMA/ionomycin. Representative CD4+ T cell clone stimulated with PMA/ionomycin and the level of PD-1 and TIM-3 monitored by flow cytometry before the stimulation (t0) and after 18-48-72 and 96h from the stimulus with PMA/ionomycin.

4.4 Functional analyses on CD4+ T cell clones expressing PD-1 and TIM-3 at high levels after 18h from stimulus with PMA/ionomycin

To verify that the expression of PD-1 and TIM-3 markers leads to an exhausted phenotype on CD4+ T cell clones and that this phenotype is associated with reduced ability in the cytokine production, we tested CD4+ T cell clones that already after 18h from the stimulus with PMA/ionomycin expressed high levels of both PD-1 and TIM-3 markers. As shown in figure 4.4.1 the analyses on 3 CD4+ T cell clones showed that unstimulated T cells clones (t0) expressed PD-1 and TIM-3 at middle levels (30% and 18% respectively) and also the percentage of CD4+ T cells coexpressing both PD-1 and TIM-3 was low (10%), but after 18h from the stimulation with PMA/ionomycin the percentage of CD4+ T cell expressing PD-1,

TIM-3 and coexpressing both markers increased, reaching percentages of 65%, 68% and 58%. For the percentage of CD4+ T cells expressing TIM-3 markers, these differences compared to the unstimulated condition were statistically significant (p=0.0250).

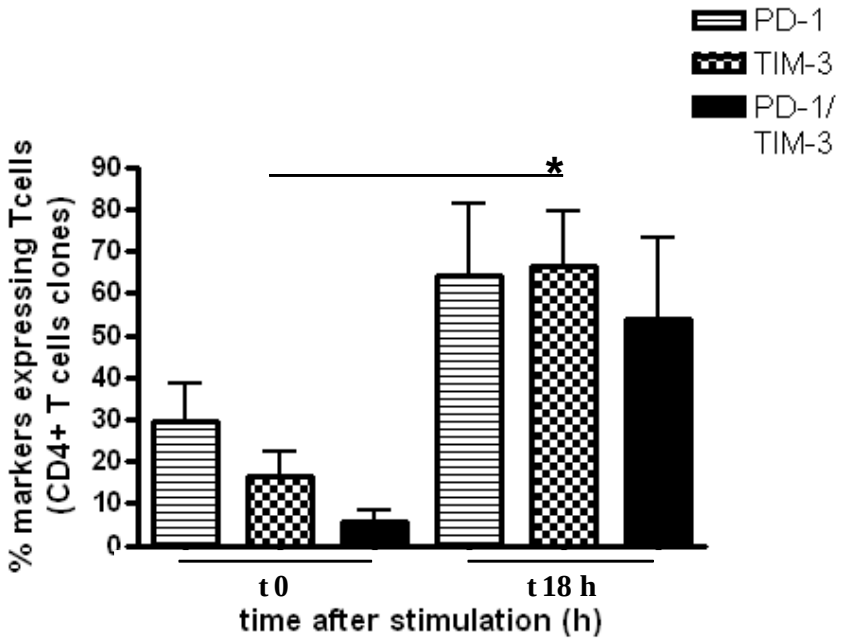


Figure 4.4.1 Expression of PD-1 and TIM-3 on 3 CD4+ T cell clones stimulated with PMA/ionomycin. CD4+ T cell clones were tested for the expression levels of PD-1 and TIM-3 at t0(unstimulated condition) and after 18h from the PMA/ionomycin stimulus by flow cytometry. The + SD mean of 3 independent experiments. The percentage of CD4+ T cells expressing PD-1, TIM-3 or coexpressing both markers at time t18h after stimulation was compared to that relative at time t0 (* p=0.0250; unpaired t test).

CD4+ T cell clones that expressed PD-1 and TIM-3 at high levels after 18h from the stimulus and as control CD4+ T cells in the t0 condition (in which the marker expression levels were lower) were stimulated with PMA/ionomycin for 12 h at 37°C. Cells stained with labeled mab against IL-2 and IFN-γ and then the secretion of these cytokines was tested by intracellular cytokine staining and then analized by flow cytometry. Unstimulated cells were used as negative controls.
As shown in figure 4.4.2 the analyses of the mean of cytokine production of 3 CD4+ T cell clones showed that T cells expressing PD-1 and TIM-3 at middle levels (t0 condition) are good producers of both IL-2 and IFN-γ (60% and 70% of T cells produced IL-2 and IFN-γ respectively, and more than 50% of the same CD4+ T cells were co-producers of both cytokines).
At 18h from the stimulation, the analyses of cytokine production showed that the percentage of CD4+ T cells producing IL-2, IFN-γ and co-producing both cytokines was greatly diminished, with less than 10% of producer cells therefore these differences was statistically significant ($p<0.05$).

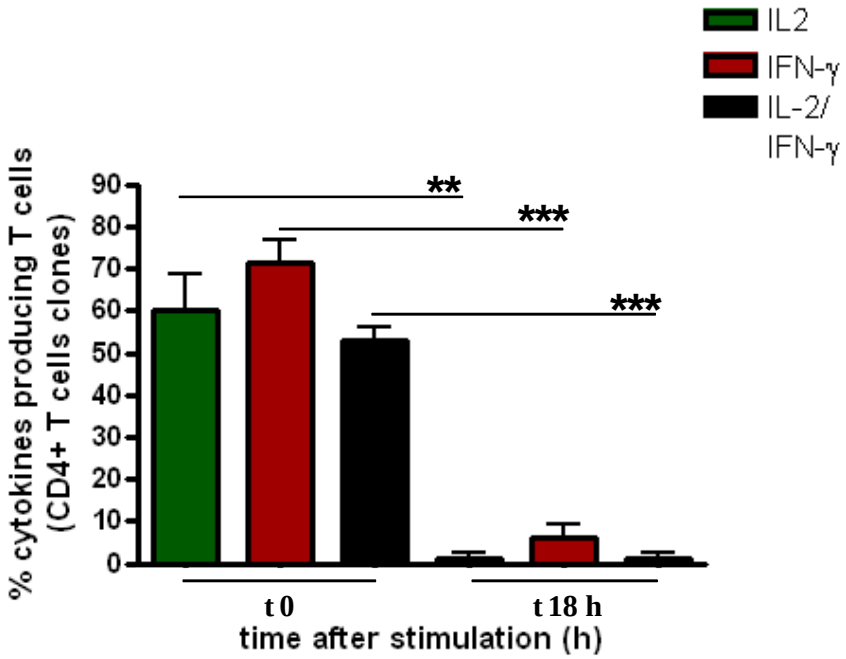


Figure 4.4.2 Cytokine production (IL-2 and IFN-γ) by 3 CD4+ T clones expressing PD-1 and TIM-3 at low and high levels (t0 and t 18h from the stimulus).
CD4+ T clones coexpressing low levels and high levels of PD-1 and TIM-3 (t0 and t18h respectively) were tested in a intracellular cytokines assay and the production of IL-2 and IFN-γ was assessed stimulating the CD4+ T cells with PMA/ionomycin. Unstimulated cells are used as negative controls. In the graph the +SD mean of cytokine production was reported for 3 independent experiments and the background was subtracted. ($P<0.005$ unpaired t test).

Figure 4.4.3 shows the average performance of a sample CD4+ T cell clone in which there is an increase in the percentage of T cells expressing PD-1, TIM-3 and coexpressing both markers that correspond to an impaired cytokine production.

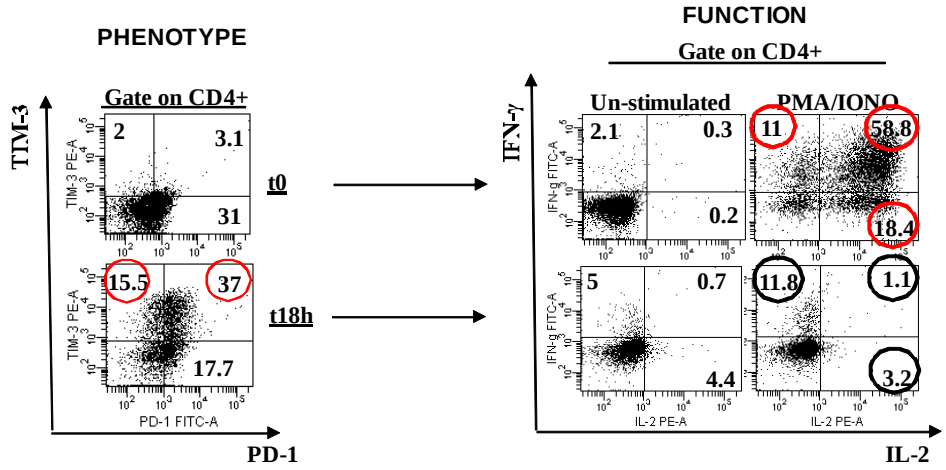


Figure 4.4.3. CD4+ T cell clones after 18h from the stimulus expressed PD-1, TIM-3 and coexpressed both markers at high levels and this phenotype was associated with reduced cytokine production and with an exhausted phenotype.

4.5 In vitro induction of PD-1 and TIM-3 expression with feeders, PHA and IL-2 and functional analyses in T cell clones

To confirm that clones of T lymphocytes are a good model to study the T cell exhaustion phenomenon *in vitro* we stimulated CD4+ and CD8+ T cells clones with another stimulus to mimic the antigenic stimulus that determines the exhaustion. T cells clones obtained by limiting dilution were stimulated with feeders, as antigen presenting cells (APC), PHA and IL-2, twice every 10 days. We monitored the expression of both PD-1 and TIM-3 markers and also the intracytoplasmic cytokine production by flow cytometry.
As shown in figure 4.5.1, the analyses on another 3 CD4+ T cell clones showed that T cells clones a 10 days after the first stimulation, upregulated both PD-1 and TIM-3. For TIM-3 markers the difference of expression levels between the unstimulated condition (day 0) was statistically significant ($p=0.0238$). At day 20 (10 days after the second stimulation) the percentage of CD4+ T cells expressing PD-1 and TIM-3 decreased even though the expression levels were higher than the unstimulated condition. The differences of expression levels of TIM-3 after 20 days was statistically significant compared to day 10 ($p=0.0179$).

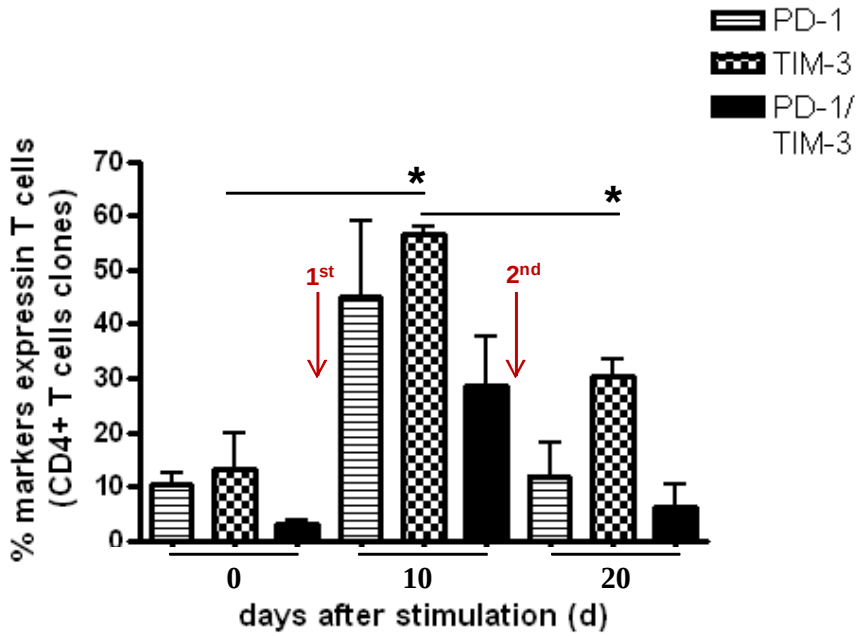


Figure 4.5.1 Expression of PD-1 and TIM-3 on 3 CD4+ T cell clones stimulated with feeders (as APC, presenting-antigen cells), PHA and IL-2. CD4+ T clones were tested for the expression levels of PD-1 and TIM-3 at day 0(unstimulated condition) and at day 10(after first stimulation) and at day 20 (10 days after second stimulation) by flow cytometry. The +SD mean of 3 independent experiments. The percentage of CD4+ T cells expressing PD-1,TIM-3 or coexpressing both markers at days10 and 20 after stimulation was compared to that relative at day 0. (* $p<0.05$; unpaired t test).

As shown in figure 4.5.2, the analyses of the mean of cytokine production of these 3 CD4+ T clones showed that T cells at 10 days after first stimulation the percentage of CD4+ T cells producing cytokines was decreased compared to the un-stimulated condition (day 0), but after 20 days (10 days after second stimulation), when both PD-1 and TIM-3 decreased, the percentage of these CD4+ T cells producing IL-2

and IFN-γ increased. We observed this with both stimuli, anti-CD3/CD28 and also PMA/iono, even though the differences were not statistically significative (p>0.05).

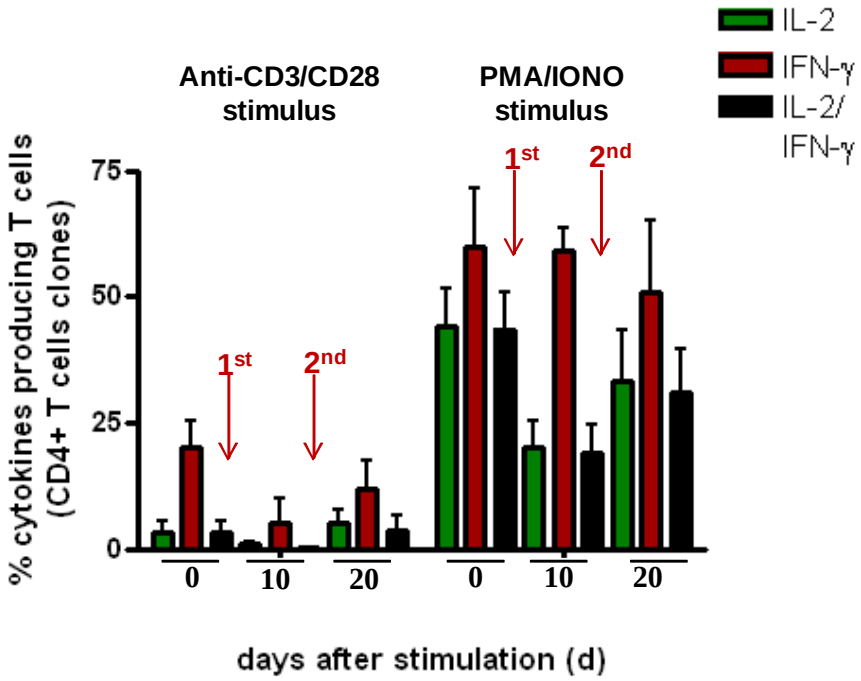


Figure 4.5.2 Cytokine production (IL-2 and IFN-γ) by 3 CD4+ T cell clones stimulated with feeders, PHA and IL-2. CD4+ unstimulated T clones (day 0), stimulated at day 10 (after first stimulation) and at day 20 (10 days after second stimulation) was tested in an intracellular cytokine assay. The production of IL-2 and IFN-γ was assessed stimulating the CD4+ T cells with anti-CD3/CD28 stimulus and also with PMA/ionomycin as a positive control. Unstimulated cells are used as negative controls. In the graph the +SD mean was reported of cytokine production for 3 independent experiments and the background was subtracted. p>0.05 unpaired t test.

Figure 4.5.3 shows a sample CD4+ T cell clone in which an increase in the percentage of T cells expressing PD-1 and TIM-3 correspond to impaired cytokine production.

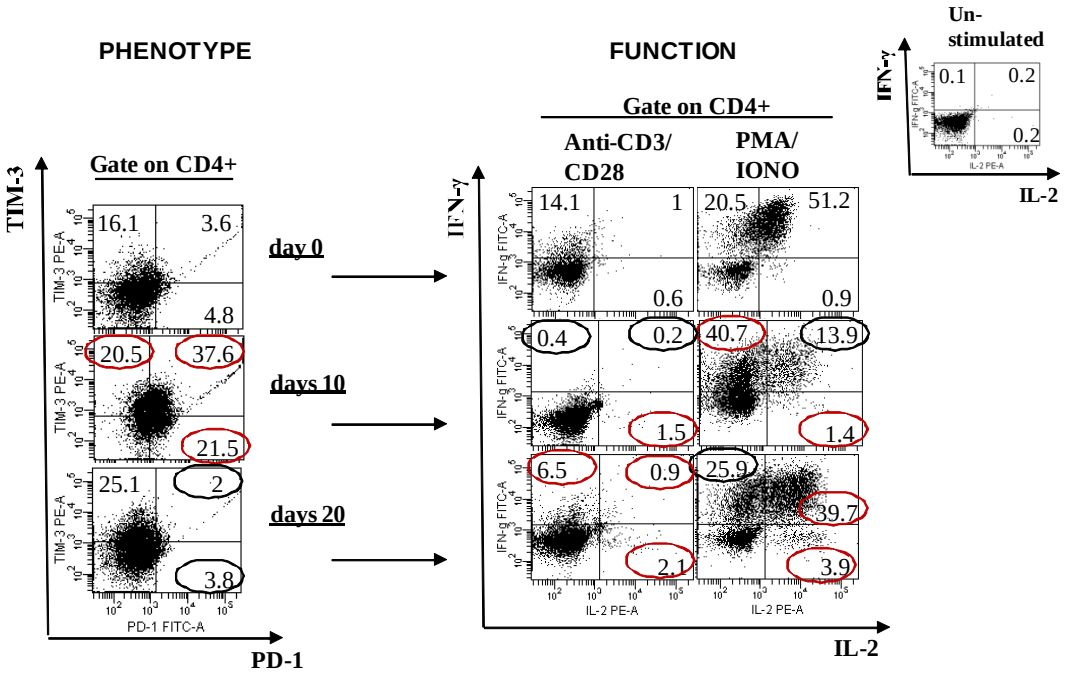
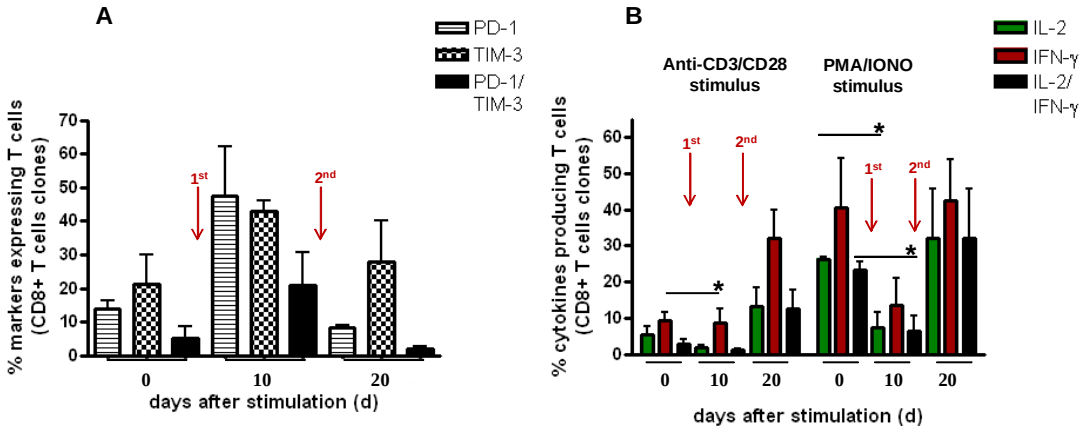
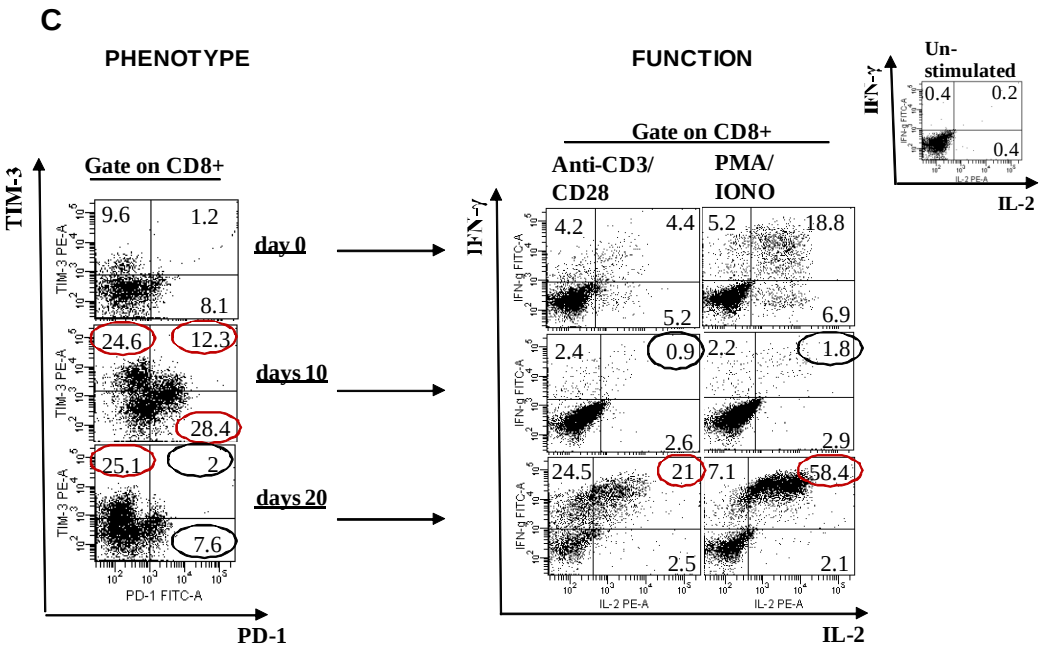


Figure 4.5.3. CD4+ T cell clone that at 10 days from the stimulus (feeders, PHA and IL-2) expressed PD-1 and TIM-3 at high levels. This phenotype was associated with reduced cytokine production. When the expression levels of both PD-1 and TIM-3 decreased (at 20 days) the cytokine production increased. Top righ: unstimulated condition.

Similar results were also obtained for CD8+ T cell clones with the same protocol of stimulation (see figures 4.5.4 A, B and C).



Figures 4.5.4 A-B. (A)Expression of PD-1 and TIM-3 on 3 CD8+ T cell clones stimulated with feeders (as APC, presenting-antigen cells), PHA and IL-2. The +SD mean of 3 independent experiments. The percentage of CD8+ T cells expressing PD-1,TIM-3 or coexpressing both markers at days 10 and 20 after stimulation was compared to that relative at day 0. $p>0.05$; unpaired t test). **(B) Cytokine production (IL-2 and IFN-γ) by 3 CD8+ T cell clones stimulated with feeders, PHA and IL-2.** In the graph the +SD mean was reported for cytokine production of 3 independent experiments and the background was subtracted. *$p<0.05$ unpaired t test.



Figures 4.5.4 C. Representative experiment of one CD8+ T clone. Top right: unstimulated condition.

4.6 MicroRNAs profiling on PBLs-PD-1+ and PD-1- in HD

The transcriptome analysis of 667 human miRNAs was performed on sorted PBLs-PD-1+ and PD-1- in healthy donors.
To study the microRNA expression profiling on T cell expressing PD-1 we selected HD sample 3-4-5-6 in which the PD-1 expression on positive fractions were higher

and in which the analysis of cytokines produced by the CD4+ and CD8+ T cells expressing PD-1 indicated that these T cells were in early exhaustion (table 1). Than we performed the taqman low density miRNA array to study the transcriptome on the early phase of the exhaustion phenomenon.
For the transcriptome analysis we used the TaqMan ® technology Low Density Array (TLDA) in which 384-wells micro-fluidic cards allow to make up to 384 reactions simultaneous of real-time PCR using the probe technology TaqMan.
We analyzed an array of 667 microRNAs, looked for a high variability between individuals analyzed and therefore we compute PD1+/PD1- values for each sample and we selected microRNAs changing consistently across 4 individuals. We considered the microRNAs as deregulated in which the ratio of level of expression in PD-1+ cells respect to PD-1- cells were >1 (and these was considered as upregulated microRNAs) and <-1 (considered as downregulated).
As shown in figure 4.6 A e B in the PBLs-PD-1+ 23 microRNAs resulted deregulated compared to PBLs-PD-1-: 15 resulted upregulated and 8 resulted downregulated.

1. MiR-15b

miR-15, one of the miR-16/15/195/424/497 family members, has been shown to play an important role in tumorigenesis, a tumor suppressor. MiR-15 and -16 were the first for which the link between miRNAs and cancer was established. They are transcribed as a cluster (*miR-15a–miR-16-1*) that resides in the 13q14 chromosomal region. Deletions or point mutations in region 13q14 occur at high frequency in CLL, lymphoma, and several solid tumors. Bcl-2 is the first target described for these miRNAs and when these miRNAs are deleted or downregulated, as in colorectal and lung cancer, the levels of Bcl-2 increase, protecting the cells from apoptosis. Both these miRNAs were also proposed to be involved in hematopoietic cell lineage differentiation that may block differentiation of later progenitor cells in CD34+ hematopoietic stem-progenitor cell.

2. MiR-21

miR-21 was one the first miRNAs detected in the human genome and it shows a strong conservation across vertebrate species. MIRN21gene is located on 17q23.1 chromosomic region, within the tenth intron of the gene encoding the transmembrane protein-49. The primary transcript pri-miR-21 is independently transcribed from a conserved promoter, located within the intron of the overlapping protein-coding gene TMEM49. Many studies suggest that this microRNA is oncogenic, in fact, it is abundantly expressed in various tumors but it may act as antiapoptotic factor.

3. MiR-27b

miR-27b is one of miRNAs of the miR-27 family. MIRN27b gene is located on 9q22.32 chromosomic region, and studies suggest that this microRNA is induced during the innate immune response both in mouse and in human following exposure to lipopolysaccharide (LPS).

4. MiR-93

MIRN93 gene is located on chromosome 7: miR-93, one of the microRNAs within the miR-106b~25 cluster (a paralog of the miR-17~92 cluster) possesses oncogenic activities. Studies in mice showed that this cluster is required for both B and T-cell development through downregulation of the proapoptotic protein Bim. The overexpression on this cluster in both T and B lymphocytes in transgenic mice resulted in the development of lymphoproliferative desease and autoimmunity, because these mutant mice exhibited spontaneous activation and pronounced expansion of both B, CD4+ and CD8+ T cells by downregulating Bim and the tumor suppressor PTEN.

5. MiR-146a

miR-146 family consists of two genes: miR-146a on chromosome 5 and miR-146b on chromosome 10. miR-146a was one of the first miRNA identified as to be

involved in the regulation of immune function, in fact in human monocytic cell line was identified as LPS-responsive miRNA.

6. MiR-155

In vitro and *in vivo* studies implicated miR-155 in various immune functions, such as innate and adaptative immune responses and also in development of immune cells. For example this miRNA is important for the development and function of CD4+CD25+Foxp3 T cells (Treg).

7. MiR-339-5p

miR-339-5p is one of microRNAs that reported a downregulated expression in the more aggressive breast cancer cell lines and tissue samples. One putative target is BCL-6, a protein that acts as a sequence-specific repressor of transcription, found to be upregulated in breast cancer. MiR-339-5p, downregulating BCL-6 in breast cancer cells could decrease tumor cell migration and invasion capacity.

miR-339-5p expression was lower in breast cancer tissues than in benign breast tissues, and its altered expression has reported in other solid tumors, including gastric cancer, lung cancer, and cervical cancer. Another target is ICAM-1 (Intercellular Adhesion Molecule-1) that is an endothelial- and leukocyte-associated transmembrane protein, long known for its importance in stabilizing cell to cell interactions and facilitating leukocyte endothelial transmigration. (ICAM)-1 is also involved in the susceptibility of tumor cells to antigen-specific lysis by cytotoxic T-lymphocytes (CTLs). In glioblastoma miR-339 suppressing ICAM-1 expression on tumor cells down-regulates the susceptibility of tumor cells to CTL-mediated cytolysis.

8. MiR-579

MIRN579 is located on chromosome 5.

This microRNA has been recently identified in THP1 cells stimulated with LPS. After the stimulus the mRNA of TNF-α, a cytokine involved in inflammation and

one that stimulate the acute phase reaction, increased and following this initial activation phase, (which incites the "cytokine storm" observed at the onset of Severe Systemic Inflammation), both mRNA and protein levels were rapidly decreased and reached background levels by 12 h. This coincides with the induction of LPS tolerance, as cells were unable to respond to a second dose of LPS. miR-579 levels became elevated during the LPS-dependent evolving phase of tolerance; thereafter a second LPS stimulus markedly augmented its expression. Expression profiling revealed that miR-579 was selectively induced in LPS-tolerant cells and that this microRNA acted on TNFα mRNA for translational repression.

9. MiR-597

For miR-597 the functions in T lymphocytes are not know. These microRNAs has been recently identified as a novel deregulated microRNA in colonrectal cancer.

10. MiR-625

Also for this microRNA the functions in T lymphocites are not known. MIRN625 gene is located on chromosome 14, in position 14q23.3.

11. miR-629

MIRN629 is located on position 15q23 on chromosomes 15. This microRNAs were identified as tumor-specific microRNA. It was found upregulated in various types of cancer such as breast cancer, colon, liver, lung, ovary, prostate and also in testicular cancer. miR-629 can serve as biomarkers for detection of common human cancer type. For the immune system there are no findings for this microRNA.

12. miR-636

MIRN636 is located on position 17q25.1 on chromosome 17. This microRNA were recently identified as a diagnostic marker for myelodisplastic syndromes (MDS) in bone marrow mononuclear cell samples. MDS are aging-associated disorders characterized by ineffective maturation of hematopoietic elements. This microRNA also plays a role in the host response to enterovirus infection. This microRNA was

found expressed in EV71-infected Hep2 cells (Human epidermoid Carcinoma) and can act on predicted target genes that have been identified to be involved in virus entry, replication and propagation.

13. miR-642

MIRN642 is located on position 19q13.32 and based on current literature there are no documented functions in T lymphocytes.

14. miR-885-5p

miR-885-5p has been recently identified as a potential marker for detecting liver pathologies. This microRNA was found upregulated in serum pools from patients with HCC (hepatocellular carcinoma), LC (liver cirrhosis) and CHB (chronic hepatitis B).

15. miR-886-5p

Recently miR-886-5p has been shown to regulate apoptosis of cancer cells. miR-886-5p inhibits apoptosis by down-regulating the pro-apoptotic protein Bax expression in human cervical carcinoma cells.

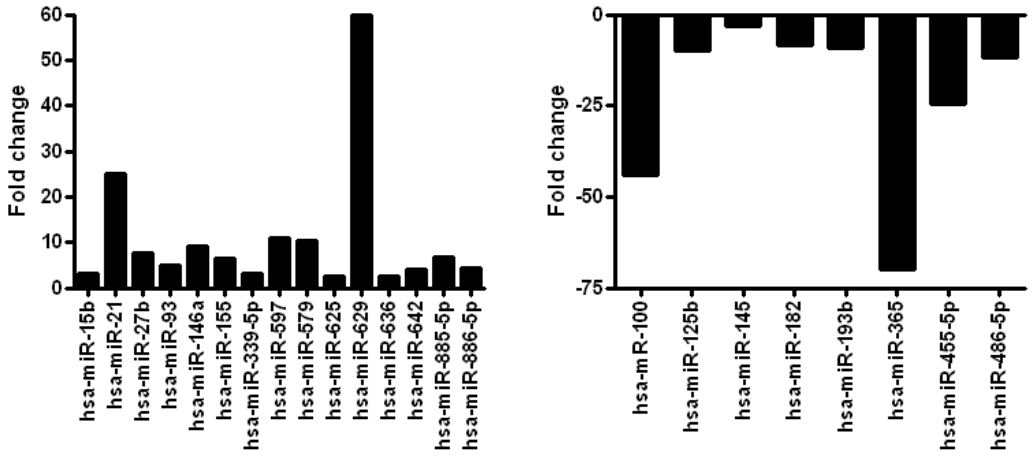


Figure 4.6 A,B. Deregulated microRNAs in PBLs- PD-1+ cells compared to PBLs- PD-1- cells. **(A):**upregulated miRNAs and **(B)** downregulated miRNAs in PBLs-PD-1+ cells compared to PD-1- cells.

And as shown in figure 4.6 B in the same PBLs-PD-1+ 8 microRNAs are instead shown downregulated:

1. miR-100

miR-100 is one of the miR-99 family members that has been recently associated with the process of metastasization of prostate carcinoma. For this microRNA there are not finding in T cells.

2. miR-125b

miR-125b was first implicated in human disease by expression profiling studies of cancer. MiR-125b is one of the miRNAs highly upregulated in germinal center (GC) B cells, in which it acts on two transcription factors necessary for the maturation of B cells, IRF4 and BLIMP1, suggesting that miR-125b regulates B cell differentiation upon encounter with an antigen. Mir-125b is also expressed in T cells and appears to play a role in controlling HIV infections by regulating latency in resting primary CD4+ T lymphocytes. High constitutive expression of miR-125b in these cells inhibits HIV-1 production through their interaction with 3' end of HIV-1 RNA and it may be pivotal in sustaining HIV-1 latency.

3. miR-145

MiR-145 functions as a protective miRNA identified in tumor tissues of lung adenocarcinoma patients and also in esophageal squamous cell carcinoma.
MiR-145 can inhibit the proliferation of lung cancer stem cells down-regulating OCT4 expression. MiR-145 is a potential protective miRNA of lung cancer.

4. miR-182

miR-182 has been identified as upregulated in activated helper T lymphocytes. Its expression is induced by IL-2 and TCR signaling and down-modulating the transcription factor Foxo1, a well-known suppressor of clonal expansion allowing helper T cell clonal expansion to occur.

5. miR-193b

miR-193b was demonstrated to be upregulated in HCV infected cells compared to control cells. This microRNA targets anti-apoptotic protein Mcl-1 and indeed lower levels of Mcl-1 were found in miR-transfected cells. Moreover, these cells were more sensitive to drug-induced apoptosis, showing that miR-193b could potentially be used to combat viral infection. Recently, this microRNA has been demonstrating a role in the maintenance of the naiveness state in T lymphocytes.

6. miR-365

Recently, miR-365 has been identified as novel negative regulator of IL-6. Overexpression of miR-365 mimics decreased activity of a luciferase reporter containing the IL-6 3′-UTR and led to repression of IL-6 protein. In contrast, ectopic expression of a miR-365 inhibitor elevated IL-6 expression. The negative regulation of miR-365 was strictly dependent on a microRNA binding element in the 3′-UTR of IL-6 mRNA.

7. miR-455-5p

This microRNA recently has been identified as upregulated miR in macrophages stimulated with LPS and pathogen agents.

8. miR-486-5p

This microRNA has been demonstrated to be modulated during normal hematopoietic differentiation and in leukemic hematopoietic. MiR-486-5p has a regulatory role in the growth of hematopoietic stem cells and their erythroid differentiation by inhibiting Foxo1 expression.

Given the role of miR-21, miR-146a, miR-27b and miR-155 in the immune system we decided to focus on these microRNAs because they are involved in immune responses to inflammation.

5. **Discussion and conclusions**

Functional exhaustion of antigen-specific T cells is a defining characteristic of many chronic infections and is the main reason why the host fails to eradicate the infection. Despite the many advances made in the control and treatment of infectious deseases, chronic viral infections remain an important cause of mortality.

Exhausted T cells are characterized by impaired effector functions such low proliferative potential and decreased ability of cytokines production such IL-2, TNF-α and IFN-γ.

Studies both in mouse and human models have demonstrated that exhaustion is a gradual process, with range of dysfunctions from relatively mild to more severe (167); phase I of partial exhaustion is characterized by little IL-2 and poor TNF-α and cytotoxicity; phase II of partial exhaustion is characterized by decreased IFN-γ production and finally in full exhaustion there is a total loss of IFN-γ production and T cells can be physically deleted. Proliferative potential decreases concomitantly with the loss of the other functions. The exhaustion was more severe for the CD8+ T cells in the absence of CD4+ T cells that play the helper role, sustaining the functions of CTLs.

These defects in the functions of impaired T cells have been associated with upregulation of inhibitory receptors. The first inhibitory receptor correlated with T cell exhaustion is programmed death 1 (PD-1;also known as CD279) receptor that is inducibly expressed on CD4+ and CD8+ T cells, NKT cells, B cells and monocytes upon activation (168,169). PD-1 regulates pathways of memory CD8+ and CD4+ T cells differentiation: both in mice and in human PD-1 expression was upregulated after the effector T cells stage in chronically infected individuals, and was rapidly downregulated when the infection was cleared. In the exhausted T cells and in chronic viral infection the PD-1expression remains high and this inhibitory receptor is used as an exhaustion markers.

In this study we analyzed healthy individuals to verify if the inhibitory receptor PD-1 is an exhaustion marker also in healthy donors. We screened 90 HD and we observed

the PD-1 expression on only 20 HD. In only 6 individuals of 20 we observed a sufficient PD-1 expression level to test this exhaustion marker.
The analysis of PD-1 expression in CD4+ and CD8+ total T cells expressing PD-1 demonstrated that PBLs-HD expressed PD-1 at a level that was too low and that the CD4+ T cells expressed more PD-1.
We analyzed the functional ability of CD4+ and CD8+ total T cells expressing or not expressing PD-1 in 6 sample of healthy donor stimulating these T cells with physiologic stimulus anti-CD3/CD28 and with PMA/ionomycin as positive control. On two samples we noted that the population of enhriched PD-1+ T cells (CD4+and CD8+ T cells expressing PD-1 at average level of 60.5%) produced less IL-2 and IFN-g with both stimuli compared to depleted PD-1+ T cells (CD4+ and CD8+ T cells PD-1-) that instead produced more cytokines with both stimuli. In 4 other samples analyzed, we observed that CD4+ and CD8+/PD-1+ T cells (expressing PD-1 at average level of 82 %) only produced less IL-2 compared to CD4+ and CD8+/PD-1- T cells when stimulated with anti-CD3/CD28 stimulus; conversely the same cells expressing PD-1 when stimulated with PMA/ionomycin produced more IL-2 and these cells were better producers of IFN-g with both stimuli.
It is known that exhaustion is a gradual process in which the IL-2 production is the first function to be lost.
Our data demonstrates that the first hd samples analyzed (HD1 and HD2) are characterized by PBLs-PD-1+ that are in terminal exhaustion because these T cells expressing PD-1 produced less cytokines (both IL-2 and IFN-γ) when stimulated with both stimuli compared to T cells PD-1-. The 4 other samples examined are characterized by T cells in early exhaustion, because both CD4+ and CD8+ T cells PD-1+ only produced less IL-2 when stimulated with physiological stimulus antiCD3-CD28.
In addition, the sample with T cells in which the PD-1 were expressed at lower levels, (56 and 63%), showed characteristics of terminally exhausted T cells, instead samples with PD-1 expression at higher levels were constituited by CD4+ and CD8+

T cells in early exhaustion. This demonstrated that PD-1 expression correlates with different degrees of exhaustion.
In T cell exhaustion other markers have been involved, such as TIM-3. In this study we tested T cell clones (CD4+ and CD8+) *in vitro* stimulating these clones with mitogenic stimuli and tested the expression levels of both PD-1 and TIM-3 and also cytokine production. CD4+ and CD8+ T cell clones upregulated at high levels both of the markers and when this occurred, they showed impaired cytokine production. TIM-3 was easily upregulated in CD4+ T clones and this is coherent with its function in regulating Th-1 responses, whereas PD-1 is more easily upregulated in CD8+ T clones. This demonstrated that T cells clones are a good model to study *in vitro* T cell exhaustion and these models both PD-1 and TIM-3 are markers of T cells exhaustion. In addition, we demonstrated that the exhaustion is a reversible process because when we observed downregulation of these markers the cytokine production increased.
To identify candidate microRNAs in the early phases of the exhaustion process we selected the 4 samples of healthy donors in which CD4+ and CD8+ T cells /PD-1+ produced less IL-2 with anti-CD3/CD28 stimulus and their relative conterpart PD-1-. We analyzed the microRNAs expression profile analyzing a panel of 677 microRNAs by taqman low density array. The analyses showed that PD-1+ T cells deregulated only 23 microRNAs compared to PD-1- T cells: 15 were upregulated and 8 were downregulated.
Our attention was directed towards microRNAs involved in inflammation, such as miR-21, miR-27b, miR-146a and miR-155 that resulted upregulated in CD4+ and CD8+/PD-1+ T cells. This panel of microRNAs upregulated was similar to expression profiling of that observed for the central memory CD4+ and CD8+ T cells compared to naive CD4+ and CD8+ T cells (Fulci et al., manuscript in preparation) suggesting that PBLs-PD-1+ in early exhaustion have been in contact with antigen.

Recent studies have demonstrated that miR-27b is induced during the innate immune response both in mouse and in human following exposure to lipopolysaccharide (LPS) (170, 171).
miR-146a has shown to be involved in innate immunity (164). It was also demonstrated that Toll/Il-1 receptor-mediated miR-146a expression is preferentially driven by NF-kB and it has been speculated that miR-146a might fine-tune negative regulation feedback of inflammation downregulating IRAK1 and the tumor necrosis factor receptor-associated factor 6 (TRAF6), two proteins involved in Toll/Il-1 receptor signaling (172). In 2010 a study in collaboration with Fulci et al. has demonstrated that miR-146a is involved in the modulation of adaptative immunity: has showed that miR-146a is upregulated during T cell differentiation, in fact the levels of this microRNA were lower in human naive CD4+ and CD8+ T cells compared to levels in memory CD4+ and CD8+ T cells. It has been speculated that miR-146a could be induced upon TCR stimulation. By stimulating primary T lymphocytes with PMA and ionomycin, it has been observed that levels of miR-146a gradually increased parallel to the increase of CD45RO, a transition marker to a memory phenotype. Also in Jurkat cells, TCR stimulation induced a similar increase of this microRNA. The overexpression of miR-146a in Jurkat cells impaired IL-2 production and activator protein 1 (AP-1) induced by TCR engagment. These finding suggested that miR-146a plays a role in the timing of different T-cell activation steps, by contributing to switch off the IL-2 signaling. In samples PD-1+ miR-146a is also upregulated and CD4+ and CD8+ T cells produced less IL-2 compared to CD4+ and CD8+ T cells PD-1 -.
miR-155 is rapidly induced in B and T lymphocytes after engagment of the antigen receptor and in leukocytes after exposure to inflammatory mediators (173). A central role for this microRNA in the regulation of T- and B-cell responses during the acquired immune response has emerged from studies in knockout mice, that were to be immunodeficient and failed to develop a protective response to bacteria after immunization due to reduction in IgG1 antibodies downregulating the expression of the transcription factor PU.1 (158).

Mir-155 is also involved in T cell differentiation: naive T cells derived from miR-155 knockout mice were shown to have an increased propensity to differentiate into Th2 rather than Th1 cells, with the concomitant production of Th2 cytokines. This bias results from miR-155 targeting of c-Maf, a transcription factor known to be a transactivator of a IL-4 promoter, a key cytokine in the development of Th2 cells. In addition, in acute immune response, T lymphocytes had an impaired response and showed attenuated IL-2 and IFN-γ release in response to antigens (174).

In a recent study, it has also showed that miR-155 promotes Th1 differentiation in mice when overexpressed in activated CD4+ T cell by inhibiting IFN-γ signaling (175). miR-155 downregulating IFN-γRα enhances Th1 maturation by decreasing sensitivity of the cells to the antiproliferative effects of IFN-γ, inhibiting cytokine signal trasduction in CD4+ T cells.

In a recent study in human T lymphocytes by the multidimensional approach to integrate genome-wide miRNA, mRNA and protein expression have demonstrated that differentially upregulated miRNAs regulate T lymphocyte activation by targeting highly differentially expressed genes involved in networks critical for cell activation, proliferation and survival. In this study they analyzed microRNAs expression after T lymphocyte activation and also they profiled mRNA and protein expression. A target of miR-155 and also miR-21 is PI3KR1, that belongs to the phosphoinositide 3-kinase family that phosphorylates phosphatidylinositol-(4,5)-biphosphate to phosphatidylinositol-(3,4,5)-triphosphate to regulate cell proliferation and cytokine production. This could confirm that miR-21 and miR-155 play an inhibitory role in T cells in early exhaustion (176).

They also showed that inhibition of these microRNAs in CD4+ T cells increased proliferation by removing suppression of four target genes involved in proliferation and survival.

miR-193 and miR-125b were recently identified as microRNAs upregulated in human naïve CD4+ T cells and downregulated in memory CD4+ T cells (177). In this study they showed that miR-125b regulates genes involved in the differentiation of naïve CD4+ T cells into effector-memory lymphocytes. Infact naïve CD4+ T cells

activated in the presence of ectopic expression of miR-125b had less effector function, such us lower production of IFN-g and IL-13. The samples in early exhaustion expressing PD-1 downregulated miR-193 and miR-125b suggest that these cells have encountered antigen. A target of miR-125b is BLIMP1 that is involved in differentiation terminal of T cells. Amount Blimp-1 are associated with the exhausted phenotype: small amount of BLIMP-1 promote the formation of memory T cells, moderate amounts favor the terminal differentiation of function effector T cells, but high amounts foster the exhaustion. The downregulation of miR-125b could be consistent with increased levels of Blimp-1 and with an early exhausted phenotype.

MiR-182 has been recently identified as a negative regulator of Foxo1 in activated helper T lymphocytes (178).

After being activated by the antigen, helper T lymphocytes switch from a resting state to clonal expansion. This switch requires the inactivation of the transcription factor Foxo1, a suppressor of proliferation expressed in resting helper T lymphocytes. They had demonstrated that in the late phase of expansion, Foxo1 was no longer post-translationally regulated but was inhibited post-transcriptionally by the interleukin 2 (IL-2)-induced microRNA miR-182. Specific inhibition of miR-182 in helper T lymphocytes limited their population expansion *in vitro* and *in vivo*. This microRNA is downregulated in T cells PD-1+ and this could be consistent with a minor proliferative potential of these cells and with an early exhausted phenotype.

Other deregulated microRNAs will be studied valuating the role in T lymphocytes during exhaustion, especially for miR-629 and miR-365 the most up- and downregulated; for miR-629 there are no finding on its role in T lymphocytes.

For the functional characterization of a microRNA accurate identification is the primary determinant of its mRNA targets. The fact that in animals, miRNAs are conducting their action only through perfect complementarity of nucleotides 2-7 or 2-8 of the seed sequence, greatly complicates the study for the identification of target genes of each miRNA, because such short sequences might theoretically be complementary to a huge number of sequences in the 3'UTR of the entire genome,

leading to the identification of many false positives. In recent years, to try to reduce and properly address this study a series of bioinformatics prediction programs based on mathematical algorithms have developed such as TargetScan, PICTar, and Miranda, which identify the hypothetical mRNA target for any specific microRNAs.
These computer systems, network-accessible, take into account, as well as complementarity, some important parameters such as evolutionary conservation among different species and the thermodynamic stability of eteroduplex formed by the interaction between microRNAs and 3'UTR. Even if the target must always be validated later by specific experimental methods, this type of analysis is certainly the first step in studying the functional role of a microRNA.
Just through the use of bioinformatics prediction it has been estimated that for each microRNA there may exist even more than two hundred different target genes, including transcription factors, secreted proteins, receptors, transporters and regulators of intracellular pathways. This enormous variability suggests that microRNAs may be involved in almost all physiological processes of the cell, defining a regulatory control of about one third of the messenger RNA of the whole human genome (179).
Using the TargetScan to control target of any deregulated microRNA in early exhaustion of T cells, we noted that for example miR-15b have CCNE1 (Cyclin E1) as a target gene. The protein encoded by this gene belongs to the highly conserved cyclin family, whose members are characterized by a periodicity in protein abundance through the cell cycle. Cyclins function as regulators of CDK kinases.
Different cyclins exhibit distinct expression and degradation patterns which contribute to the temporal coordination of each mitotic event. This cyclin forms a complex and functions as a regulatory subunit of CDK2, whose activity is required for cell cycle G1/S transition. This protein accumulates at the G1-S phase boundary and is degraded as cells progress through the S phase. During T cell exhaustion PD-1 transduces an inhibitory signal leading to inhibition of T cell activation; signalling through this receptor causes growth arrest and the loss of cytokine production and cytotoxic ability. Inhibitory receptor signalling mediates growth arrest through

inhibition of the AKT signalling pathway (as a result of inhibition of phosphoinositide 3-kinase (PI3K), the activity of which is required for AKT phosphorylation). This in turn lifts the block on forkhead box O (FOXO) transcription factors and activates the transcription of p27 (also known as KIP1), thereby preventing the transition from G1 to S phase in the cell cycle (180-182).
Considering that miR-15b is one of the upregulated microRNAs in T cells in early exhaustion downregulating the cyclin E1 could be a microRNA pro-exhaustion.
The most upregulated microRNA in this study, miR-629 have CD28 and HDAC5 as target genes. CD28 is directly involved in T cell exhaustion in fact this receptor, expressed on naive T cells, provides co-stimulatory signals, necessary and required for T cell activation. CD28 is the receptor for CD80 (B7.1) and CD86 (B7.2). CD28 is the only B7 receptor constitutively expressed on naive T cells. Stimulation through CD28 in addition to the TCR can provide a potent co-stimulatory signal to T cells for the production of various interleukins (IL-2 and IL-6 in particular). Association of the T cell receptor of a naive T cell with MHC:antigen complex without CD28:B7 interaction can result in a T cell that is anergic.
Another target gene of miR-629 is HDCA5 (Histone deacetylase 5)**.** Histones play a critical role in transcriptional regulation, cell cycle progression, and developmental events. Histone acetylation/deacetylation alters chromosome structure and affects transcription factor access to DNA. The protein encoded by this gene belongs to the class II histone deacetylase/acuc/apha family. It possesses histone deacetylase activity and represses transcription when tethered to a promoter. MiR-629 inhibiting HDAC5 can leads to an major transcription of PD-1 on T cells and could be responsible of upregulation of this inhibitory receptor and therefore to the exhausted phenotype. For miR-629 we proposed a role as pro-exhaustion microRNA.
Mir-579, another microRNA upregulated in T cells/PD-1+ has Foxo3 as a target gene.
FOX (Forkhead box) proteins are a family of transcription factors that play important roles in regulating the expression of genes involved in cell growth, proliferation, differentiation, and longevity. Foxo 3 is a suppressor of proliferation

on T cells and miR-579 cuold be an anti-exhaustion microRNA because inhibiting foxo3 the proliferation of T cells PD-1+ could be encouraged.
The most downregulated **,** miR-365 has been recently demostrated to be a negative regulator of IL-6 in human cells (183).
IL-6 is an cytokine that has pleiotropic effect and is involved in the regulation of the immune response, hematopoiesis, and inflammation. It was previously considered to be a regulator of acute-phase responses and a lymphocyte-stimulatory factor (184,185).
Overexpression of miR-365 mimics decreased activity of a luciferase reporter containing the IL-6 3′-UTR and lead to repression of the IL-6 protein. In contrast, ectopic expression of a miR-365 inhibitor elevated IL-6 expression. The negative regulation of miR-365 was strictly dependent on a microRNA binding element in the 3′-UTR of IL-6 mRNA. This microRNA is the most downregulated in CD4+ and CD8+ T cells/PD-1+ and therefore could be proposed to be an anti-exhaustion microRNA in this system.
Another target gene of miR-629 is PI3K. PI 3-Kinases (phosphoinositide 3-kinases, PI3Ks) is a family of lipid kinases capable of phosphorylating the 3'OH of the inositol ring of phosphoinositides. They are responsible for coordinating a diverse range of cell functions including proliferation, cell survival, degranulation, vesicular trafficking and cell migration. This downregulated miR also negatively regulated this kinase and could be coherent with its role of being downegulated and being an anti-exhaustion microRNA.
These targets will be validate and studied in future experiments in T cell clones model systems.
miR-21 is another microRNAs involved in immunity. In the immune system this microRNA is one of the most abundant miRNAs in T cells with critical functions for T cell homeostasis (186).
The history of miR-21 in fact began in 2007 when it was showed that miR-21 was upregulated in mouse CD8+ T cells upon in vitro activation. Similar results were

recently showed also for human CD8+ T cells demonstrating that expression of this miRNA is dynamically regulated during antigen-induced T cell differentiation (187).
Mir-21 is specifically induced upon T cell receptor (TCR) stimulation and is a negative regulator of RASGRP1, a key player in the TCR signal transduction, suggesting that this miRNA could negatively affect TCR signaling strength (188).
In addition, the overexpression of miR-21 results in lower T cell activation. From data in collaboration, results show that miR-21 activation via TCR stimulation reacts to negatively modulate TCR signaling strength (fulci et al., manuscript in preparation). In 2007 a study showed that mice RasGRP1-/- exhibit decreased T cell responses and delayed pathogen clearance, which suggests that they could be prone to developing chronic infection. In particular, RasGRP1-/- CD4 T cells showed expression markers of acute activation and memory like exhausted phenotypes, limited self renewal capacity, and also strongly expressed PD-1: this study demonstrated that these cells shared attributes with functionally exhausted memory T cells (189).
In 2009 it was shown that miR-21 was the most highly induced miRNA in an IL-13-induced asthma model and its expression was validated also in other asthma models (induced by OVA- and *Aspergillus fumigatus* antigens). MiR-21 was expressed in leukocytes in asthmatic lung tissue (190).
In this study the authors demonstrated that miR-21 modulates IL-12, a key cytokine derived from macrophages and dendritic cells involved in the adaptative immune responses involving TH1 cell polarization and the inhibition of miR-21 drived TH-1 polarization. This is consistent with a role of miR-21 in the Th-1 responses switching off. This was also recently demonstrated in murine models of hypersensitivity in the lungs and skin (191). Targeted ablation of miR-21 in mice led to reduced eosinophilia after allergen and increased levels of the Th1 cytokines IFN-γ. By the biological network-based transcriptome analysis of OVA-challenged miR-21-/- mice these authors identified dysregulation of IL-2/IFN-g pathway consistent with a functional miR-21 binding site in IL-12p35. This was demonstrated also in dendritic and CD4 T cells: miR-21 deficiency led dendritic cells to produce more IL-

12 after LPS stimulation and OVA-challenged CD4+ T lymphocytes to produce increased IFN-g and decreased IL-4. Loss of miR-21 significantly enhanced the TH-1-associated delayed-type hypersensitivity cutaneous responses, demonstrating that miR-21 plays a role in the inhibition of TH-1 responses.

Our data has demonstrated that miR-21 is the most upregulated microRNA involved in the inflammation in CD4+ and CD8+ Total T cell expressing PD-1 and thus could be a candidate microRNA in the early phases of exhaustion of CD4+ and CD8+/PD-1+ T cells. In PD-1+ T cells, during early exhaustion the microRNA expression profile revealed an upregulated panel of microRNAs that inhibits the activation of T cells, which could be called pro-exhaustion microRNAs and an downregulated panel of microRNAs that instead could be considered an anti-exhaustion microRNA and could foster the activation. This is consistent with an exhausted phenotype in early phases.

The miR-21 will also be studied in T cell clones that are a good model for studying T cell exhaustion *in vitro*. In addition, the dilution limiting used for T cell cloning and the subsequent stimulation with mitogens agents can help with an adeguate cell number. Future studies will be carried out to verify the upregulated expression of miR-21 in T cell in terminal exhaustion and also in T cell clones. By studying *in vitro* the effects of inhibition of miR-21 will be tested on these cells as well as miR-21 targets in exhausted state. If the reversion of T cell exhaustion is possible with inhibitors of miR-21 this microRNA will be used for therapy.

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