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Regulação da Expressão de Moléculas MHC Classe I em Linfócitos T em Divisão

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Resumo

As moléculas do Complexo Major de Histocompatibilidade (MHC) Classe I são glicoproteínas transmembranares compostas por uma cadeia pesada de 44-49 kDa, uma cadeia leve de 12 kDa denominada β 2-microglobulina e um péptido de 9-11 aminoácidos. Estas moléculas apresentam péptidos que são principalmente originados de péptidos intracelulares a células T citotóxicas e são necessárias para a indução e desenvolvimento de respostas imunes adaptativas. As moléculas de MHC Classe I são expressas por todas as células nucleadas. Contudo, estas moléculas podem ser expressas numa conformação diferente, na qual as cadeias pesadas de MHC classe I não se encontram associadas com o péptido e/ou com a β 2-microglobulina (denominados conformações abertas) que se podem associar com uma grande variedade de recetores expressos à superfície celular como por exemplo, o recetor de insulina, recetores de células Natural Killer (NK) e recetores de CD8, entre outros, modulando assim a sua ativação. Estas são também capazes de associar entre elas com implicações em doenças como a espondiloartrite. Estudos anteriores mostraram que estas conformações abertas são expressas na superfície celular de linfócitos ativados.

Neste trabalho, as células mononucleares do sangue periférico foram isoladas de amostras de sangue de pessoas saudáveis e de pacientes com excesso de ferro e eritrocitose, os glóbulos vermelhos lisados, as células marcada com CFSE, tendo-se adicionado diferentes estímulos capazes de induzir divisão celular e colocaram-se as células em cultura durante 6 dias. Depois deste período, as células foram marcadas como diferentes anticorpos e analisadas por citometria de fluxo. Células do sangue total de pacientes com Policitemia Vera foram isoladas de amostras de sangue de 3 mL. Esta doença caracteriza-se por elevado número de glóbulos vermelhos, aumento o volume do sangue, tornando-o assim mais espesso, sendo que este excesso de glóbulos vermelhos pode levar a outras complicações. Os glóbulos vermelhos foram lisados, as células lavadas e marcadas com os anticorpos W6/32 e HC-10. As amostras foram analisadas por citometria de fluxo.

Os nossos resultados mostram claramente que os estímulos aplicados nas células resultaram em diferentes níveis de proliferação e que em linfócitos T em divisão existe uma tendência para a manutenção ou um ligeiro decréscimo na expressão de moléculas MHC classe I na sua conformação fechada, ao passo que as conformação abertas aumento, tendo isto sido verificado tanto para linfócitos T CD4⁺ como para linfócitos T CD8⁺. No caso dos doentes de Policitemia Vera, os resultados mostram que a expressão de moléculas MHC classe I na sua conformação fechada encontra-se aumenta nas três diferentes populações leucocitárias estudadas: linfócitos, monócitos e granulócitos. Em relação à expressão de moléculas MHC classe I na sua conformação aberta, a sua expressão encontrava-se aumenta nos monócitos e

granulócitos ao passo que no caso dos linfócitos se encontrava diminuída em relação aos controlos.

Os resultados obtidos neste estudo indicam que a proliferação e divisão de células T conduz a uma alteração na expressão de moléculas MHC classe I à superfície das células, resultando numa alteração que aparenta estar a levar a um equilíbrio entre as conformações fechadas e abertas, o que poderá ter várias implicações imunes e não-imunes, tendo em conta o tipo de associações a que as conformações abertas têm sido implicadas. Ainda assim, mais investigações são necessárias, sendo que existe a necessidade de aumentar o número de indivíduos estudados. O alargamento do tempo de cultura das células poderá levar a um melhor conhecimento sobre os mecanismos de expressão destas moléculas, visto que o número de divisões será maior, o que poderá mostrar qual a verdadeira tendência associada à expressão destas conformações de moléculas MHC classe I. No caso do estudo da expressão destas moléculas em pacientes com Policitemia Vera será necessário aumentar o número de controlos saudáveis, com uma idade superior a 65 anos, assim como monitorizar os doentes de modo a perceber possíveis alterações que estejam a ocorrer, visto que os dados apresentados correspondem apenas a resultados preliminares.

Palavras-chave

Moléculas MHC classe I, Conformações Abertas, Linfócitos T em Divisão, Policitemia Vera

Abstract

MHC Class I molecules are transmembrane glycoproteins composed by a 44-49 kDa heavy chain, a 12 kDa light chain named β 2-microglobulin and a 9-11 amino acid peptide. These molecules present peptides originating mainly from intracellular peptides to cytotoxic T cells and are mandatory for the induction and development of adaptive immune responses. MHC Class I molecules are expressed by all nucleated cells. However, these molecules can be expressed in a different conformation, where MHC class I heavy chains are not associated with peptide and/or β 2-microglobulin (called open conformers) that can associate with a variety of cells surface receptors, resulting in modulation of their activation. Previous studies showed that open conformers are expressed at the cell surface of proliferating lymphoid cells.

In this work, we isolated peripheral blood mononuclear cells, from blood samples of healthy patients and iron overload and erythrocytosis patients, lysed red blood cells, labeled cells with CFSE, added different stimulus and cultured the cells for 6 days. After this period of time cells were labeled with different antibodies and analyzed by flow cytometry. Whole blood cells from Polycythemia Vera patients were isolated from 3 mL blood samples, red blood cells were lysed and cells were labelled with W6/32 and HC-10 antibodies.

Our results clearly demonstrate that the stimulus applied to the cells resulted in different proliferation rates and that in dividing T cells there's a tendency for the maintenance or a slightly decrease in the expression of closed MHC Class I molecules and an increase in the expression of open conformers, for both $CD4^+$ and $CD8^+$ T lymphocytes. Regarding, Polycythemia Vera patients, expression of closed MHC class I molecules is increased, while the levels of open conformers expression are increased in monocytes and granulocytes.

Keywords

MHC class I molecules, Open Conformers, Dividing T cells, Polycythemia Vera

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List of Abbreviations

APC	Antigen Presenting Cells
B₂m	Beta-2 microglobulina
CFSE	5-(and -6)-Carboxyfluorescein diacetate succinimidyl ester
DC	Dentritic Cell
EGF	Epidermal Growth Factor
ERAD	Endoplasmic Reticulum-associated degradation
FBSi	Fetal Bovine Serum inactivated
HC	Healthy Controls
HLA	Human Leukocyte Antigen
IL	Interleukin
MHC	Major Histocompatibility Complex
NK	Natural Killer
PHA	Phytohemagglutinin
PBS	Phosphate-Buffered Saline
PBMC	Peripheral Blood Mononuclear Cells
PV	Polycythemia Vera
RBC	Red Blood Cells
WBC	Whole Blood Cells

Chapter 1 - Introduction

1.1. Brief Historical Perspective

The Major Histocompatibility Complex (MHC) is a chromosomal region containing genes that play key roles in the immune response. The MHC locus was identified as a result of transplantation studies in mice during the 1940-1960s, initially performed by Peter Gorer and subsequently by George D. Snell and Baruj Benacerraf, that led to the identification of the group of genes responsible for transplant rejection that were designated Histocompatibility group 2 genes (thereafter H-2). The equivalent of the H-2 locus in humans was identified by Jean Dausset on leukocytes and designated Human Leukocyte Antigens (thereafter HLA) (1, 2). The MHC locus was shown to encode two classes of molecules: class I and class II. In 1970s, while studying the immune response of mice to viruses, Doherty and Zinkernagel demonstrated that the immune response mediated by T cells was restricted by the own MHC, thus introducing the concept of MHC restriction (3). In the following years the structure of the MHC class I and class II molecules was characterized and defined as classical and non-classical based on polymorphism and pattern of expression.

1.2. MHC Class I Molecules: from structure to function

Classical MHC class I molecules are composites of a 44-49 kDa α heavy chain, a 12 kDa light chain, called beta-2 microglobulin ($\beta 2m$) and a 9-11 amino acid peptide (4-6). These composites are also designated as closed MHC-class I conformers. The heavy chain consists of an extracellular part constituted by three domains ($\alpha 1$, $\alpha 2$ and $\alpha 3$), a transmembrane part and a cytoplasmic tail containing tyrosine and serine motifs. While the $\alpha 3$ domain is conserved, the $\alpha 1$ and $\alpha 2$ domains are highly polymorphic and form a groove/cleft where the peptide binds. These composites are assembled in the endoplasmic reticulum (ER) with the help of a number of molecular chaperones, including calnexin, calreticulin ERp57 and TAP (7). The peptides that are part of the final structure of MHC class I molecules are generated in the cytoplasm, mainly from endogenous proteins, by the enzymatic activity of a multimeric complex called proteasome. The peptides are then transported to the ER by the transporters associated with antigen processing (TAP) a complex formed by two subunits, TAP1 and TAP2, where they bind to the peptide binding cleft formed by the $\alpha 1$ and $\alpha 2$ domains. Once the structure is stabilized it is transported to the plasma membrane, via the Golgi apparatus where the α -chain is glycosylated in the $\alpha 1$ domain, where its main known function is to present peptides to CD8⁺ T lymphocytes. MHC class I molecules that fail to assemble correctly and become misfolded, due to deficiency in any of the components involved, are

retrotranslocated into the cytosol for their subsequent degradation by the proteasome. The process involves the use of the Sec61 pore complex that removes unstable or accumulating misfolded proteins from the ER, a process called ER-associated degradation (ERAD) (8).

Contrary to MHC class II molecules, which typically present peptides derived from exogenous proteins acquired by endocytosis or from internalized plasma membrane proteins, MHC class I molecules generally present peptide antigens derived from endogenous proteins. MHC class I molecules are expressed by the vast majority of cells, allowing circulating CD8⁺ cytotoxic T cells to survey them for possible infection or improper protein expression (9). Before becoming competent effector cells, T cells must first be primed against specific antigens by professional antigen presenting cells (thereafter APCs), namely mature dendritic cells (DCs). DCs express abundantly MHC class I and II molecules at the cell surface, akin to costimulatory and adhesion molecules and are thought to be the main “priming” cell *in vivo* (10). Besides presenting peptides derived from intracellular proteins, MHC class I molecules can also present peptides originated from exogenous antigens, a phenomenon called cross-presentation and that allows immune responses against tumor cells or extracellular pathogens.

1.3. MHC Class I Cell Surface Expression: endocytosis and beyond

At the cell surface, MHC-class I molecules are subjected to continuous and spontaneous cycles of endocytosis. This process was initially described on T cells by the group of Benvenuto Pernis (11) and subsequently found to take place in other cell types (12, 13). This endocytosis is thought to be regulated by conserved motifs located in the cytoplasmic tail of the majority of MHC-class I alleles (14) and proposed to have important implications for peptide presentation and viral escape. On the other hand, cell surface MHC class I molecules are also subjected to proteolytic cleavage by membrane proteases leading to shedding of soluble MHC-class I molecules which are thought to have immunoregulatory properties (15-18). Interestingly, these studies revealed that cell surface MHC class I molecules could lose the peptide and/or the light chain and become misfolded MHC class I heavy chains, also designated as β 2m-free and peptide-free heavy chains. However, to differentiate these heavy chains from truly misfolded ones resident in the ER, they were later designated as open MHC-class I conformers, in the sense that they still keep an ordered structure and in opposition to the closed MHC-class I conformers (19). These open conformers are thought to be originated after endocytosis and have been described mostly in activated T cells and cell lines (20-22) thanks to the production of a monoclonal antibody that was specific for a linear aminoacid sequence containing Arg and Asn on positions 62 and 63 of the α 1 domain, that is only detected in the absence of peptide (21).

Years later, these open MHC-class I conformers, more specifically open HLA-B27 heavy chains, were shown to self-associate and form homodimers (23). These finding brought about much interest on the biology of cell surface MHC-class I molecules and their relationship with disease beyond antigen presentation. In fact, these “unconventional” structures (HLA class I dimers) have been shown to interact in *trans* with a variety of receptors expressed by NK cells (24, 25), therefore regulating their function and with implications for autoimmune disorders, such as spondyloarthritis (26, 27).

On the other hand, a number of studies carried out in the 1980s and 1990s showed that cell surface MHC class I molecules could interact with a variety of receptors present on the same plasma membrane (*cis* interactions). Although these studies were controversial, they showed that by associating with receptors for hormones, such as the insulin receptors, MHC class I molecules were capable to fine-tune intracellular signaling (28), with implications for tumor cell survival and escape (29). More recently, this contextual framework has been revived, again in the context of cancer escape, by showing that expression of open MHC-class I conformers by medulloblastoma cells favor cancer progression due to interference with cell survival signals (30, 31).

1.4. Non-immunological Functions of MHC Class I Molecules: guilty by *cis*-association

As already mentioned, MHC class I molecules have the ability to physically *cis*-interact with various receptors. One of the first interactions discovered and studied was with the insulin receptor. MHC class I molecules were found to physically interact with the insulin receptor in mouse liver membranes (32) and raised the hypothesis that MHC class I molecules could be a partner involved in the regulation of receptor-associated kinases and therefore cell growth and proliferation. Indeed, these studies showed that the MHC class I heavy chain and the insulin receptor are structurally associated in the cell membrane, due to replacement of B2m by the insulin receptor, which results in a higher affinity binding of insulin to its receptor (33). This interaction was shown to be involved in hormone signaling (34). MHC class I molecules, most likely open conformers, have also been shown interact with a variety of other receptors, including the epidermal growth factor (EGF) receptor, the interleukin (IL)-2 in low affinity receptor, with the CD8 receptor, among others, with implications for the signaling pathways mediated by these receptors (35-39).

This novel role of MHC-class I molecules, that is *cis*-associations with other receptors, has been also observed in another contexts as well as for non-classical MHC class I molecules, including HFE, HLA-F and M10. HFE has been shown to *cis*-interact with the transferrin receptor and regulates iron homeostasis (40, 41). HLA-F also *cis*-associates with open MHC-I conformers association, contributes to a novel and unrecognized model of antigen

presentation in on activated lymphocytes and monocytes which may significantly contribute to the regulation of the immune system mediated by these lymphocytes (42). Finally, M10 *cis*-interacts with pheromone receptors and regulates neuronal functions (43, 44). Importantly, classical MHC-class I molecules have also been shown to modulate neuronal signaling both through *cis*-interactions (45) and *trans*-interactions after shedding (46). Similar finding have been reported on the *cis*-associations between MHC-class I molecules and NK receptors (47).

1.5. MHC Class I Molecules on T Cells

Expression of open MHC class I conformers in activated, but not resting, T cells was first described more than 25 years ago (20-22). Although the molecular mechanisms responsible for the generation of these open conformers have not been completely elucidated, there is evidence that endocytosis and tyrosine phosphorylation of the cytoplasmic tail are involved (14, 15, 20, 48, 49). Activated T cells and cell lines were then shown to self-associate via unpaired cysteines resulting in the formation of disulphide-linked homodimers (23). These homodimers are part of larger clusters MHC class I molecules that have been described at the cell surface of activated T cells and other metabolically active cells by some authors (50-52). The physiological significance of these homotypic interactions has not been solved but could be related to early studies showing that crosslinking of cell surface MHC-class I molecules on activated T cells modulated signal transduction pathway (53-56).

Expression of open MHC-class I conformers by T cells in the context of chronic disorders is scarce or lacking. Indeed, there are only reports showing anomalies in the expression of these forms on peripheral blood cells types of patients with spondyloarthropathies (57-59). On the other hand, a number of studies have reported the existence of anomalies in the expression of open MHC-I class I conformers in solid tumors (30, 60). In this regard, it is important to refer that in patients with Polycythemia Vera (PV), a myeloproliferative malignancy there is a significant downregulation of several HLA class I genes, which may be of major importance for defective tumor immune surveillance (61). However, and to our knowledge, there are no studies examining the expression level of open MHC-class I conformers in blood cell populations in PV patients.

Searching the literature, few investigation have been made to characterize the expression of MHC-I molecules in PV patients. One of the few works found relating this matter, studied the expression of HLA class I and II genes in PV. Since no work could be found about the expression of MHC-I molecules in their closed and open conformation, we studied the expression of these molecules in lymphocytes, monocytes and granulocytes of PV patients.

Chapter 2 - Aims of the Study

Given our interest in studying the functional role of open MHC class I conformers present at the cell surface of activated T cells we went to characterize the expression of HC-10-reactive (i.e., open conformers) and W6/32-reactive (i.e., closed conformers) in T cells activated in vitro with combinations of stimuli that we have previously shown to induce different levels of T cell proliferation and survival (62, 63). In addition, we performed a throughout characterization of the expression of open and closed MHC-class I conformers on peripheral blood leukocyte populations (bulk lymphocytes, monocytes and granulocytes) from Polycythemia Vera patients. The specific objectives were as follows:

1. To monitor the expression of closed and open MHC class I conformers in dividing T cells
2. To characterize the expression of these conformers on three leukocytes population of Polycythemia Vera patients

Chapter 3 - Materials and Methods

3.1. Cells and Reagents

Phytohemagglutinin (PHA, from *Phaseolus vulgaris*), bovine serum albumin, trizma base, ammonium chloride, RPMI-1640 medium and the antibiotic-antimycotic solution were obtained from Sigma-Aldrich (Madrid, Spain). Lymphoprep was from STEMCELL Technologies (Genobre, France). Sodium azide was obtained from Amresco (Solon, USA). CellTrace Carboxyfluorescein diacetate succinimidyl ester (CFSE) Cell Proliferation Kit was from eBioscience (San Diego, USA). W6/32 was from eBioscience (San Diego, USA), anti-CD4 and CD8 antibodies were purchased to Immunotools (Germany), while HC-10 antibody was a special gift from Dr. Simon Powis (St. Andrews University, Glasgow, Scotland, UK).

Fresh human peripheral blood mononuclear cells were obtained from buffy coats and phlebotomy from hereditary haemochromatosis and polyglobulia patients and buffy-coats after centrifugation over Lymphoprep. Peripheral blood mononuclear cells (PBMC) were washed twice in Phosphate-Buffered Saline (PBS) and contaminating RBC lysed in red cell lysis solution (10 mM Tris, 150 mM NH₄CL, pH 7.4) for 10 minutes at 37°C. RBC were collected from the pellet region after Lymphoprep centrifugation and diluted 1:10 in RPMI supplemented with 2.5% Fetal Bovine Serum inactivated (FBSi) and 1% antibiotic-antimycotic. Whole Blood Cells (WBC) were isolated from 3 mL bloods samples of PV patients. RBC were lysed in red cell lysis solution (described above) for 10 minutes at 37°C one time. Then, cells were washed twice in PBS.

3.2. CFSE Labelling

Ten million PBMCs were labelled with CFSE at a final concentration of 5 µM for 10 minutes at 37°C, with occasional mixing. Then, cells were washed twice with Washing Buffer (PBS and 20% FBSi) and resuspended in culture medium.

3.3. Culture Conditions

PBMCs (1.5×10^6) were cultured in six-well plates, in a final volume of 5 mL for up to 6 days in an incubator at 37°C, 5% CO₂ and 99% humidity. PBMCs were cultured in three different conditions: unstimulated, stimulated with PHA and stimulated with PHA plus RBC at a ratio of 1:10 in RPMI medium.

3.4. Flow Cytometry

Staining steps of resting and activated T cells for flow cytometry studies were performed at 4°C for 45 minutes in staining buffer (PBS, 0.2% Bovine Serum Albumin, 0.1% NaN₃) in 96-well round-bottom microtiter plates with approximately 0.5x10⁶ cells per well. At the end of culture, cells were harvested and washed with PBS. In cultures that received RBC, erythrocytes were lysed twice with red cell lysis solution and washed again with PBS. First, cells were labelled with W6/32 and HC-10 antibodies, followed by a Goat anti-Mouse secondary antibody. Finally, anti-CD4 and anti-CD8 antibodies were used. CFSE halving was used to determine rounds of cell divisions. For each sample at least 10000 viable lymphocytes were acquired using forward scatter/side scatter characteristics in a BD Biosciences Accuri C6 Flow Cytometer and analyzed using BD Accuri C6 software. For leukocyte populations of PV patients, Staining steps of resting and activated T cells for flow cytometry studies were performed at 4°C for 45 minutes in staining buffer (PBS, 0.2% Bovine Serum Albumin, 0.1% NaN₃) in 96-well round-bottom microtiter plates with approximately 0.5x10⁶ cells per well. Cells were labelled with W6/32 and HC-10 antibodies, followed by a Goat anti-Mouse Secondary antibody. For each sample, 15000 viable lymphocytes were acquired were acquired using forward scatter/side scatter characteristics in a BD Biosciences Accuri C6 Flow Cytometer and analyzed using BD Accuri C6 software.

3.5. Statistical Analysis

Statistical analysis was performed using Graph Pad Prism 6 software. Independent Student's T-test was used to determine the differences between group means. Spearman's correlation was used to assess the correlations between the proliferation index of T cells and the ratio of the expression between closed and open MHC class I conformers. Statistical significance was defined as P<0.05.

Proliferation index was calculated according to this formula:

$$\frac{1}{(CFSE\ MFI\ in\ activated\ cells \div CFSE\ MFI\ in\ non - activated\ cells)}$$

Chapter 4 - Results

4.1. T Cell Proliferation under Different Activation Conditions

The objective of this study was to ascertain the existence of a possible relationship between the expression of MHC class I molecules at the cell surface and the extent of T cell division. For that objective we cultured PBMC in vitro with stimuli previously known to induce different levels of proliferation that is PHA and PHA plus red blood cells (RBC). PBMC were cultured for 6 days in the absence of stimulus (NONE), with PHA or with PHA+RBC. At the end of the culture the percentage of dividing in each culture condition was calculated by adding the percentage of cells in the upper- and lower-left dot-plots of CFSE/W6/32 or HC-10 characteristics. As shown in Figure 1, T cells proliferated at different levels depending on the stimulus, being the culture condition with PHA+RBC much better than PHA ($P < 0.0001$).

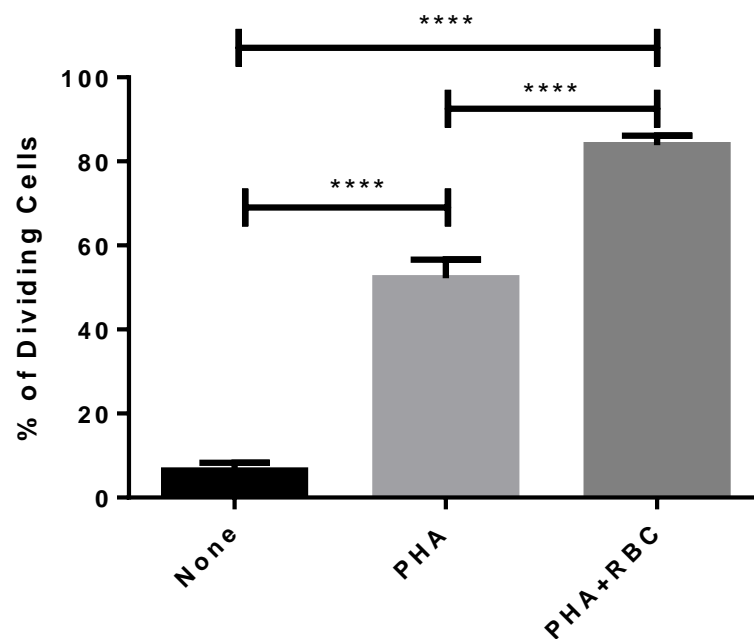


Figure 1. Proliferation of PBMC. After isolation over Lymphoprep, cells were labelled with CFSE and culture in three different conditions: None, PHA and PHA+RBC, for six days. At the end of this time period, cells were harvested, washed and cultures with RBC were lysed twice in RBC lysis solution. PBMC were labelled with antibodies previously referred and analyzed by flow cytometry. The expression of MHC class I molecules was analysed in the lymphocyte region, according to FSC/SSC characteristics using BD Accuri C6. The percentage of proliferating lymphocytes in the different culture conditions was calculated by determining the fraction of cells with CFSE lower than the observed in unstimulated cultures. Student's t-tests were performed. **** $P < 0.0001$.

4.2. Monitoring the Expression of Closed/Open MHC Class I Conformers on Proliferating/Dividing T Cells

Next, we wanted to analyze the expression of closed and open MHC class I conformers on T dividing Cells. For that purpose, we labelled PBMC with the dye CFSE to monitor cell division cycles and at the end of the culture, cells were labelled with W6/32 or HC-10 antibodies to study expression of these conformational states on CD4⁺ and CD8⁺ T cells.

Figure 2 illustrates a representative experiment where the expression of closed (W6/32) and open (HC-10) MHC class I conformers along the proliferation process (determined by CFSE halving) is shown in the different culture conditions. The level of proliferation as well as the expression of open MHC class I conformers in unstimulated PBMC (NONE, left dot-plots) were very low. Unstimulated PBMC, however, showed expression of closed MHC class I conformers. In cultures of PBMC stimulated with PHA there was an apparent entrance into the cell cycle (middle dot-plots) akin to an increase in the expression of open MHC class I conformers. However, a large fraction of T cells did not divide. The level of expression of closed MHC class I conformers remained steady. In cultures of PBMC stimulated with PHA+RBC, the percentage of non-dividing T cells was very low with the majority of T cells entering the cell cycle and dividing up to six times (right dot-plots), thus corroborating the data shown in Figure 1. Interestingly, the expression of open MHC class I conformers increased further in the PHA+RBC culture conditions in comparison to PHA alone or to unstimulated cultures. In contrast, the expression of closed MHC class I conformers slightly decreased.

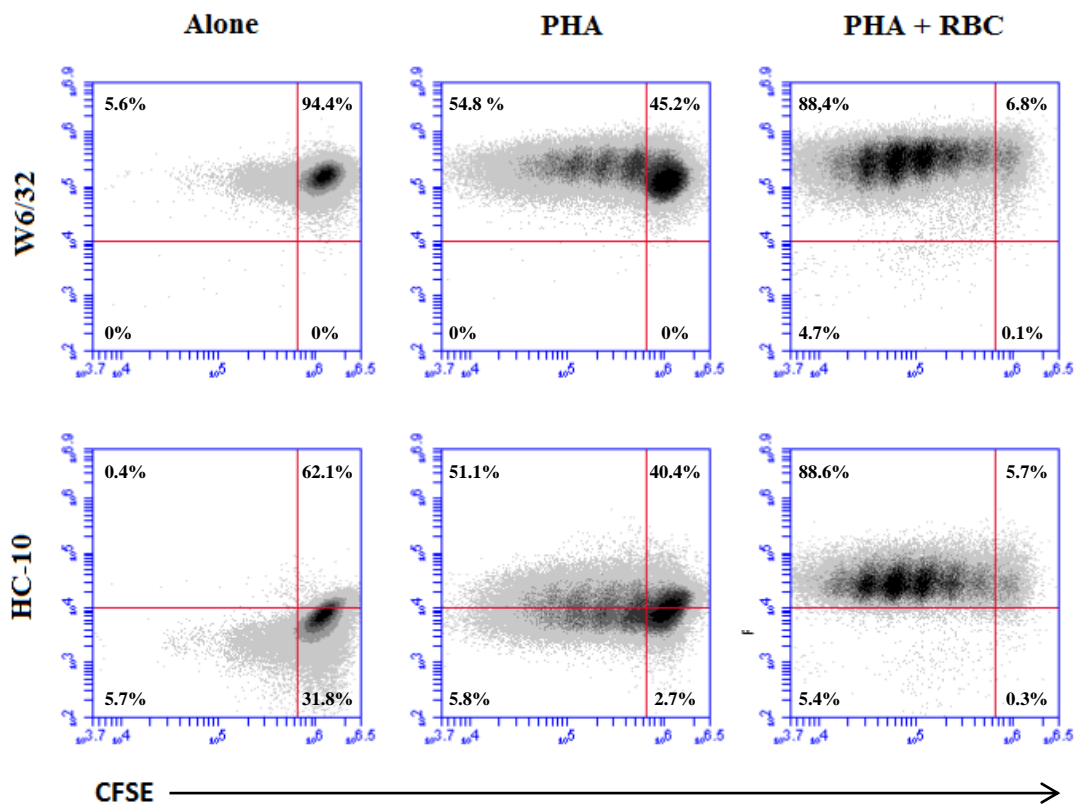


Figure 2. Proliferation of PBMC in response to different stimuli. After isolation over Lymphoprep, cells were labelled with CFSE and culture in three different conditions: None, PHA and PHA+RBC, for six days. At the end of this time period, cells were harvested and cultures with RBC were lysed twice in RBC lysis solution. PBMC were labelled with antibodies previously referred and analyzed by flow cytometry. The expression of MHC class I molecules was analysed in the lymphocyte region, according to FSC/SSC characteristics. Dot-plots show CFSE halving in the different culture conditions *versus* expression closed (W6/32, upper dot-plots) and open (HC-10, lower dot-plots) MHC class I conformers.

In view of these results, we wanted to ascertain whether there was a correlation between the levels of expression of MHC Class I conformers on dividing T cells at the end of the six days culture and the extent of proliferation. To that end, we performed a cross-relation analysis between the proliferation index (a measure of the extent of proliferation, see Material & Methods) and the ratio of the expression of the closed to open MHC class I conformers (as a measure of the oscillations in the expression of these conformers, see Materials and Methods) in CD4⁺ and CD8⁺ T cells. As illustrated in Figure 3, there was an inverse relationship between the extent of cell proliferation and the ratio of the expression of closed to open MHC class I conformers, both in CD4⁺ and CD8⁺ T cells. These results indicate that the overall level of open MHC class I conformers at the cell surface of dividing T cells increases, while the level of closed conformers is maintained or slightly decreases, at least in the culture conditions with PHA+RBC.

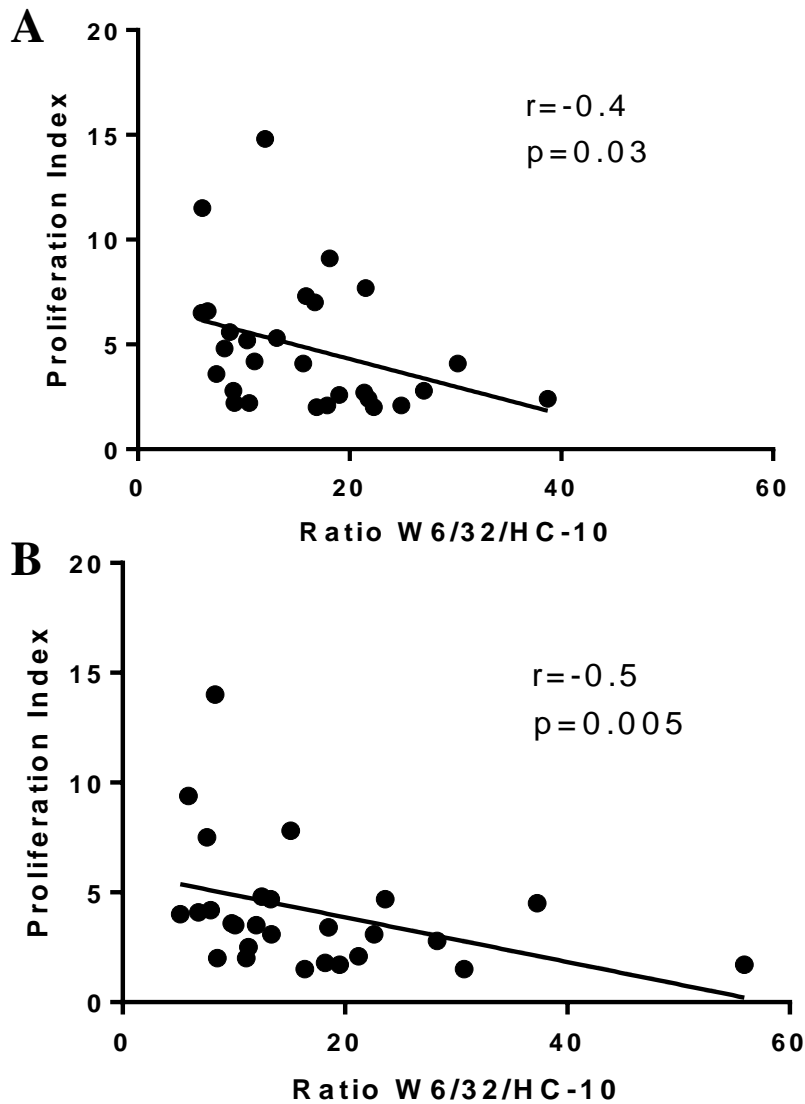


Figure 3. Correlation between cell proliferation and expression of MHC class I conformers in T lymphocytes. After isolation over Lymphoprep, PBMC were labelled with CFSE and culture in three different conditions: None, PHA and PHA+RBC, for six days. At the end of this time period, cells were harvested and cultures with RBC were lysed twice in RBC lysis solution. PBMC were labelled with W6/32, HC-10, and anti-CD4 and CD8 antibodies and analyzed by flow cytometry. The expression of MHC class I molecules was analysed in the lymphocyte region, according to FSC/SSC characteristics. Graph shows an inverse relationship between the extent of proliferation and the ratio of closed/open MHC Class I conformers (as determined by W6/32 and HC-10 expression levels) in CD4⁺ T lymphocytes (A) and in CD8⁺ T lymphocytes (B) in stimulated cultures. Coefficient of correlation (Spearman's correlation) and P-values are indicated. N=28

4.3. Impact of Cell Division Number on the Level of Expression of MHC Class I Conformers

Since the use of CFSE allows to monitor the different cycles of cell division along the six-day culture (see Figure 2), we went to analyze the expression of closed and open MHC Class I conformers in non-dividing cells and in each division cycle. As shown in Figure 4, PBMC cultures stimulated with PHA did not show significant differences in the expression of closed MHC class I conformers. In marked contrast, in cultures of PBMC stimulated with PHA+RBC, in addition to higher expression levels when compared to cultures with PHA ($P < 0.05$), there was an evident decrease in the expression of closed MHC class I conformers from cell division to cell division, that was statistically significant ($r = -1$, $P = 0.0004$).

A similar analysis for the expression of open MHC class I conformers revealed a similar pattern as for closed MHC class I conformers in cultures of PBMC stimulated with PHA. However, a marked decrease was observed between the 5th and 6th cell division cycle (see Figure 5). In PBMC cultures stimulated with PHA+RBC there was only a slight decrease in the level of open MHC class I conformers that did not reach statistical significance. Noteworthy, the level of expression of open MHC class I conformers in cultures with PHA+RBC were statistically significantly higher when compared to cultures with PHA ($P < 0.0001$) in each division cycle but the last.

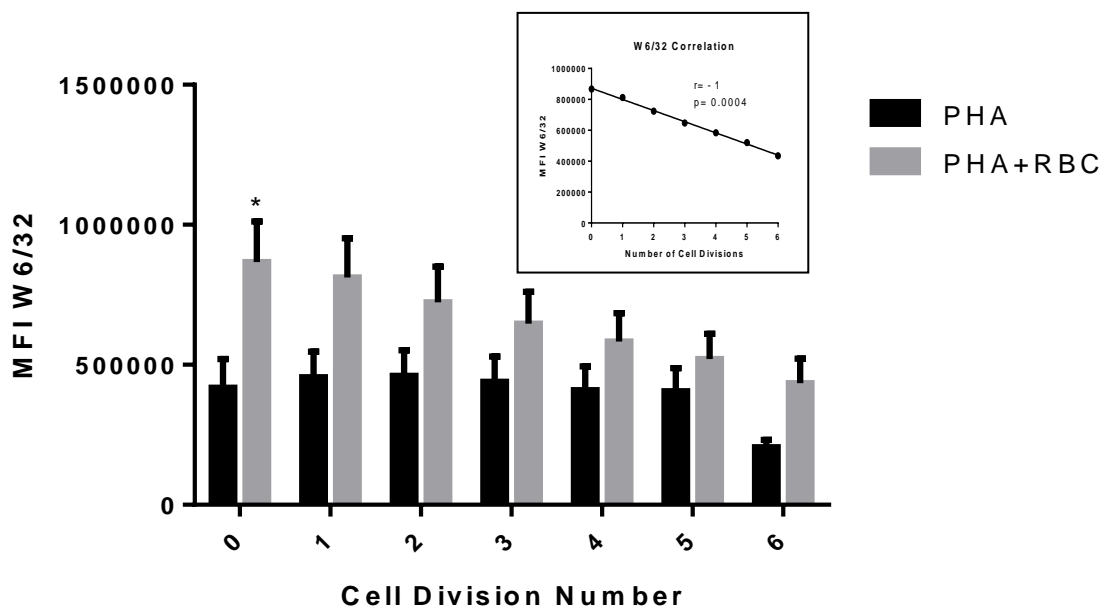


Figure 4. Expression of closed MHC class I conformers (W6/32) in each cell division of stimulated cultures. After isolation over Lymphoprep, cells were labelled with CFSE and culture in three different conditions: None, PHA and PHA+RBC, for six days. At the end of this time period, cells were harvested and cultures with RBC were lysed twice in RBC lysis solution. PBMC were labelled with W6/32 and HC-10 antibodies and analyzed by flow cytometry. The expression of MHC class I molecules was analyzed in the lymphocyte region, according to FSC/SSC characteristics. Graphic shows the expression of closed MHC Class I conformers in non-dividing cells and each cell division. Expression levels in stimulated cultures are shown (Mean \pm SEM). It is also showed an inset correlation between the expression of closed MHC class I conformers and the number of cell division (spearman's Correlation * $P < 0.05$)

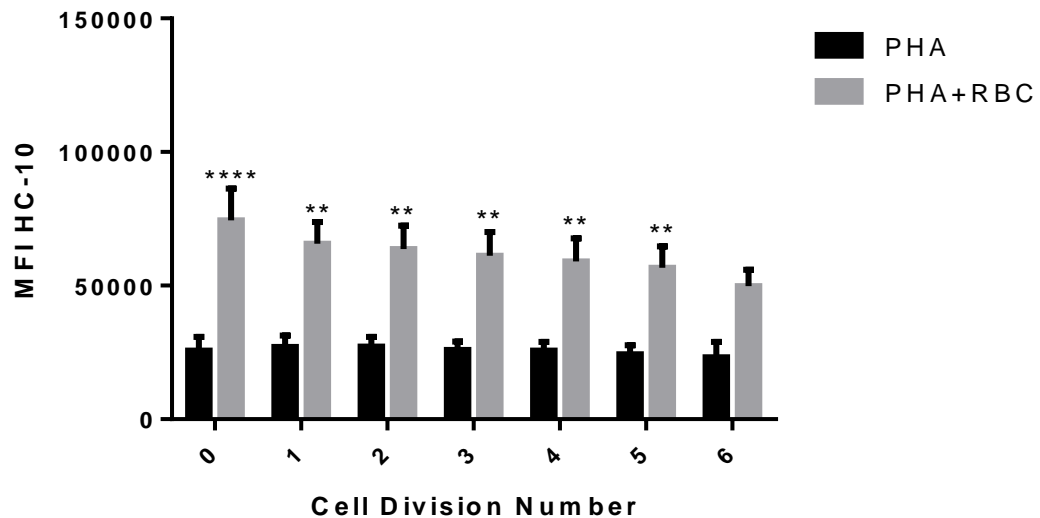


Figure 5. Expression of open MHC class I conformers (HC-10) in each cell division of stimulated cultures. After isolation over Lymphoprep, cells were labelled with CFSE and culture in three different conditions: None, PHA and PHA+RBC, for six days. At the end of this time period, cells were harvested and cultures with RBC were lysed twice in RBC lysis solution. PBMC were labelled with W6/32 and HC-10 antibodies and analyzed by flow cytometry. The expression of MHC class I molecules was analyzed in the lymphocyte region, according to FSC/SSC characteristics. Graphic shows the expression of open MHC Class I conformers in non-dividing cells and each cell division. Expression levels in stimulated cultures are shown (Mean±SEM). *P<0.05

4.4. Expression of Closed and Open MHC Class I Conformers on Leukocyte Populations of Patients with Polycythemia Vera

Since the main focus of this work was to monitor the expression of closed and open MHC Class I conformers in dividing T lymphocytes, we took advantage of having access to blood samples of Polycythemia Vera (PV) patients to analyze the expression of MHC class I molecules on the three main leukocyte populations; lymphocytes, monocytes and granulocytes (mostly neutrophils). Most of these patients are considered elderly (74 ± 2.4) and a number of previous studies have shown that T cell populations, namely CD8⁺ T cells, in the elderly have undergone many cycles of cell division (64). Likewise, these PV patients are characterized by a chronic inflammatory status (65). Therefore, we went to characterize the expression of closed and open MHC class I molecules.

Total leukocytes were obtained from 3 mL blood samples after RBC lysis, as indicated in Material and Methods. Cells were then stained with W6/32 and HC-10 antibodies followed by secondary goat anti-mouse antibody. After staining, cells were acquired using a BD Accuri C6 and expression of closed and open MHC class I conformers was analyzed in the three major leukocyte populations shown in Figure 6.

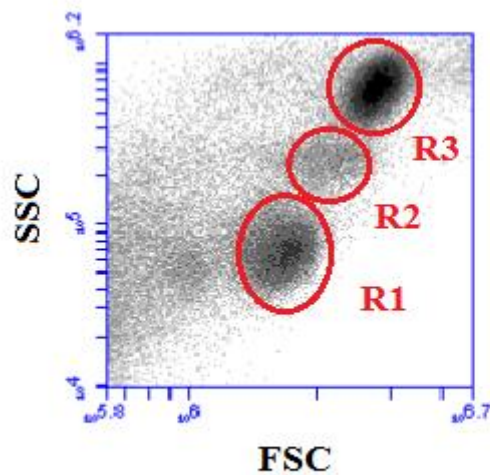


Figure 6. Leukocyte Populations studied in Polycythemia Vera patients. WBC were isolated from 3 mL blood samples. RBC were lysed in REB lysis solution and washed twice in PBS. Then cells were labelled with W/6/32 and HC-10 antibodies for 30 minutes on ice followed by labelling with Goat anti-Mouse secondary antibody. Cells were acquired by flow cytometry in BD Accuri C6 and analyzed in BD Accuri C6 software. Study of closed and open MHC Class I conformers expression was performed in Polycythemia Vera patients in different leukocyte populations. R1 corresponds to the lymphocytes region, R2 to the monocytes region and R3 to granulocytes region.

Figure 7 shows the expression (MFI, Mean±SEM) of closed MHC Class I conformers in the different leukocyte populations in patients and healthy controls (HC). Differences in the expression of both conformers between PV patients and HC were observed. However, only in monocytes the difference in the expression of W6/32-reactive conformers reached statistical significance (185699 in PV vs. 99238 in HC, P<0.001).

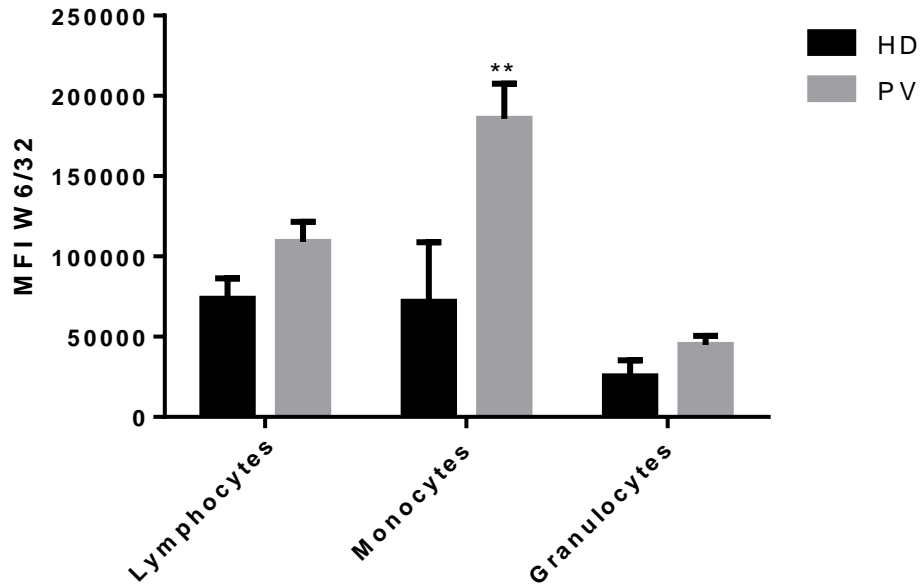


Figure 7. Expression of closed MHC Class I conformers (W6/32) in the different leukocyte populations of Polycythemia Vera patients and healthy controls. . WBC were isolated from 3 mL blood samples. RBC were lysed in REB lysis solution and washed twice in PBS. Then cells were labelled with W/6/32 and HC-10 antibodies for 30 minutes on ice followed by labelling with Goat anti-Mouse secondary antibody. Cells were acquired by flow cytometry in BD Accuri C6 and analyzed in BD Accuri C6 software. Graphic shows the different expression levels of closed MHC Class I conformers (W6/32) between Polycythemia Vera patients (PV) and healthy controls (HD) in lymphocytes, monocytes and granulocytes (Mean±SEM). **P<0.001

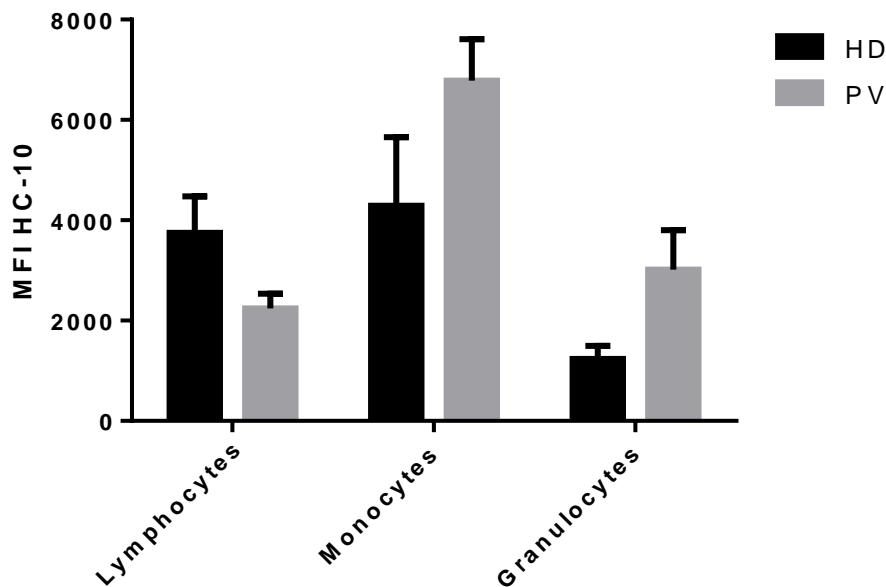


Figure 8. Expression of closed MHC Class I conformers (W6/32) in the different leukocyte populations of Polycythemia Vera patients and healthy controls. . WBC were isolated from 3 mL blood samples. RBC were lysed in REB lysis solution and washed twice in PBS. Then cells were labelled with W/6/32 and HC-10 antibodies for 30 minutes on ice followed by labelling with Goat anti-Mouse secondary antibody. Cells were acquired by flow cytometry in BD Accuri C6 and analyzed in BD Accuri C6 software. Graphic shows the different expression levels of open MHC Class I conformers (HC-10) between Polycythemia Vera patients (PV) and healthy controls (HD) in lymphocytes, monocytes and granulocytes (Mean±SEM). **P<0.001

Chapter 5 - Discussion

The main objective of this work was to monitor the expression of closed and open MHC Class I conformers in dividing T lymphocytes. For this purpose, T cells were stimulated with previously described stimulus known to induce different rates of proliferation.

Previous experimental studies showed that open MHC class I conformers are expressed at the cell surface of activated, but not resting, lymphoid cells (20-22, 48, 66). These studies were performed in various cell lines and in activated PBMC of diseased individuals. From this point, we went to investigate the expression of closed and open MHC class I conformers in activated PBMC, isolated from iron-overload/erythrocytosis patients and buffy-coats from healthy individuals, on dividing T cells. These were cultured for six days and different stimuli were applied to induce T cell proliferation. Expression of W6/32-reactive molecules and HC-10-reactive molecules follows different trends. In the present study, a correlation between the extent of proliferation and the decrease in the ratio closed to open MHC class I conformers was found to occur for both CD4⁺ and CD8⁺ T lymphocytes, within the six-day culture, pointing to an increase in the expression of open MHC class I conformers, while the expression of closed MHC class I conformers was maintained or slightly decreasing. A recent work (49), showed that open conformers are expressed at the cell surface of normal human T cells. Cells were activated for 5 days and the expression of open conformers was analyzed at different time points (days 0, 1, 3 and 5), showing an increase in the expression of open MHC class I conformers. On the other hand, in our work, expression of both closed and open MHC class I conformers was monitored in six-day cultures, where different stimulus were applied and the expression of these molecules was studied in each cell division cycle, extending the knowledge about this subject. When stimulated with PHA, PBMC expression of closed conformers increases after cell division, slightly decreasing after some division cycles, whereas the expression of open conformers is maintained from cell division to cell division, being higher than in non-dividing cells, in the first cell division cycles. Cell surface expression of open MHC class I conformers in activated T cells is associated with tyrosine phosphorylation, which may be responsible for the increase in open conformers expression, since shedding of closed MHC class I conformers is reduced, possibly leading to a closed to open equilibrium (49). However, PHA+RBC stimuli induced an evident decrease in the expression of closed MHC class I conformers from cell division to cell division, while expression of open MHC class I conformers slightly decreases in each cell division cycle. Furthermore, expression of both conformers was increased in PHA+RBC-stimulated cells, when compared to PHA-stimuli alone, which may be explained by the known ability of RBC to promote survival, growth and cell cycle progression of T lymphocytes (62, 63).

Regarding the expression of closed and open MHC Class I conformers in PV patients, the data presented represents only preliminary results. Expression of closed conformers is increased in

the three leukocyte populations of PV patients in comparison to HC, with statistically significance on monocytes. Although the expression of open conformers is also increased in monocytes and granulocytes of PV patients, the same cannot be said about lymphocytes. In this case, HC express higher levels of open MHC Class I conformers. A recently published paper (61), revealed that HLA genes are downregulated in myeloproliferative neoplasm, in which PV is included. One can speculate that since MHC class I molecules are being overexpressed in this patients, HLA genes may be repressed, in order to decrease the expression of these molecules. Since one of the features of PV patients is an uncontrolled production of RBC, higher expression of open MHC class I molecules may be related with it, allowing cells to divide more often and in higher rates. Nevertheless, it important to take in consideration that the number of HC used is very low, so few conclusions can be taken concerning this matter. More importantly, for our knowledge, this is the first work describing the expression of open MHC class I conformers in PV patients.

This work contributes to the knowledge about the expression of MHC class I conformers, in particular open conformers that have been implicated in a variety of immune and non-immune functions, reviewed in (19). As shown in this work, expression of open conformers increases with the extent of cell proliferation, contributing to a closed to open equilibrium. Considering the immune and non-immune functions of open MHC class I conformers, further studies addressing the formation of these molecules as well their ability to *trans*- and *cis*-associate with cell surface receptors are required, in order to disclose novel insights about a possible relation between open MHC class I conformers and several diseases.

Chapter 6 - Conclusion

In the present work we were able to monitor the expression of MHC class I conformers on dividing T cell for a short period of time (six-day cultures). The main finding is that the levels of expression of W6/32-reactive molecules and HC-10-reactive molecules at the cell surface of activated T cells follow different trends. This was more evident in PHA+RBC-stimulated cultures, where it is known that T cells divide much more and dye much less. Thus, closed MHC class I conformers appear to decrease their levels of expression with the increase of cell divisions, whereas open MHC class I conformers seem to maintain or slightly decrease their expression levels. Of note, expression of open conformers increases sharply after activation, being more evidence, once again, in cultures stimulated with PHA+RBC, in contrast to closed conformers.

This study comprises a preliminary characterization of the expression of closed and open MHC class I conformers in leukocyte populations of PV patients. Expression levels of W6/32-reactive molecules are increased in the three populations studied, whereas HC-10-reactive molecules expression is increased in monocytes and granulocytes. The first steps were taken for a better understanding of the expression of MHC class I molecules, which may have implications for the disease development.

Chapter 7 - Future Perspectives

Further studies are required in order to confirm our results and to ameliorate our understanding about the expression of MHC class I conformers. Considering that we monitored the expression of these molecules in six-day cultures, it would be interesting to perform the same experiences in long-term cultures of PBMC from patients and healthy blood donors. A bigger time window would clarify what happens to the expression of W6/32-reactive molecules with an increase of the number of cell cycle division and if HC-10-reactive molecules would maintain or not, their levels of expression. Since the expression of open MHC class I conformers is increased at the cell surface of activated T cells, leading to a closed to open equilibrium, studies about the association of these molecules with other cell surface receptor and with themselves could contribute for a better understanding of several diseases that may be related with the non-immunological functions of open conformers. Furthermore, investigations addressing tyrosine phosphorylation of MHC class I molecules may help understand the influence of open MHC class I conformers in several, non-related, diseases. Regarding PV patients, it is very important to increase the number of HC with a mean age above 65 years so that our results can be confirmed, or not, improving our knowledge about the disease. It would be interesting to extend our investigations to CD4⁺ and CD8⁺ T lymphocytes, considering their importance to the immune system. Finally, follow-up studies should be performed so possible correlations between the expression of closed and open MHC class I conformers and the progression of the disease may be established.

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