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Phenotypic and Functional Aspects of Lymphocyte Populations in Patients with Blood Dyscrasias Undergoing Phlebotomy

Ana Raquel Costa Brito

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Orientador: Prof. Doutor Fernando Arosa
Coorientadora: Profª. Doutora Elsa Cardoso

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Resumo

As discrasias sanguíneas são patologias associadas a alterações funcionais ou estruturais de um qualquer constituinte do sangue. A Policitemia Vera (PV) e a Policitemia Secundária (SP), inseridas neste grupo, são doenças associadas com o aumento da quantidade de eritrócitos (eritrocitose) na corrente sanguínea. O aumento da quantidade de glóbulos vermelhos (RBC) na PV parece estar maioritariamente associado à presença de uma mutação na cinase Janus 2 (JAK2) que culmina na ativação constitutiva desta cinase nas células hematopoiéticas e consequente produção excessiva de RBC. Contrariamente à PV, a SP resulta de mecanismos que não envolvem alterações nas células progenitoras da medula óssea, estando maioritariamente associada à presença de outras patologias. Por sua vez, a Hemocromatose Hereditária (HH) e a Hemocromatose Secundária (SH) são doenças associadas ao aumento da quantidade de ferro e também elas são inseridas no grupo das discrasias sanguíneas. Enquanto a HH é uma doença associada a mutações no gene *HFE* ou a alterações nos mediadores envolvidos na absorção e transporte de ferro, a SH é uma patologia que surge maioritariamente como consequência da necessidade de transfusões sanguíneas recorrentes verificada em algumas doenças. Atualmente, e tendo em conta as evidências experimentais, pensa-se que os RBC estão envolvidos em várias funções para além do transporte de gases metabólicos e nutrientes para os tecidos. De facto, a presença de RBC em culturas com linfócitos humanos isolados do sangue periférico e estimulados com mitogénios é responsável pelo aumento da proliferação e sobrevivência de linfócitos T. Nos poucos estudos prévios onde foram estudadas populações de linfócitos em doentes com PV e SP, foi descrito um aumento do rácio $CD4^+/CD8^+$, assim como um aumento da percentagem e da capacidade supressora de linfócitos T $CD4^+$ reguladores ($CD4^+CD25^+FoxP3^+$) no sangue periférico de doentes com PV. A nível da HH e da SH, o panorama é distinto, tendo sido realizados estudos imunológicos com maior frequência, sendo que a maioria reporta anomalias a nível das populações de linfócitos T $CD8^+$, incluindo a diminuição da população T $CD8^+$ e o aumento da percentagem de células $CD8^+CD28^-$.

O objetivo primordial deste trabalho foi avaliar se doentes com anomalias no número de RBC apresentavam alterações fenotípicas e funcionais a nível das diferentes populações de linfócitos e se estas alterações poderiam ser influenciadas pelo tratamento com flebotomias. Para isso, foi realizado o estudo fenotípico das populações de linfócitos em doentes com PV, SP, HH e SH, assim como num grupo controlo constituído por dadores regulares de sangue. Para além disso, foi também avaliada a influência da presença de RBC de doentes e controlos na proliferação e sobrevivência de linfócitos T, recorrendo à realização de culturas com células mononucleares do sangue periférico (PBMC). As PBMC usadas neste estudo foram isoladas a partir de amostras de sangue periférico de dezanove dadores saudáveis, dezassete doentes com HH, três doentes com SH, três doentes com PV e dez doentes com SP. As

percentagens de populações de linfócitos, assim como a percentagem de proliferação e sobrevivência das células em cultura foram determinadas através da análise por citómetria de fluxo. Várias condições de cultura foram incluídas neste estudo, mais especificamente a estimulação das células com um mitogénio de linfócitos T ou a presença de RBC autólogos ou heterólogos.

Quando as percentagens das diferentes populações de linfócitos foram analisadas, foi possível observar que a percentagem de linfócitos NK se encontrava elevada em todos os grupos de doentes em comparação ao grupo de indivíduos saudáveis, no entanto este aumento só era estatisticamente significativo nos grupos de doentes com HH e SP. Em contraste, a percentagem de linfócitos B encontrava-se significativamente diminuída nos doentes com HH, PV e SP em relação ao grupo controlo. Outras diferenças, nomeadamente o aumento das populações de linfócito T $CD8^+$ e $CD8^+CD28^+$ nos doentes com SH e SP, assim como a diminuição destas populações em doentes com PV, foram também observadas. Em relação à monotorização das percentagens de linfócitos dos doentes ao longo do tratamento com flebotomias, foi verificada uma tendência para a diminuição da percentagem de linfócitos NK em cinco dos sete doentes seguidos. Em relação à influência dos RBC nas culturas de PBMC, apenas ligeiras diferenças em termos de proliferação e sobrevivência foram observadas, com a excepção das culturas com PBMC de doentes com PV onde, de modo geral, se registou uma menor percentagem de proliferação em todas as condições de cultura em comparação com os restantes grupos de doentes e com o grupo controlo, provavelmente devido ao aumento da população de linfócitos T $CD4^+$ reguladores no sangue periférico descrito recentemente em doentes com PV. No entanto, e talvez uma das mais inesperadas observações relativa às experiências de proliferação, foi o facto de ser constantemente verificado que os próprios RBC, principalmente os RBC isolados de dadores saudáveis, eram capazes de induzir proliferação de uma população de linfócitos T na ausência de estímulo e sem a observação do aumento de tamanho e complexidade desta população (blastos), independentemente da origem dos PBMC onde eram adicionados.

Os resultados obtidos neste estudo sugerem que o excesso de RBC, bem como o excesso de ferro em circulação, podem estar associados com alterações a nível dos linfócitos NK e B. Futuramente, é necessário realizar mais estudos com vista a determinar se para além das alterações a nível da quantidade de linfócitos NK e B associadas às discrasias sanguíneas, também se encontram anomalias noutros parâmetros destas populações, como por exemplo na expressão de recetores superficiais. Existe também a necessidade de alargar o período de monotorização dos doentes tratados com flebotomias, assim como a necessidade de aumentar o número de indivíduos estudados, tendo em conta que este foi apenas um estudo de carácter preliminar. É ainda imprescindível realizar estudos exaustivos sobre as implicações que a idade e o tempo de armazenamento dos RBC podem ter na sobrevivência e proliferação de células T, provavelmente responsáveis pelos inesperados resultados obtidos neste estudo.

Palavras-chave

Linfócitos, Policitemia, Hemocromatose, glóbulos vermelhos

Abstract

Blood dyscrasias are pathological conditions in which any of the constituents of the blood are structuraly or functionally abnormal. Polycythemia Vera (PV) and Secondary Polycythemia (SP) are two disorders characterized by the increase of the amount of red blood cells (RBC) while hereditary hemochromatosis (HH) and secondary hemochromatosis (SH) are associated with iron overload. It is currently believed that RBC play an important role in T cells growth and survival, which may suggest that anomalies in red blood cells could be associated with lymphocyte alterations. The few immunological studies conducted in erythrocytosis patients reported an increase of $CD4^+/CD8^+$ ratio as well as an increase in the percentage of T $CD4^+CD25^+FoxP3^+$ cells in patients with PV. A number of earlier studies reported anomalies in $CD8^+$ T populations in patients with HH. The main objective of this work was to ascertain if patients with anomalies in RBC numbers have alterations in lymphocyte populations and whether therapeutic phlebotomy influences these populations. In order to do that, blood samples from nineteen healthy donors, seventeen HH patients, three SH patients, three PV patients and ten SP patients were used as a source of peripheral blood mononuclear cells and RBC. The percentages of lymphocyte populations as well as the extent of T cell proliferation and survival were determined by flow cytometry. It was observed that the percentage of NK cells was higher in all groups of patients when compared to controls. However, this increase was statistically significant only in HH and SP patients. In contrasts, B cells were significantly decreased in HH, PV and SP in relation to controls. When the influence of RBC on T cell proliferation and survival was ascertained, it was possible to note that cells from PV patients tended to proliferate less than the cells from the other groups of patients and controls. Unexpectedly, it was also observed that RBC by themselves were able to induce T cell proliferation in the absence of any mitogen stimulus. This effect was most striking with RBC obtained from buffy coats of regular blood donors. These results suggest that an excess of circulating RBC and/or iron are associated with alterations in NK and B populations. Further studies are needed in order to ascertain if the anomalies in NK and B cells include alterations of other parameters, like the expression of surface receptors. More importantly, based on the evidences of the effect of the addition of RBC from controls to cultures, it is necessary to perform extensive studies to elucidate the implications of RBC storage and aging on T cell proliferation and survival.

Keywords

Lymphocytes, Polycythemia, Hemochromatosis, Red blood cells

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Abbreviations

BSA	Bovine serum albumin
CFSE	5-(and -6)-Carboxyfluorescein diacetate succinimidyl ester
COPD	Chronic obstructive pulmonary disease
DNA	Deoxyribonucleic acid
EPO	Erythropoietin
FBS	Fetal bovine serum
FSC	Forward scatter
HH	Hereditary hemochromatosis
HLA	Human leukocyte antigen
IL	Interleukin
JAK2	Janus kinase
KIR	Killer immunoglobulin receptor
MAP	Mitogen-activated protein
MCH	Mean corpuscular hemoglobin
MCV	Mean corpuscular volume
MHC	Major histocompatibility complex
NK	Natural killer
NO	Nitric oxide
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline solution
PHA	Phytohemagglutinine
PI	Propidium iodide
PI3K	Phosphatidylinositol 3 kinase
PV	Polycythemia Vera
RBC	Red blood cells
RBCA	Autologous red blood cells
RBCH	Heterologous red blood cells
SH	Secondary hemochromatosis
SP	Secondary Polycythemia
SSC	Side scatter
STAT5	Signal transducer and activator of transcription protein 5
Treg	T regulator lymphocytes
Tf	Transferrin

I. Introduction

I. Introduction

1. Blood dyscrasias

Blood dyscrasias are pathological conditions in which any of the constituents of the blood are structuraly or functionally abnormal. Sereveral hematological diseases may be included in this group like leukemias, iron-related disorders and myeloproliferative neoplasms. Iron-related disorders are characterized by alterations in iron-related parameters, which may lead to the development of iron overload, as seen in hereditary hemochromatosis (HH) or secondary hemochromatosis (SH) [1], or to the development of iron deficiencies [2]. In contrast, myeloproliferative neoplasms are a group of diseases that include Polycythemia Vera (PV), Essential Thrombocythemia and primary myelofibrosis and are characterized by clonal expansion of an abnormal hematopoietic stem cell [3].

1.1. Polycythemia Vera

Polycythemia Vera is a bone marrow disorder characterized by an excessive production of blood cells in the marrow, including red blood cells (RBC), granulocytes and megakaryocytes (responsible for the production of platelets) [4]. Hemoglobin values above 18.5 g/100 mL in men or 16.5 g/100 mL in women [5], or hematocrit values over 52% in men or 48% in women [6] are indicative of erythrocytosis. Nonetheless, PV individual manifestations may differ greatly between subjects. Some subjects present a moderate elevation in the amount of erythrocytes with an extreme degree of thrombocytosis, while in others the leukocytes count may be at or close to leukemia levels, with only a slight increase in RBC and platelets [4].

Polycythemia Vera has an estimated incidence of about 2.3 per 100,000 every year with a median age of presentation of approximately 60 years old [7], with rare juvenile cases [8]. The incidence of PV is slightly higher in men than women (2.8 versus 1.3 cases/100,000 per year), and is highest for men aged 70–79 years (24 cases/100,000 persons per year)[9]. Common symptoms associated to this disease include headache, vertigo, visual disturbance, vascular disturbances of the extremities, hemorrhage after minor trauma, venous and arterial thrombosis [4], weight loss, pruritus after a warm bath and arthritis [10]. PV is associated with significant morbidity and mortality [7]. This disease may evolve into myelofibrosis, anemia, or less commonly into acute leukemia [7], but the primary causes of morbidity and mortality are thrombotic complications and bleeding [11].

In 2005, the discovery of an acquired Janus kinase 2 (JAK2) mutation was considered as the cause of classical myeloproliferative neoplasms [4,12,13]. Patients with these disorders had a homozygous G→T transversion that cause a phenylalanine-to-valine substitution at the position 617 of *JAK2* (V617F)[12]. The *JAK2*V617F mutation is present in more than 95% of PV

patients [14,15]. PV patients without the *JAK2*V617F mutation virtually all have a *JAK2* exon 12 mutation [14], another *JAK2* mutation associated with this disease [9]. However, some studies suggest that the *JAK2*V617F mutation may not entirely account for the development of PV [16]. The homozygous *JAK2*V617F mutation is the result of two steps. The first step is an acquired point mutation which results in a heterozygous state. Then, loss of heterozigosity occurs as the result of mitotic recombination between homologous chromosomes 9p [12,14]. Homozygous *JAK2*V617 patients (about half of PV patients) tend to have a longer duration of the disease, higher hemoglobin levels, increased incidence of pruritus and are more likeliness to progress to myelofibrosis [17].

The *JAK2*V617F somatic mutation results in constitutive activation of JAK2 leading to cytokine hypersensitivity and erythrocytosis [7]. This mutation is located in the JAK2 auto-inhibitory domain that results in the autonomous activation of the JAK2 downstream signaling pathways including signal transducer and activator of transcription protein 5 (STAT5), phosphatidylinositol 3 kinase (PI3K), Akt serine threonine kinase and mitogen-activated protein (MAP) kinase (Figure 1)[5,8]. Under normal conditions JAK2 signaling is initiated by the biding of erythropoietin (EPO), a glycoprotein hormone produced predominantly in the kidney that controls erythropoiesis, to its receptor [5]. Experiments performed in murine cell lines showed that the mutated JAK2V617F was constitutively active and able to activate the EPO receptor-dependent signaling pathways in the absence of EPO [8]. Genetic evidences and in vitro functional studies suggest that V617F gives hematopoietic precursors proliferative and survival advantages [12]. The dominance exerted by the *JAK2*V617F erythroid clone over polyclonal erythroid precursors could be due, in part, to its ability to complete differentiation more efficiently in a low EPO milieu [18]. This dominance may also involve transforming growth factors produced by PV patients mononuclear cells and the hypersensitivity of polycythemia erythroid progenitor cells to interleukin (IL)-3, granulocyte macrophage–colony-stimulating factor, stem cell factor, and insulin like growth factor [4,9,18].

The *JAK2*V617F mutation is present in granulocytes, erythroblast and in all the EPO-independent erythroid colonies. Initially it was thought that the *JAK2*V617F mutation was not present in B and T cells [19], however, Ishi et *al*. demonstrated that the *JAK2*V617F mutation is also present in B and T lymphocytes in a subpopulation of patients with PV [20]. Two possible mechanisms of the *JAK2*V617F mutation are suggested. In the first *JAK2*V617F mutation alone or in combination with one or more preexisting somatic mutations may cause the myeoloproliferative disorder [12]. The second model suggests that the *JAK2*V617F mutation is a secondary genetic event that occurs after the onset of the myeloproliferative phenotype, which may be caused by mutation(s) in a gene or genes that remain unknown [12,21]. In any case, the detailed mechanism involved in the abnormal activation of JAK2 and its transforming activity remain to be elucidated [13].

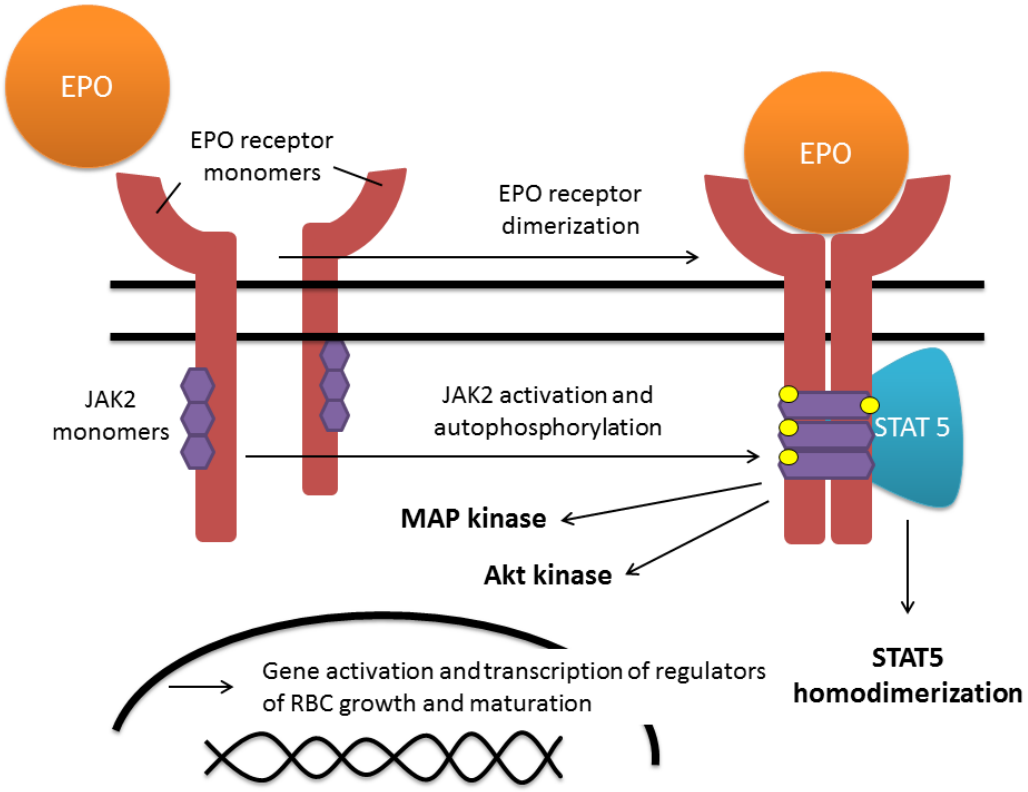

Figure 1. Erythropoietin receptor signaling. Binding of Epo to its receptor results in receptor homodimerization and autophosphorylation of the receptor-associated JAK2. Activated JAK2 in turn mediates the phosphorylation of key tyrosine residues on the distal cytoplasmic region of EpoR, which then serve as docking sites for downstream effectors, including STAT5 and PI3K. Activated STAT5 translocates to the nucleus to affect gene transcription. Adapted from [5].

Besides the alterations in the amount of RBC, other RBC anomalies have been associated with PV. The removal of excessive RBC from the circulation in PV patients as therapy may imply changes in RBC morphology. The reduction of iron stores in PV due to the continuous need of this metal for hemoglobin production, may lead to the production of smaller and hypochromic RBC [4]. Alterations in the adhesion properties of RBC from PV patients have also been reported [8]. In a study conducted by Wautier et *al*. RBC from PV individuals were abnormally adherent to resting human umbilical vein endothelial cells under static and flow conditions [22]. A later study from the same group confirmed that the increased adhesion levels were due to a constitutive phosphorylation of an adhesion molecule that correlated with the presence of *JAK2*V617F mutation [23].

1.2. Secondary Polycythemia

Secondary Polycythemia (SP), previously designated as polyglobulia, is a condition that comprises a group of diseases characterized by an increase in RBC mass or RBC amount like PV, but that results from a mechanism other than alterations in bone marrow progenitors [6]. SP is differentiated from PV by the absence of *JAK2* mutations and by raised EPO levels

[10,24,25]. SP can be classified as congenital, which are associated with genetic abnormalities, or acquired, usually related to a secondary disorder or external factors that lead to an increase of erythrocyte production [6,9].

Congenital SP are associated with genetic abnormalities which include mutations in genes from the oxygen-sensing pathway that culminate in the increase of EPO levels (e.g. mutations in the *von Hippel Lindau* gene), heterozygous mutations in the prolyl hydroxylase 2 gene or mutations in the hypoxia inducible factor [5,6,24,26]. High oxygen-affinity hemoglobin and rare enzyme deficiencies, such as bisphosphoglycerate mutase deficiency, are other congenital causes that can culminate in SP [5,24].

The causes of acquired SP belong to three main categories: hypoxia, pathological EPO production and exogenous erythropoietin. The hypoxic process may be associated with conditions that limit oxygen availability in the arterial circulation, such as chronic obstructive pulmonary disease (COPD), cyanotic heart disease or obstructive sleep apnea [27,28]. High altitude habitat is another hypoxia-related factor that may lead to the development of SP [5,24,28]. Pathological EPO production is mainly a consequence of renal alterations like local renal hypoxia or tumors. The presence of exogenous EPO may also drive to the over production of RBC and is generally associated with the administration of androgens or corticosteroids or with the use of EPO to increase the red blood cell mass in athletes [24,28].

1.3. Hereditary Hemochromatosis

Hereditary Hemochromatosis (HH) is an inherited autosomal recessive disorder [29] characterized by the progressive accumulation of iron [30] that is caused by dysregulated intestinal iron absorption [31]. Some of the symptoms associated with the disorder include fatigue, depression, weight loss, joint pain, decreased libido, hair loss and abdominal discomfort [32]. The iron deposition in parenchymal organs that takes place in this disease can be responsible for serious organ dysfunctions leading to liver failure and cirrhosis, hepatocellular carcinoma, atherosclerosis, arthritis, various endocrinopathies including diabetes, hypermelanotic pigmentation of the skin, or compromised immune defense [29].

Hereditary Hemochromatosis is considered a disorder with slow progression with typical age of presentation for HH related symptoms usually occurring between 40 and 60 years old for males and after menopause in females [32]. Men tend to develop symptoms and signs of organ injury at a rate of 5 to 10 times higher than women, a particularity that is likely related with the recurrent blood loss in women due to menses and childbirth and varying dietary habits among men and women [31].

The most common type of HH is associated with mutations in the *HFE* gene [29]. The *HFE* gene was first identified in 1996 as a major histocompatibility complex (MHC) class I-like gene in which homozygosity for a missense mutation that results in cystein-to-tyrosine substitution

at amino acid 282 of human HFE protein (C282Y), was responsible for most cases of HH [33]. C282Y is the most prevalent mutation in Western populations affecting approximately one out of 250 individuals [34,35] being much less common in Hispanic, Asian American, Pacific Islander, and black persons [36].

The HFE protein interacts with transferrin (Tf) receptors 1 and 2, senses iron body status, and activates downstream signaling pathways that regulate iron homeostasis [37]. *HFE* mutations result in low levels of hepdicin, a hormone produced in the liver that plays a major role in the regulation of systemic iron homeostasis, acting as a negative regulator of iron absorption from the gut and iron egress from macrophages [31]. The C282Y mutation disrupts a disulphide bond apparently required for proper folding of the HFE molecule, preventing its association with ß2-microglobulin as well as its surface expression [38]. Approximately 80% of HH patients are homozygous for C282Y while only a few cases of patients are heterozygotes for a C282Y and H63D mutation, a second mutation in the *HFE* gene associated with HH [29]. Other factors that affect iron absorption, transport, and mobilization include hepdicin, hemojuvelin, Tf receptor-1 and -2, ferroportin [30], and $CD8^+$ T cells [39]. Mutations in some of these genes (e.g. *haemojuvelin* gene, *hepdicin* gene and *Tf receptor-2* gene) have been associated with non-*HFE*-related forms of HH [1], namely aggressive juvenile subtypes [2]. These mutations are much less frequent than *HFE* mutations. However, in contrast to *HFE* mutations, they are spread in different parts of the world and are not linked to a specific race [35].

Morphological alterations of RBC have been described in hemochromatosis patients and individuals with high serum ferritin levels relative to those from normal controls [40]. RBC from these individuals presented a significantly elongated shape than RBC from controls (Figure 2). Alterations in fibrin network morphology from RBC were also observed. These differences could largely be reversed in the presence of some iron chelating agents [40].

Figure 2. RBC morphological alterations related with iron overload disorders. RBC scanning electron microscope images from a normal healthy individual (A), a HH patient (B), and an individual with high ferritin levels (C). Figure adapted from [40].

1.4. Secondary Hemochromatosis

Secondary Hemochromatosis (SH), also known as secondary iron overload, is mainly associated with recurrent blood transfusions in patients with hereditary or acquired anemia subtypes that arise from mutations in genes that lead to insufficient or malfunctioning erythrocytes, such as myelodysplastic syndrome, thalassemia or sickle cell disease [2]. Although blood transfusions may be able to correct the anemia state in patients with myelodysplastic syndrome, thalassemia, and sickle disease, chronic transfusion therapy remains one of the most important causes of SH [1,41–43].

Other causes of secondary iron overload include liver diseases [44] and ingestion of excessive dietary amounts of iron [42]. The release of iron from injured hepatocytes is one of the main causes of iron overload in chronic liver diseases like hepatitis C, alcoholic liver disease and non-alcoholic fatty liver disease [1]. Hepdicin dysregulation and *HFE* mutations may also contribute to iron overload in these particular diseases, however further studies are necessary to confirm this association [1].

1.5. Therapeutic phlebotomy: implications for RBC anomalies

Phlebotomy, also known as bloodletting or venesection, is a major therapeutic procedure that has been performed in various civilizations since antiquity up to the present [10]. Currently, therapeutic phlebotomy is one of the main treatments in PV, SP, HH and SH. Classical phlebotomy is a simple, safe, and low-cost method [45] that allows reduction of RBC mass and depletion of iron stores. The amount of blood that is removed at each session as well as the phlebotomy frequency depend of the hematocrit values in PV and SP patients and serum ferritin levels in patients with iron overload, but in the majority of the cases approximately 450 to 500 mL of blood are removed at each session [4,10,30].

As most therapeutic approaches, phlebotomy is associated with positive and negative effects. Several studies demonstrated that hepatic fibrosis in HH patients can be reversed by phlebotomy therapy [30]. Also, phlebotomy has been associated with a reduced risk of the development of leukemia, an increased risk of vascular events and a better overall median survival in PV patients when compared to other treatment strategies [9,14]. Some negative aspects referenced by patients undergoing phlebotomy include venous access problems, time wasted (for travel, waiting, and the procedure) and the fact that in many cases the blood is not used for donation [30]. The lack of long term follow-up studies of patients with erythrocytosis that are treated exclusively with therapeutic phlebotomy makes it difficult to assess the clinical benefits of this treatment.

The need of recurrent venesections as the main treatment of iron overload disorders and erythrocytosis, and the fact that this blood cannot be reused for other purposes like blood

donation, makes phlebotomy samples an ideal source for the study of lymphocyte populations.

2. Cross-talk between RBC and T cells

In the past decades evidence has been accumulated supporting the view that RBC are more than just mere carriers of gases and nutrients. Marked alterations in the immune system after transfusions of RBC suggest that these cells may be associated with immunomodulation [46,47]. Previous studies conducted by our group also indicate that RBC play an important role in T cell growth and survival [48,49]. The characteristics of RBC, as well as their functions are further discussed in the subsequent sections of this chapter.

2.1. RBC and erythropoiesis

Erythrocytes represent the most common cell type in adult blood. Human blood contains approximately $5x10^6$ erythrocytes per microliter (normal range $4.7x10^6$ to $6.1x10^6$ for males and $4.2x10^6$ to $5.4x10^6$ for females) and have an average life span of 120 days [50]. In normal adults, approximately 200 billion of the oldest erythrocytes (about 1% of the total number) are replaced every day by an equal number of newly formed erythrocytes [51]. The small size of RBC, usually 6 to 8 μm of diameter, and its biconcave shape creates a large surface area for gas exchange and allows their entry into the micro capillaries of the tissues [50]. Mature RBC in the bloodstream lack the nucleus and cytoplasmic organelles, including mitochondria and ribosomes. As RBC age, their surface area and volume, but not hemoglobin, are progressively lost [52].

Erythropoiesis is a tightly regulated process by which the hematopoietic tissue of the bone marrow produces RBC. It consists of several developmental stages: hematopoietic stem cell, burst-forming unit-erythroid, colony-forming unit-erythroid, proerythroblast, basophilic erythroblast, polychromatic erythroblast, orthochromatic erythroblast, reticulocyte and ultimately to mature RBC [25,51–53]. In humans, the new red cells enter the circulation as reticulocytes that need approximately one week to complete the maturation process [50], which include the complete elimination of traces of DNA, mitochondria, endoplasmic reticulum, and ribosomes [54]. EPO is the main regulator of erythropoiesis [51]. Alterations in this process, like mutations in the hematopoietic cells or the increase of EPO levels, have been associated with the development of classical myeloproliferative neoplasms and SP, respectively, as described previously.

2.2. RBC functions and transfusion-related immunomodulation

Red blood cells have been considered almost exclusively as transporters of metabolic gases and nutrients for the tissues. Currently, a number of clinical and experimental evidence support the notion that these are not the only functions played by erythrocytes. RBC are one of the major components of blood antioxidant capacity and one of the cells more resistant to

oxidative stress [55]. Within inflamed areas, RBC can contribute to detoxify reactive oxygen and nitrogen species and thus to rescue or partially "protect" cells under intense oxidative stress. In some physiological conditions, for instance when RBC become targets of xenobiotics or parasites, or when they pass through tissues where an intense production of reactive oxygen or nitrogen species occurs, RBC can also act as sources of reactive oxygen species [55].

Red blood cells may also play a pivotal role in the regulation of the local vascular processes matching oxygen supply and demand in active skeletal muscle in humans [56–58]. It is believed that RBC contribute to these local regulatory processes releasing vasoactive substances into the vascular lumen in response to several metabolic and mechanical stimuli [57]. Indeed, several lines of evidence have implicated RBC as modulators of nitric oxide (NO) signaling by effecting both NO formation and inhibition of NO signaling [59]. Under hypoxic conditions it has been demonstrated that RBC induce NO-dependent vasodilatation [60]. It is thought that nitrite is reduced to NO in a manner that is regulated by the fractional saturation of hemoglobin and hence regulated by mechanisms that control oxygen affinity [61]. In addition, Srihirun et *al*. reported that nitrite in the presence of RBC had an inhibitory effect on platelet aggregation, but nitrite alone at physiological concentrations did not have the same effect [62]. This inhibitory effect was promoted by deoxygenated hemoglobin [62]. Despite these evidences, the mechanisms of release and potential sources of NO in RBC are still a matter of debate [60], since RBC can act both as NO scavengers and NO producers.

Most of the current knowledge on the influence of RBC in the immune system is the result of the investigation of the consequences of RBC transfusions. Immune mediated reactions to transfusion include hemolytic reactions, febrile non-hemolytic reactions, allergic reactions, transfusion related acute lung injury, bacterial contamination, transfusion related immunomodulation, and transfusion associated graft versus host disease [46]. Transfusion-related immunomodulation consists of both immunostimulatory and immunosuppressive phenomena. Proinflammatory responses, like systemic inflammatory responses and multiple organ failure, are often seen after massive transfusions, whereas the immunosuppressive effects are seen in lower-volume transfusion [47]. Transfusion-related immunomodulation persists despite leukoreduction (removal of the contaminating donor white cells and platelets in red cell concentrates) [63], which implicates RBC in the production of mediators during storage that may be responsible for transfusion related outcomes [64].

Allogeneic (but not autologous) transfusion has been associated with down regulation of cellular immunity and dysregulation of inflammatory innate immunity. Experimental evidence support the hypothesis that immune deviation towards type 2 immune responses and corresponding inhibition of type 1 responses may explain post-operative infection and cancer recurrence after transfusion [64]. Additionally, increases in T regulatory (Treg) cells have

been demonstrated after allogeneic exposure to stored RBC [65] which may explain, in part, the immunosuppression outcomes observed sometimes after RBC transfusion [66].

Recent studies suggest that the consequences of RBC transfusion are dependent of the time of storage of the RBC units [67]. Most storage lesion effects appear to worsen with increased storage duration. Inflammatory response, mediators of oxidative injury and risk of hyper-coagulation appear to increase when RBC with a long period of storage are compared with fresh RBC [68]. The excessive activation of the innate immune system by danger associated molecular pattern molecules generated throughout RBC storage may explain some of the adverse outcomes of transfusion [46,69].

In a study conducted by Bernard et *al*. it was reported that banked human RBC suppressed mitogen-stimulated human and antigen-stimulated mouse T-cell proliferation by mechanisms independent of arginine depletion (associated with the generation of suppressor cells) or cell death [70]. More recently, the same group demonstrated that B cell proliferation was also suppressed by blood bank RBC. They also concluded that T cells and B cells suppression was abrogated when fresh RBC were used [47]. The authors suggested that the lack of suppression observed with fresh RBC could be related with alterations of RBC properties during the isolation procedure [47].

2.3. RBC and T cell growth and survival

Previous studies performed by our group have demonstrated that the presence of RBC in cultures of mitogen-stimulated human peripheral blood lymphocytes was able to increase T cell proliferation and survival [48]. Moreover, the presence of RBC in cultures without serum allowed T cells to proliferate to levels very similar to those observed in cultures with serum, literally rescuing the activated T cells from cell death [71]. The increase in T cell survival was associated with the inhibition of activation-induced T cell death by RBC. This finding was correlated with a decrease in oxidative stress within the activated T cells and the inhibition of the formation of acrolein, a lipid-derived product that function as a marker of oxidative stress. In this case, the RBC protection-effect required the presence of intact RBC and RBC/T cells contact and was independent of monocytes [48]. It is also believed that RBC may have a different impact on $CD4^+$ and $CD8^+$ T cells. Indeed, some studies suggest that RBC support the preferential expansion of $CD8^+$ T cells in cultures [48,72].

Several attempts to identify the molecular mechanisms responsible for the protective effect exerted by RBC on T cells have been performed [73]. It is currently known that RBC are capable to augment the percentage of T cells that enter cell division and also the percentage of cell survival both after T cell receptor-dependent and T cell receptor-independent stimuli [71]. However, many aspects related with the molecular mechanisms associated with the cross-talk between RBC and T cells remain to be elucidated.

3. Lymphocyte anomalies associated with blood dyscrasias: a role for RBC?

Few immunological studies have been conducted in SP and PV. In 1987 Laurence and collaborators reported that $CD4^+/CD8^+$ T lymphocyte ratios were significantly increased in patients with PV. The finding of reduced $CD8^+$ T lymphocytes, and hence increased $CD4^+/CD8^+$ ratios in PV, seemed to be a persistent feature of the condition, remaining uncorrected by previous treatment. They also demonstrated that these alterations were not verified in patients with SP [74].

More recently, alterations in T cell subpopulations have been associated with PV. Zhao et *al*. showed that the percentage of T $CD4^+$ regulator cells ($CD4^+CD25^+FoxP3^+$) in peripheral blood of PV patients was significantly higher compared to healthy controls. Moreover, when co-cultured with activated $CD4^+CD25^-$ T cells, the $CD4^+CD25^+$ Treg cells showed enhanced suppressive function in PV [7]. In a more recent study, an increase in $CD4^+$ Treg cells was also demonstrated in PV patients undergoing long term treatment with interferon alpha. However, this increase was not observed in untreated patients [75]. T cells from PV patients also seem to produce growth factors that contribute to endogenous erythroid and megakaryocyte colony formation [16]. Decrease in natural killer (NK) cells activity in patients with PV was also reported [76].

The immunological studies performed in hereditary and secondary hemochromatosis patients are much more abundant and mostly describe alterations in T cell subsets. Interestingly, like in PV, high $CD4^+/CD8^+$ T cell ratios were also observed in some HH patients. In HH, high $CD4^+/CD8^+$ T cell ratios were associated with high levels of iron overload [77]. Other T cell anomalies reported in HH patients include expansion of $CD8^+CD28^-$ T cells linked with the HLA-A3 haplotype, an increase of the relative percentage of $CD8^+HLA\text{-}DR^+$ cells (a marker of recent activation) and a decrease in the cytotoxic activity of $CD8^+$ T lymphocytes [78]. In another study, Fabio et *al*. described a decrease on total lymphocyte counts of several lymphocyte populations, including $CD3^+CD4^+$, $CD4^+$, $CD28^+$, $CD8^+CD28^+$ and NK cells in HH. They also reported a major increase in the production of IL-4, IL-5 and IL-10 by the $CD3^+CD8^+$ T cell subset [79]. More recently, the low numbers of $CD8^+$ lymphocytes observed in HH were associated with a decrease of the most mature $CD8^+$ effector memory T cells [80].

Besides phenotypic alterations, functional abnormalities in $CD8^+$ T lymphocytes were also described in HH patients. The level of autophosphorylation of the CD8-associated p56lck, a protein kinase that plays a key role in T cell activation, as well as its phosphotransferase activity was significantly reduced up to three-fold in HH patients relative to healthy donors [81]. Interestingly, a recent study performed using transient HFE transfection assays in a model of antigen-presenting cells demonstrate that wild type HFE inhibits T lymphocyte

activation independently of the nature of the peptide being presented by MHC-I molecules and of MHC-I/peptide complex stability when compared to C282Y-mutated HFE [37].

The lymphocyte and RBC alterations found in PV, SP, HH and SH may suggest the existence of a cross-talk between RBC and T cells that may impact the development and survival of lymphocyte subsets. In this context, the study of phenotypic and functional lymphocyte alterations in patients with blood dyscrasias may provide further evidences that can lead to a better understanding of the cross-talk between RBC and T cells as well as the implications of RBC alterations in this interaction.

II. Aims of the study

II. Aims of the study

The main objective of this work was to ascertain if patients with anomalies in RBC numbers and/or cell mass have alterations in the four major lymphocyte populations (T, B, NK and NKT) and whether therapeutic phlebotomy influences these populations. For that purpose, patients with PV, SP, HH and SH, along with a group of healthy blood donors were studied.

In this context, the specific aims of this work were:

1. To analyze lymphocyte populations in patients and controls.

2. To evaluate if RBC from patients influence differently T cell proliferation.

III. Materials and methods

III. Materials and methods

1. Subjects

Seventeen HH patients (14 males and 3 females; mean age 57 years; range 30-77 years), three SH patients (3 males; mean age 61 years; range 48-70 years), three PV patients (3 males; mean age 74 years; range 71-78 years) and ten SP patients (9 males and 1 female; mean age 58 years; range 38-72 years) were selected to this study. Nineteen healthy regular blood donors (12 males and 7 females; mean age 40 years; range 21-55 years) from Centro de Sangue e da Transplantação de Coimbra were used as controls. All buffy coats included in this study were between 2-3 days old.

HH patients were diagnosed taking into account abnormal elevated values of serum Tf saturation (>50% to 55% in men and >45% in women) [31] and serum ferritin (>300 µg/L in men and >200 µg/L in women) [30,31] and the presence of *HFE* mutations. SH patients also presented elevated levels of Tf saturation and serum ferritin, similarly to HH patients. However, the presence of *HFE* mutations was excluded in these patients and the raised iron levels were associated in most of the cases with the presence of liver disorders, recurrent blood transfusions and the ingestion of excessive amounts of dietary iron.

The diagnosis of PV was established according to the World Health Organization diagnosis criteria. These include high hematocrit values (>52% in men and >48% in women) or raised RBC mass (>25% above predicted) and the presence of *JAK2* mutations. In *JAK2* mutations negative PV patients minor criteria like bone marrow biopsy changes, serum EPO below the normal range or *in vitro* endogenous erythroid colony formation are also required [6]. Hematocrit values and RBC mass were also raised in SP patients. However, SP diagnosis was established by the absence of *JAK2* mutations and generally by raised serum EPO. In most cases, SP were secondary pathologies associated with pulmonary disorders, like COPD and emphysema, or with smoking habits.

This study was approved by the Ethics Committee of Centro Hospitalar Cova da Beira and is part of a joint research project between CICS-UBI and the Immunohemotherapy Service from Centro Hospitalar Cova da Beira (projectnº105/2013). Informed consent was obtained from all subjects following the guidelines of local institutions.

2. Reagents and antibodies

Phytohemagglutinin (PHA, from Phaseolus *vulgaris*), bovine serum albumin (BSA), trizma base, ammonium chloride, RPMI-1640 medium, propidium iodide (PI) and the antibiotic-antimycotic solution were obtained from Sigma-Aldrich (Madrid, Spain). Biocoll (density 1.10 g/mL) and fetal bovine serum (FBS) were from Biochrom (Berlin, Germany). 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 Molecular Probes (Amesterdan, The Netherlands). The mouse fluorochrome-conjugated anti-human monoclonal antibodies used in this work and its characteristics are described in Table 1.

Table 1. Description of the mouse fluorochrome-conjugated anti-human monoclonal antibodies used in the study.

Antibody	Clone	Isotype	Conjugate	Company	Dilution
CD3	UCHT1	IgG1 κ	APC	ImmunoTools	1:10
CD3	MEM-57	IgG2a	PE	ImmunoTools	1:10
CD3	UCHT1	IgG1 κ	RPE-Cy5	Dako	1:20
CD4	MEM-241	IgG1	APC	ImmunoTools	1:10
CD4	MEM-241	IgG1	PE	ImmunoTools	1:10
CD8	MEM-31	IgG2a	PE	ImmunoTools	1:10
CD8	BW135/80	IgG2a	PE	MACS Miltenyi Biotec	1:10
CD8	MEM31	IgG2a	PE-Dy647	ImmunoTools	1:20
CD4/CD3/CD8	MT310/DK25/UCHT1	IgG1 κ	FITC/RPE/RPE-Cy5	Dako	1:20
CD19	HD37	IgG1 κ	RPE	Dako	1:20
CD19	LT19	IgG1	FITC	ImmunoTools	1:10
CD20	LT20	IgG2a	FITC	ImmunoTools	1:10
CD28	CD28.2	IgG1 κ	PE-Cy5	Biolegend	1:10
CD56	B159	IgG1 κ	Alexa Fluor 488	BD Pharmingen	1:10
CD56	MEM-188	IgG1	FITC	ImmunoTools	1:10
CD56	B-A19	IgG1	PE	ImmunoTools	1:10
CD235a	HIR2	IgG2b	PE	ImmunoTools	1:10

3. Cells

Buffy coats were obtained from the patients fresh collected peripheral blood bags (therapeutic phlebotomies) after a centrifugation at 2200xg for 15 minutes (Figure 3). Peripheral blood mononuclear cells (PBMC) were obtained from the buffy coats of healthy donors and from the buffy coats of patients by performing a gradient centrifugation at 1000xg for 30 minutes over Lymphoprep or Biocoll (Figure 3). PBMC were washed with 1x phosphate buffered saline solution (PBS) followed by centrifugation at 700xg for 10 minutes. Contaminating RBC were lysed in pre-heated lysis solution (10 mM Tizma base, 150 mM Ammonium Chloride, pH 7.2), for 10 minutes at 37 °C. After RBC lysis, PBMC were washed once again with PBS and centrifuged at 500xg for 10 minutes.

RBC were obtained from the pellet region after Lymphoprep/Biocoll centrifugation or from the pellet region after centrifugation of total blood at 2200xg for 15 minutes and diluted 1:10 in RPMI-1640 (Figure 3). The RBC solution was stored until use for a maximum of one week at 4 °C.

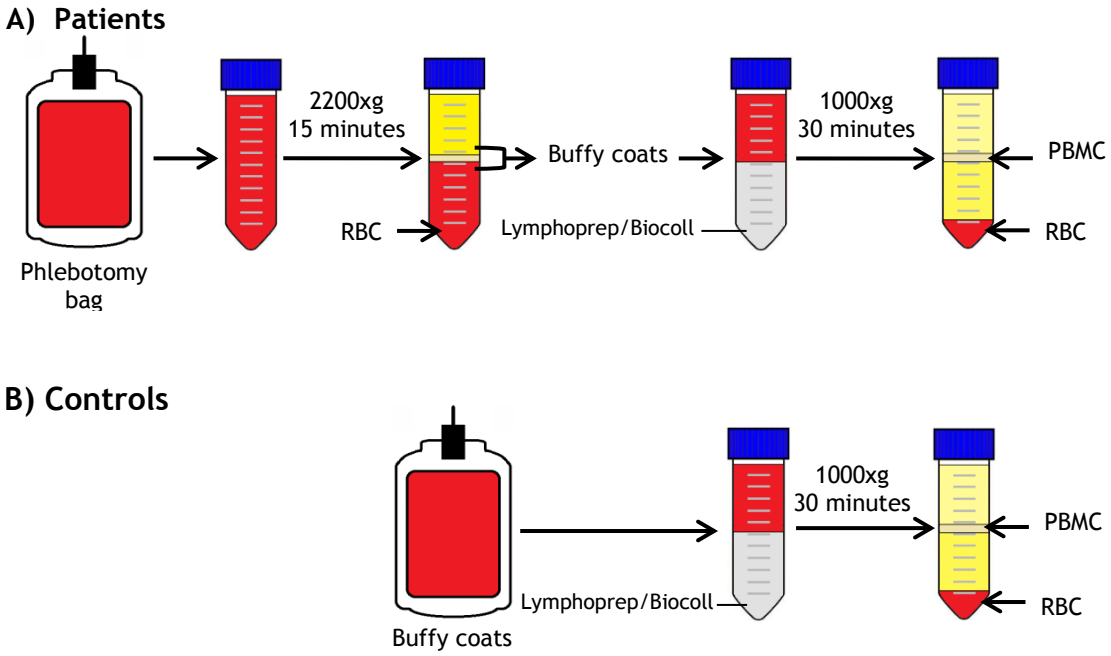

Figure 3. Scheme of PBMC and RBC isolation from peripheral blood samples from patients and controls. The figure shows the process of PBMC and RBC isolation from samples from patients (A) and from healthy donors (B). Buffy coats from patients were obtained after whole blood (phlebotomy samples) centrifugation at 2200xg for 15 minutes. In the case of healthy donors, the blood samples received from Centro do Sangue e da Transplantação de Coimbra were already in the form of buffy coats. PBMC from patients and controls were obtained after a gradient centrifugation of buffy coats at 1000xg for 30 minutes over Lymphoprep or Biocoll. RBC were collected from the pellet region after centrifugation of whole blood or after Lymphoprep/Biocoll centrifugation.

4. Culture conditions

PBMC ($1.5x10^6$) were cultured in 6-well plates in RPMI-1640 supplemented with 1% heat-inactivated FBS and 1% antibiotic-antimycotic solution in a final volume of 5 mL. Cultured cells were left in an incubator at 37 °C, 5% CO_2 and 95% humidity, between 6 to 7 days. PBMC were either left unstimulated or stimulated with 5 µg/mL of PHA, a T cell mitogen, in the absence or presence of autologous RBC. In some experiments PBMC from controls and patients were cultured in the presence of heterologous RBC. All the cultures with RBC were performed at a PBMC:RBC ratio of 1:10. Unstimulated PBMC with no RBC were used as a negative control. Samples (PBMC/RBC) obtained from different phlebotomies of the same patient were considered as independent proliferation experiments.

5. CFSE labeling

CFSE is a fluorescent dye that passively diffuses into cells and forms dye-protein adducts which are retained by the cells until they enter mitosis, where the dye-protein adducts are splitted between daughter cells. For CFSE labelling PBMC were resuspended in PBS at a concentration of $10x10^6$ cells/mL and incubated with 5 µM of CFSE for 10 minutes at 37 °C, with occasional mixing. After labeling, PBMC were washed twice with PBS/20% heat inactivated FBS and resuspended in culture media. CFSE labeling efficiency was analyzed by flow cytometry immediately after label procedure and further confirmed by the analysis of fluorescence from unstimulated PBMC at the end of cultures.

6. Lymphocyte subsets phenotyping and flow cytometry

PBMC or total leukocytes were used for lymphocyte phenotyping. When total leukocytes were used, peripheral blood samples were first treated with RBC lysis solution (10 mM Tizma base, 150 mM Ammonium Chloride, pH 7.2), for 10 minutes at 37 °C. Approximately $0.5x10^6$ PBMC or $2.5x10^6$ total leukocytes (in case of whole blood staining) were placed per well (in 96-wells round bottom plates) and washed twice with staining solution (PBS 1x, 0.2% BSA, 0.1% Sodium azide). Cells were then incubated on ice for 30 minutes, in the dark, with 50 µL of the appropriated diluted anti-human monoclonal antibodies on staining solution. Cells incubated with 50 µL of staining solution (no antibody added) were used as negative control. Afterwards, PBMC or total leukocytes were washed again twice with staining solution and once with PBS. Cells were resuspendend in 150 µL of PBS and placed in FACS tubes at a final volume of 500 µL. After labeling, cells were then acquired in a FACSCalibur (Becton Dickison S.A. Madrid). For each sample 10,000 viable lymphocytes were acquired, whenever possible, using FSC/SSC characteristics and analyzed with CellQuest Pro or CellQuest Software (Becton Dickison S.A. Madrid).

Different combinations of monoclonal antibodies were used in order to discriminate distinct lymphocyte populations (see Table 1). Lymphocyte subsets were defined as follow: $CD8^+$ T cells ($CD3^+CD8^+$), $CD4^+$ T cells ($CD3^+CD4^+$), NK ($CD3^-CD56^+$), NKT ($CD3^+CD56^+$) and B cells ($CD19^+$ or $CD20^+$). The expression of co-stimulatory molecule $CD28^+$ was also investigated among $CD8^+$ and $CD4^+$ T cells. Simultaneous staining with CD3 and CD8 demonstrated that the percentage of $CD8^{bright}$ T cells ($CD28^+$ plus $CD28^-$) approximated the percentage of $CD3^+CD8^+$ T cells. In order to avoid discrepancies between populations, only $CD8^{bright}$ cells were considered as $CD3^+CD8^+$ T cells.

7. Determination of T cell proliferation

T cell activation and division were studied by two methods: (1) determination of cell size and complexity according to FCS/SSC parameters (blasts) and (2) sequential halving of CFSE-fluorescence intensity. T cell survival was also investigated by the determination of cell size

and complexity according to FCS/SSC parameters and PI labeling (5 µg/mL). At the end of the culture period, PBMC cultured with RBC were treated with lysis solution (10 mM Tizma base, 150 mM Ammonium Chloride, pH 7.2) for 10 minutes at 37 °C to remove RBC. This treatment did not significantly alter the activation parameters studied when compared with non-treated activated T cells [48]. Before FACSCalibur acquisition, all cells were washed with PBS and centrifuged at 500xg for 10 minutes. T cell death was determined by PI staining in a couple of experiments.

In some experiments, after the treatment with lysis solution, cells were labeled with fluorochrome-conjugated monoclonal antibodies against CD3 and CD235a (glycophorin A). Glycophorin A is a major protein expressed in the human RBC membrane and it was used in this study to investigate the presence of RBC proteins in the lymphocyte region. For each sample 10,000 events were acquired in the gate encompassing both the "small cells" and "blast cells" regions using FSC/SSC characteristics by flow cytometry and analyzed using CellQuest or CellQuest Pro software.

8. Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics version 21 (SPSS Inc. USA) software. Student's independent samples T-test was used to determine the significance of the differences between group means. Mann-Whitney test was used to determine the significance of the differences between group medians when non parametric variables were compared, namely ferritin. Pearson's correlation was used to assess the correlation between the percentages of lymphocyte populations and patients' clinical data. Statistical significance was defined as $p<0.05$.

IV. Results

IV. Results

1. Clinical data

Relevant clinical data of patients and controls included in this study are summarized in Table 2. Regarding the control population, only age and gender were available. It was possible to observe that the mean age of the four groups of patients was significantly higher than the mean age of the control group. The mean age of PV patients in relation to HH patients was also statistically significant higher. Interestingly, both the percentage of lymphocytes and the total number of lymphocytes, were decreased in PV and SP patients, when compared to patients with HH and SH ($p<0.001$).

Table 2. Relevant clinical data of patients and controls[†].

		Controls [a)] (n=19)	HH (n=17)	SH (n=3)	PV (n=3)	SP (n=10)
Age		40.3±2.5	56.8±2.7**	61.0±6.7*	74.3±2.0**	58.0±2.7**
Gender	Male	12	14	3	3	9
	Female	7	3	0	0	1
Leucocytes	(10^3/µL)	4.0-10.0	7.4±0.7	7.4±1.1	6.5±1.5	7.2±0.6
Lymphocytes	(10^3/µL)	0.8-4.0	2.4±0.1	2.0±0.4	1.1±1.3	1.6±0.2
	%	25.0–40.0	33.8±2.0	27.8±6.4	19.0±6.7	23.4±2.6
Monocytes	(10^3/µL)	0.0–1.2	0.6±0.0	0.8±0.2	0.3±0.1	0.7±0.1
	%	2.0-8.0	9.3±0.9	1.3±0.7	4.8±0.4	9.3±0.8
RBC	(10^6/µL)	4.31-6.40	4.6±0.1	4.8±0.1	5.6±0.5	5.8±0.2
Hematocrit	(%)	39.8-52.0	43.4±1.0	45.2±1.3	53.3±2.4	53.0±1.6
MCV	fL	80.0–97.0	95.9±2.1	93.6±1.6	96.6±8.5	91.1±1.6
MCH	pg	26.0–34.0	32.6±0.8	31.7±0.6	32.3±2.9	30.3±0.7
Reticulocytes	(10^6/µL)	0.03–0.08	0.1±0.01	0.1±0.02	0.1[b)]	0.1±0.01
	(%)	0.50–1.50	1.5±0.20	1.5±0.52	2.0[b)]	1.2±0.15
Iron	(µg/dL)	70.0-180.0	142.4±15.6	124.3±20.9	82.1[b)]	103.4±25.1
Tf saturation	(%)	15.0-45.0	44.9±5.8	32.8±6.7	21.5[b)]	26.9±9.3
Ferritin	(ηg/dL)	21.8-274.7	138.8±11.8	162.3±23.0	119.1[b)]	89.4±29.7

[†] Values represent the mean±SEM. Differences between controls' age and the different groups of patients (Student's T-test) are indicated (**$p<0.001$, *$p<0.05$). [a)] Clinical data from controls was not available, but reference values from healthy male populations are shown, since the majority of patients were men. [b)] SEM is not applicable since this data was only available in one of the patients. MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; n, number of subjects; Tf, transferrin.

Other differences between the groups included higher hematocrit and RBC amount and lower levels of iron, Tf saturation and ferritin in patients with PV and SP when compared with HH and SH patients (Table 2). These differences were confirmed when the four groups of patients were paired in two groups, iron overload (patients with HH and SH) and erythrocytosis (patients with PV and SP). As shown in Table 3, lymphocytes, both total and in percentage, were statistically significant lower in the erythrocytosis group in relation to the iron overload group (1.5±0.1x10^3/μL versus 2.3±0.1x10^3/μL, and 22.4±2.4% versus 32.9±1.9%, respectively). In contrast, hematocrit values as well as RBC amount were statistically significant higher in the erythrocytosis group in relation to the iron overload group (53.0±1.3% versus 43.7±0.8% and 5.8±0.2x10^6/μL versus 4.6± 0.1x10^6/μL). Although the values of iron, Tf saturation and ferritin were higher in the iron overload group, no statistically significant differences were observed among the two groups.

Table 3. Differences of the relevant clinical data among iron overload disorders and erythrocytosis[†].

		Iron Overload (n=20)	Erythrocytosis (n=13)	P value
Age		57.5±2.5	61.8±2.9	NS
Leucocytes	**(10^3/μL)**	7.4±0.6	7.0±0.6	NS
Lymphocytes	**(10^3/μL)**	2.3±0.1	1.5±0.1	**<0.001**
	%	32.9±1.9	22.4±2.4	**0.002**
Monocytes	**(10^3/μL)**	0.7±0.03	0.6±0.1	NS
	%	9.4±0.7	8.3±0.8	NS
RBC	**(10^6/μL)**	4.6±0.1	5.8±0.2	**<0.001**
Hematocrit	**(%)**	43.7±0.8	53.0±1.3	**<0.001**
MCV	**fL**	95.5±1.8	92.4±2.2	NS
MCH	**pg**	33.9±0.2	33.3±0.3	NS
Reticulocytes	**(10^6/μL)**	0.1±0.01	0.1±0.01	NS
	(%)	1.5±0.2	1.3±0.2	NS
Iron	**(μg/dL)**	139.5±13.5	99.8±20.8	NS
Tf saturation	**(%)**	43.0±5.0	26.0±7.7	NS
Ferritin	**(ηg/dL)**	142.5±10.5	94.4±24.8	NS

[†] Values represent the mean±SEM. Iron overload group includes patients with HH and SH; and erythrocytosis group includes patients with PV and SP. P values of the comparison between the two groups are shown (Student's T-test and Mann-Whitney test in the case of ferritin). NS, not significant; n, number of subjects.

2. Phenotypic characterization of peripheral blood lymphocytes

In order to investigate if peripheral blood lymphocyte populations were different among healthy individuals and patients with blood dyscrasias, PBMC or leukocytes from all individuals were labeled with antibodies against selected surface receptors and analyzed by flow

cytometry, as described in material and methods. The expression of the surface markers was then analyzed in the "lymphocyte" region according to the FSC/SSC characteristics, as shown in the scheme of Figure 4.

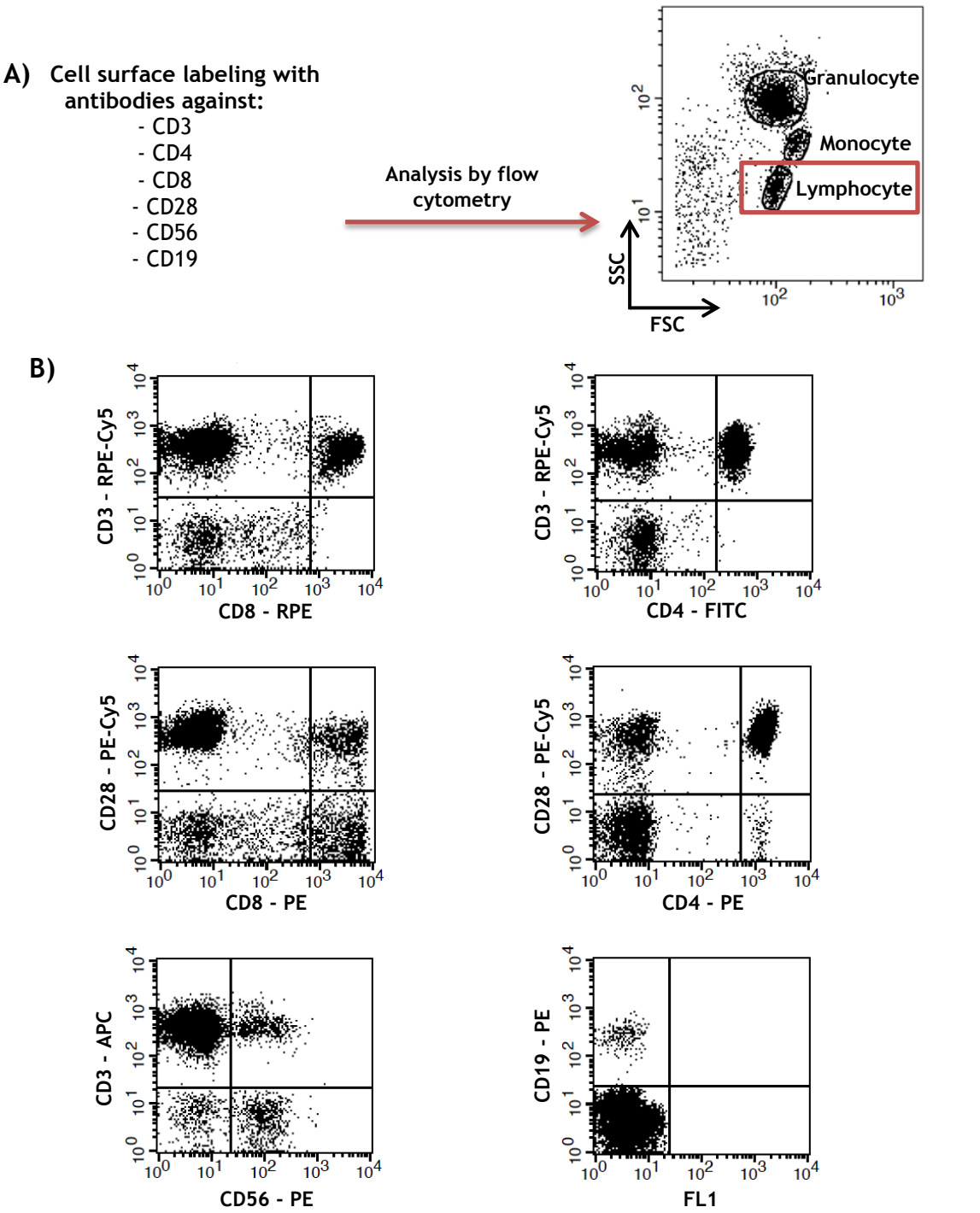

Figure 4. Schematic representation of the flow cytometry analysis of the different lymphocyte populations. As mentioned in materials and methods section, PBMC or leukocytes were labeled with combinations of different fluorochrome-conjugated antibodies against cell surface receptors and analyzed by flow cytometry. The expression of the surface markers was analyzed in the "lymphocyte" region, according to the FSC/SSC characteristics (A). A representative phenotypical experiment based in the expression of the different receptors studied in this work is shown in the dot plots (B).

Based on the surface labeling protocol described in Figure 4, the percentages of nine different lymphocyte populations were determined (Table 4). The percentages of several lymphocyte populations were different between patient groups and controls. Thus, while no significant differences were observed in the percentage of total $CD4^+$ T cells ($CD3^+CD4^+$) between patients and controls, the $CD4^+CD28^-$ population was significantly higher in HH patients (4.0±0.9% versus 1.9±0.3%, p=0.045). Regarding $CD8^+$ T cells ($CD3^+CD8^+$), a statistically significant increase was observed in the SP group when compared to controls (28.2±2.8% versus 20.7±2.0%, p=0.036). Although $CD8^+$ T cells were increased and decreased, in the SH and PV groups, respectively, when compared to controls, these differences did not reach statistical significance (see also Figure 5A). However, statistically significant differences were observed among $CD8^+CD28^+$ T cells, which were significantly higher in SH patients (18.9±1.1%, p=0.006) and markedly decreased in PV patients (6.1±0.2%, p<0.001) when compared to controls (11.2±1.0%) (see also Figure 5B).

Table 4. Total percentages of lymphocyte populations[†].

Lymphocytes	**Controls (n=19)**	**HH (n=17)**	P value	**SH (n=3)**	P value	**PV (n=3)**	P value	**SP (n=10)**	P value
$\%CD3^+CD4^+$	43.5±2.8	48.1±2.0	NS	39.1±5.3	NS	40.3±8.5	NS	48.3±2.8	NS
$\%CD4^+CD28^+$	41.5±2.9	44.2±2.1	NS	36.3±4.7	NS	37.0±9.7	NS	44.4±2.2	NS
$\%CD4^+CD28^-$	1.9±0.3	4.0±0.9	**0.045**	2.7±1.4	NS	3.3±1.8	NS	3.9±1.4	NS
$\%CD3^+CD8^+$	20.7±2.0	24.2±2.1	NS	30.4±4.2	NS	12.3±1.7	NS	28.2±2.8	**0.036**
$\%CD8^+CD28^+$	11.2±1.0	13.6±1.3	NS	18.9±1.1	**0.006**	6.1±0.2	**<0.001**	13.2±1.5	NS
$\%CD8^+CD28^-$	9.5±1.8	10.4±1.8	NS	11.5±5.3	NS	6.2±1.8	NS	15.0±2.0	NS
$\%CD56^+CD3^-$	5.8±0.5	11.5±1.6	**0.003**	9.6±3.2	NS	28.6±7.7	NS	11.3±1.9	**0.020**
$\%CD56^+CD3^+$	3.0±0.8	5.7±1.3	NS	5.1±0.8	NS	3.9±1.6	NS	7.1±2.7	NS
$\%CD19^+$	12.4±1.6	6.0±2.2	**0.021**	4.8±3.3	NS	1.2±0.3	**0.011**	3.8±0.5	**<0.001**

[†] Values represent the mean±SEM. Differences between controls and the different groups of patients (Student's T-test) are indicated. NS, not significant; n, number of subjects.

The most striking anomalies observed between patients and controls were found in two specific subsets, NK cells ($CD56^+CD3^-$) and B cells ($CD19^+$) (Table 4, Figure 5C and 5D). Thus, the percentage of NK cells was elevated in all patient groups. However, this increase was only statistically significant in HH and SP patients (Figure 5C). In contrast, B cells were decreased in all groups of patients when compared to controls. However, this reduction was only statistically significant among HH (p=0.021), PV (p=0.011) and SP (p<0.001) patients, in comparison to controls (Figure 5D). No significantly statistical differences were observed in

the percentage of NKT cells ($CD56^+CD3^+$), despite a marked increase (more than 2-fold) of this population in SP patients (see Table 4).

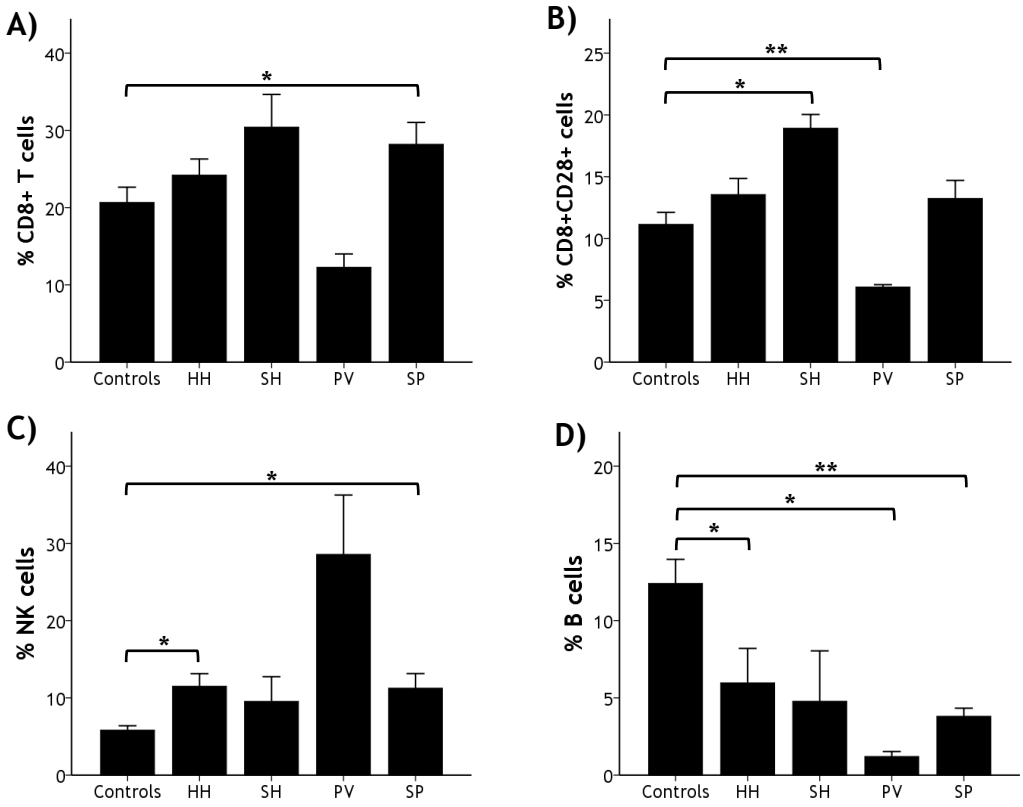

Figure 5. Percentage of lymphocyte populations among patients and controls. The graphs show the comparison between the percentages (mean±SEM) of CD8+ T cells ($CD3^+CD8^+$) (A), $CD8^+CD28^+$ T cells (B), NK cells ($CD56^+CD3^-$) (C) and B cells ($CD19^+$) (D) in HH (n=17), SH (n=3), PV (n=3) and SP (n=10) patients and controls (n=19). P values are shown (**p<0.001; *p<0.05).

Although some differences in the percentage of total $CD4^+CD28^-$ and $CD8^+CD28^+$ T cells were observed (Table 4), the relative percentage of $CD28^+$ and $CD28^-$ lymphocytes within the $CD4^+$ and $CD8^+$ T cell pools patients did not differ between patients and controls (Table 5).

Table 5. Relative percentages of lymphocyte populations[†].

	Controls (n=19)	HH (n=17)	P value	SH (n=3)	P value	PV (n=3)	P value	SP (n=10)	P
%$CD28^+/CD8^+$	58.1±4.6	58.0±4.4	NS	66.2±14.2	NS	51.6±7.9	NS	48.3±4.6	NS
%$CD28^-/CD8^-$	41.9±4.6	42.0±4.4	NS	33.8±14.2	NS	48.4±7.9	NS	51.7±4.6	NS
%$CD28^+/CD4^+$	94.7±1.1	91.7±1.8	NS	93.5±3.5	NS	90.2±5.0	NS	92.0±2.8	NS
%$CD28^-/CD4^+$	5.3±1.1	8.3±1.8	NS	6.5±3.5	NS	9.8±5.0	NS	8.0±2.8	NS

[†] Values represent the mean±SEM. Differences between controls and the different groups of patients (Student's T-test) are indicated. NS, not significant; n, number of subjects.

Finally, we noticed differences between the percentage of two NK populations, obtained by the combined use of CD3, CD8 and CD56 antibodies: $CD3^-CD56^+$ and $CD3^-CD8^+$. Thus, as shown in Figure 6, the percentage of NK cells determined by $CD3^-CD56^+$ was always above the percentage of NK cells determined by $CD3^-CD8^+$. This increase was statistically significant in the HH group (11.5±1.6% versus 6.7±1.6%, p=0.018).

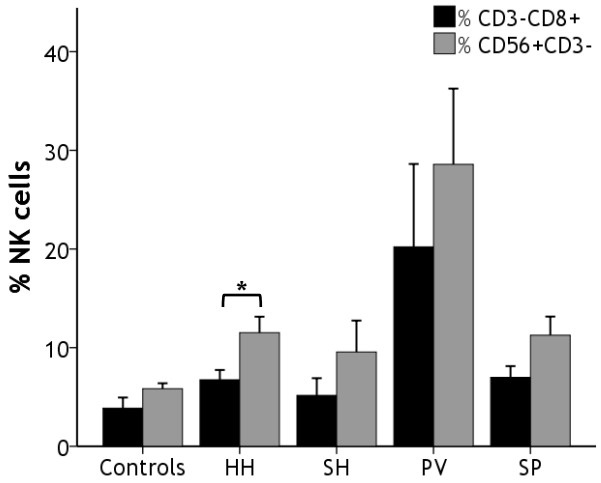

Figure 6. Comparison between the percentage of $CD3^-CD8^+$ and $CD56^+CD3^-$ cells among patients and controls. The graph shows the percentages (mean ±SEM) of $CD3^-CD8^+$ and $CD56^+CD3^-$ cells in controls (n=19), HH patients (n=17), SH patients (n=3), PV patients (n=3) and SP patients (n=10). P values are shown (*p<0.05).

3. Correlations between lymphocyte subsets and clinical data

In order to ascertain whether the differences observed in several lymphocyte populations (e.g. NK, B and $CD8^+$ T cells) could be associated with patients' clinical features we examined for the existence of correlations between these lymphocyte subsets and clinical parameters considering all patients as a whole. Interestingly, we found a negative correlation between the percentage of reticulocytes and the percentage of $CD8^+$ T cells (Figure 7A) and a positive correlation between the percentage of reticulocytes and the percentage of NK cells, as determined by $CD56^+CD3^-$ expression (Figure 7B). It was also observed that the percentage of NK cells was positively correlated with the age of the patients (Figure 7C). No correlations were found between the percentage of $CD8^+$ T cells and patients age (Figure 7D). No further correlations were found between the lymphocyte populations studied and other clinical parameters, namely RBC number, hematocrit, or iron levels (data not shown).

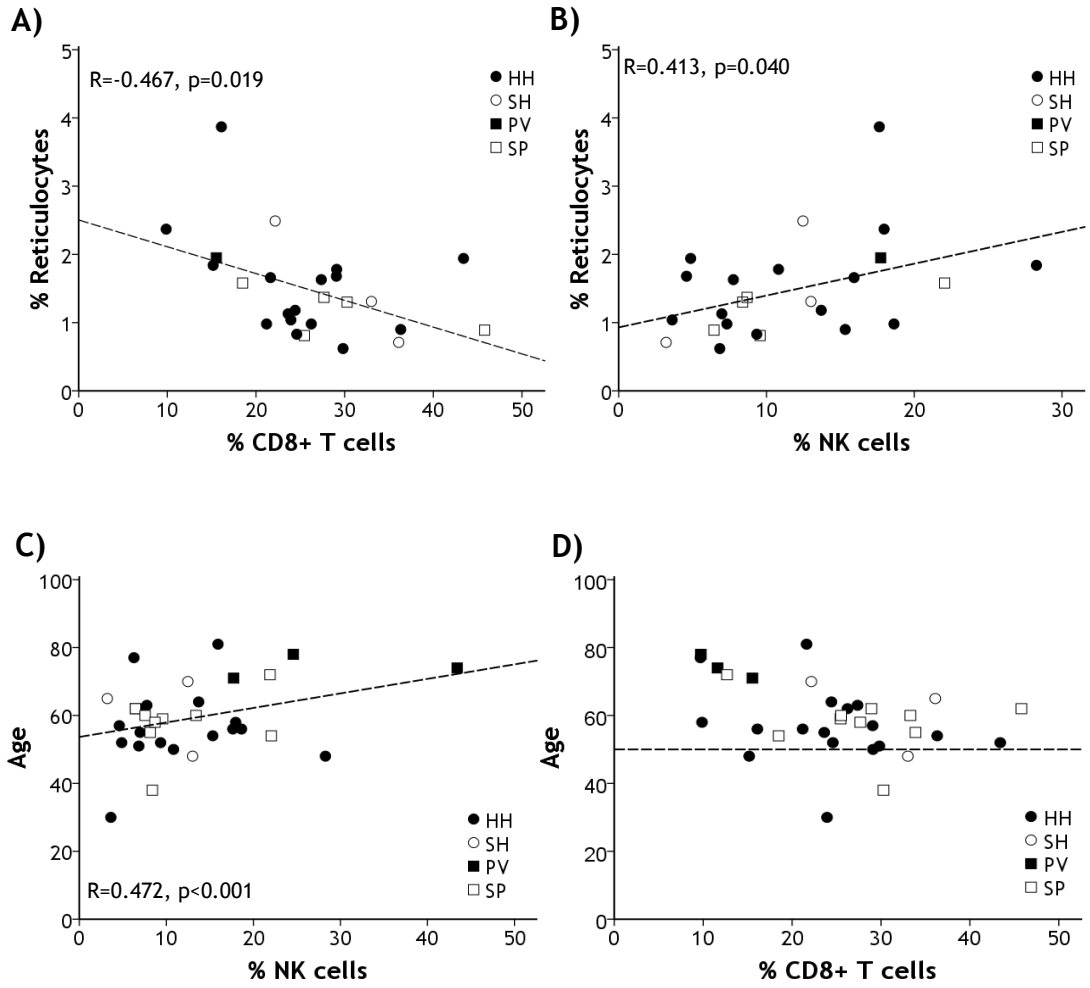

Figure 7. Correlations between clinical data and lymphocyte populations from patients. The graphs show correlations of the percentage of reticulocytes (n=25) with the percentage of $CD8^+$ T cells (A) and the percentage of NK cells ($CD56^+CD3^-$) (B), as well as the correlation of patient's age (n=33) with the percentage of NK cells (C) and the percentage of $CD8^+$ T cells (D). R and p values are shown whenever a correlation is present.

Afterwards, the four groups of patients were again paired in two groups according to iron overload or erythrocytosis. Interestingly, several relevant correlations between clinical data and lymphocyte populations were observed. Thus, correlations between the percentage of monocytes and the percentage of $CD8^+$ T cells (Figure 8A) and the percentage of B cells (Figure 8B) were found among erythrocytosis patients. Other correlations between clinical data and lymphocyte populations included a negative correlation of B cells with the percentage of reticulocytes (Figure 8C) and a positive correlation of NK cells with patients age (Figure 8D). No further correlations were observed.

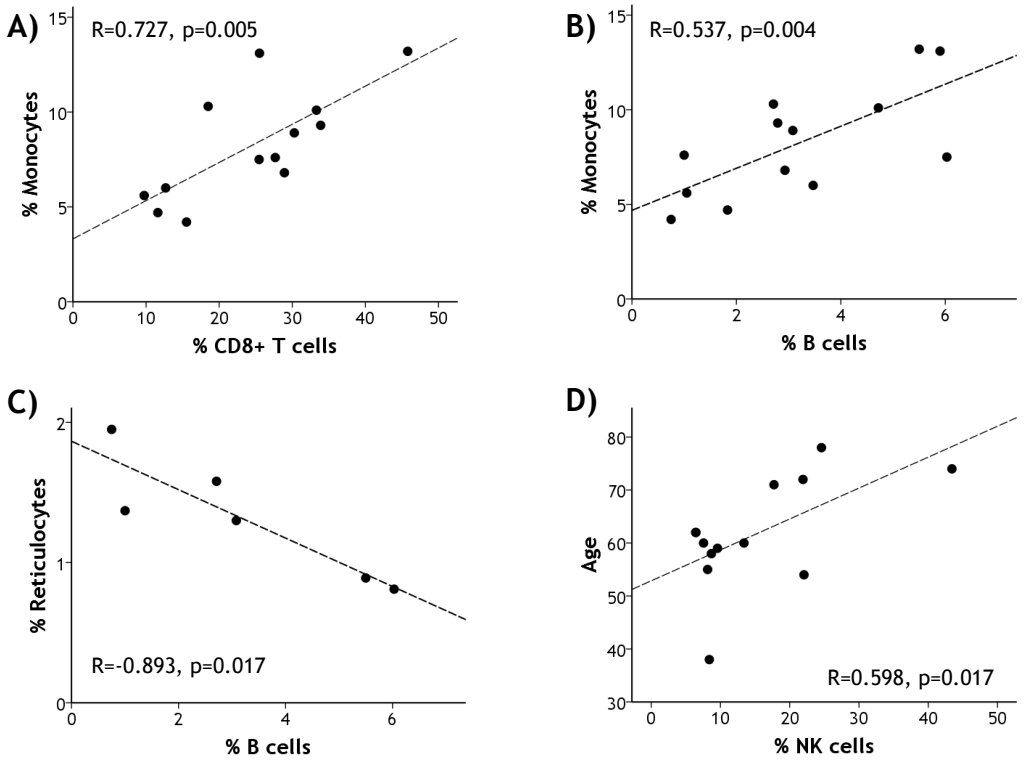

Figure 8. Correlations between clinical data and lymphocyte populations in erythrocytosis patients. The upper graphs show correlations between the percentage of monocytes (n=13) and the percentage of CD8+ T cells (A) and the percentage of B cells (B). Lower graphs show the correlation between the percentage of reticulocytes (n=6) and B cells (C), and between the age of erythrocytosis patients and the percentage of NK cells (D). R and p values are shown.

4. Follow-up studies

In order to assess whether removal of RBC and iron by therapeutic phlebotomy may influence lymphocyte populations, mainly those that revealed major differences in comparison to controls, we performed determinations of lymphocyte populations at each phlebotomy treatment session, whenever possible, following the procedure described in Figure 4. Follow-up studies with at least three determinations and encompassing a period between four and twenty weeks were possible in two HH patients (Figure 9), two PV patients (Figure 10) and three SP patients (Figure 11).

As shown in Figure 9, the percentage of NK cells tended to decrease during the follow-up period, namely in patient HH1, which was followed during 15 weeks (Figure 9A). Regarding B cells, and due to the low percentages of this population verified at the start of the follow-up study, no significant changes were observed during the follow-up period in the two HH patients (Figure 9B).

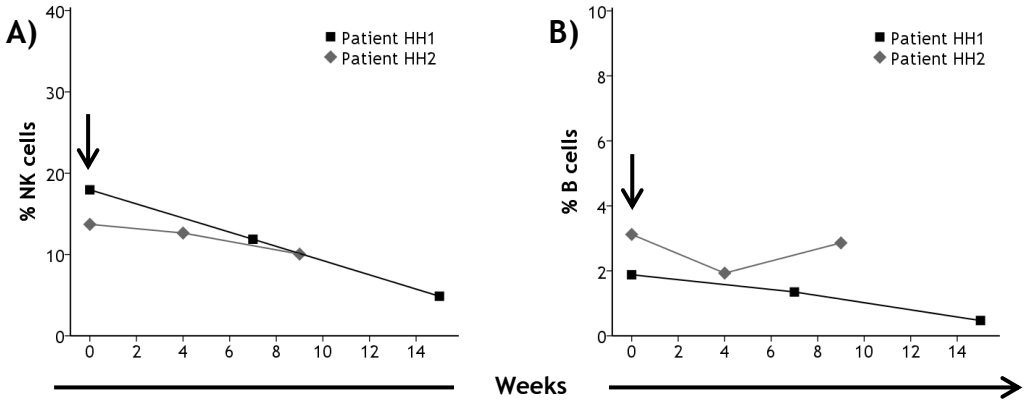

Figure 9. Lymphocyte populations follow-ups in two patients with HH. The graphs show the NK ($CD56^{+}CD3^{-}$) cells (A) and B ($CD19^{+}$) cells variations (B) during the follow-up period of patients with HH. Black arrow indicates the beginning of the follow-up study.

When the percentage of the lymphocytes was analyzed in PV patients, it was obvious that the percentage of NK cells presented opposite trends in the two patients studied (Figure 10C). NK cells tended to increase in the patient with a high percentage in the beginning of the study (patient PV2) while in the other patient it was observed a fifty percent decrease. Moreover, the increase of NK cells seemed to be associated with a decrease in the $CD8^{+}$ T cell population in the patient with a higher percentage of NK cells (Figures 10A and 10C). No obvious trends were observed with regard to $CD8^{+}CD28^{+}$ cells as well as B cells (Figures 10B and 10D, respectively).

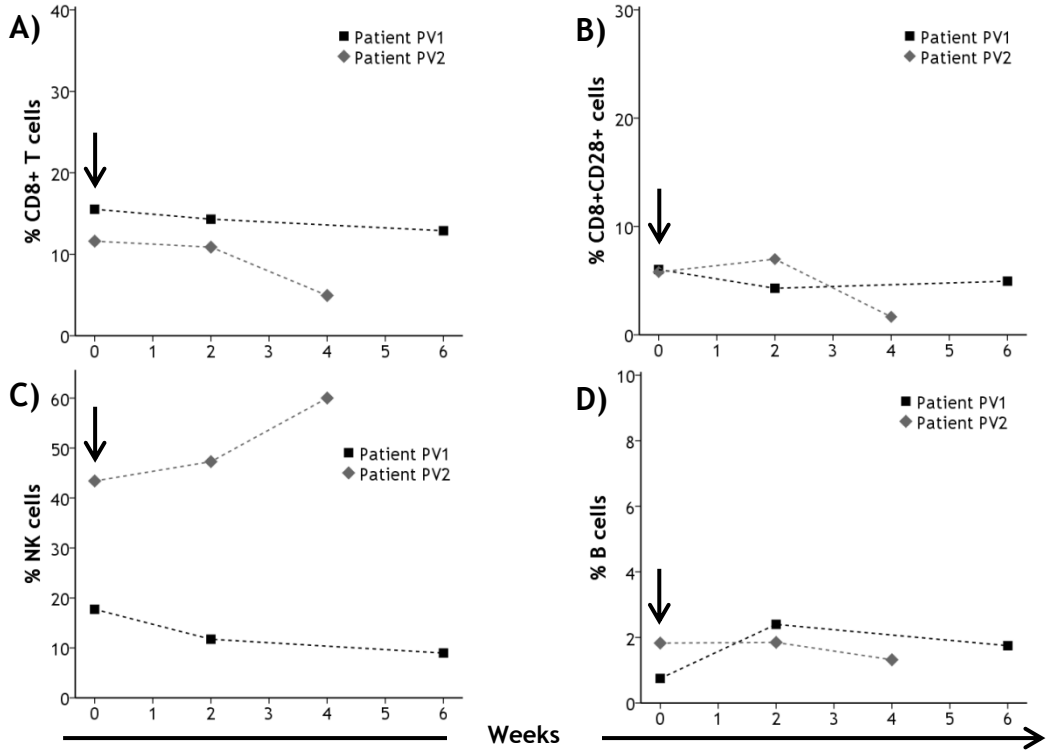

Figure 10. Lymphocyte populations follow-ups in two patients with PV. The graphs show the variation of CD8+ T cells (A), CD8+CD28+ cells (B), NK cells (C), and B cells (D) during the follow-up period of patients with PV. Black arrow indicates the beginning of the follow-up study.

In three SP patients it was possible to perform a longer follow-up study. Overall, the percentage of NK cells appeared to decrease throughout therapeutic treatment in two patients (Figure 11C). The percentages of $CD8^+$ T cells, $CD8^+CD28^+$ cells and B cells were much more inconsistent during the follow-up period (Figures 11A, 11B and 11D, respectively). Despite the increases and decreases observed in these populations throughout phlebotomy sessions the percentage of $CD8^+$ T cells and $CD8^+CD28^+$ cells tended to oscillate within a specific range. Similar values of these populations were verified at the beginning and at the end of the follow up period.

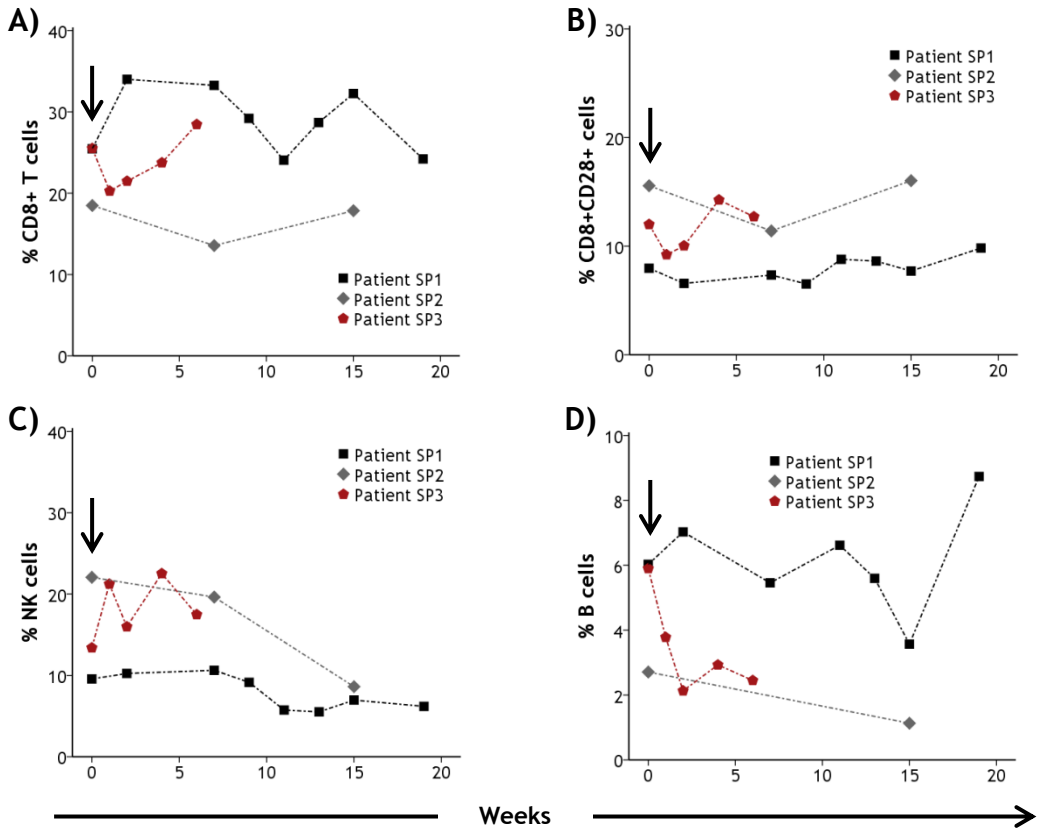

Figure 11. Lymphocyte populations follow-ups in three patients with SP. The graphs show the variation of CD8+ T cells (A), CD8+CD28+ cells (B), NK ($CD56^+CD3^-$) cells (C), and B ($CD19^+$) cells (D) during the follow-up period of patients with SP. Black arrow indicates the beginning of the follow-up study.

5. Effect of RBC on T cell proliferation and survival

In order to ascertain if RBC from patients with varied blood dyscrasias could affect differently the proliferation and survival of T cells, activation experiments with PBMC from healthy individuals and patients in the absence or presence of RBC (autologous or heterologous) were performed. T cell proliferation was determined by CFSE halving after gating in the region encompassing small and blast cells, according to FSC/SSC characteristics, as shown in Figure 12E. The presence of the T cell mitogen PHA in the cultures induced a variable percentage of

PBMC to enter blastogenesis and increase size as well as complexity, but also led to the appearance of a large percentage of dead cells, as determined by FSC/SSC characteristics, when compared to unstimulated cultures (compare Figures 12C and 12A). The presence of RBC in PHA-stimulated cultures increased the percentage of blast cells and survival (Figure 12D).

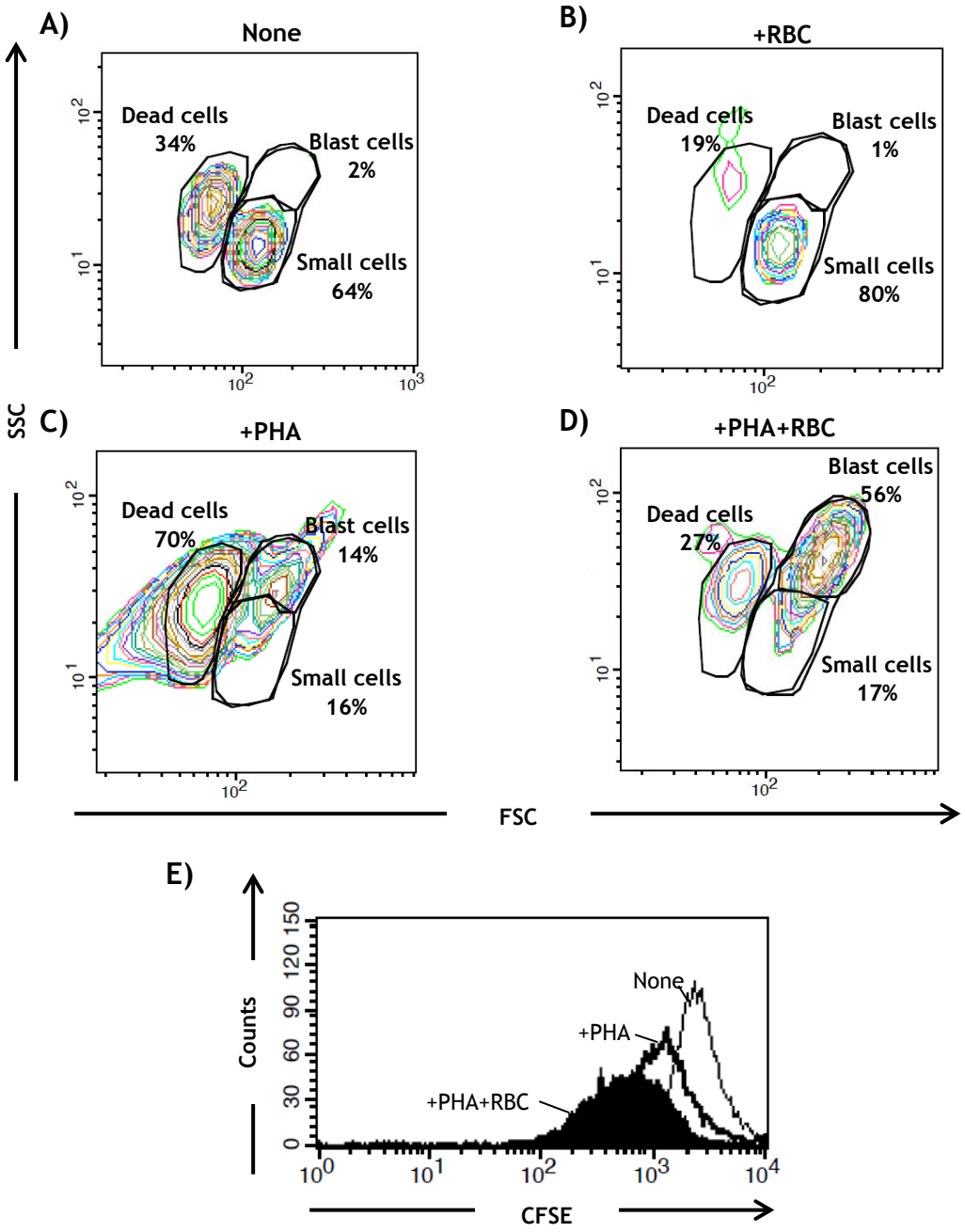

Figure 12. Analysis of T cell proliferation and survival by flow cytometry. Fresh collected PBMC were left unstimulated (A and B) or stimulated with 5 µg/mL of PHA (C and D) and cultured in 6-well plates in the absence (A and C) or presence (B and D) of autologous RBC for 6 to 7 days. The dot plots show the FSC/SCC characteristics of 6 day cultured cells under the different culture conditions used in this study. The histogram shows the loss of CFSE fluorescence of cultured cells in the region encompassing both "small cells" and "blast cells" under the different culture conditions (E).

Importantly, more than 90% of cells analyzed in the region encompassing both small and blast cells were alive, as determined by PI labeling (Figure 13). The percentage of live cells within this region was highest in PHA-stimulated cultures in the presence of RBC. T cells with a decrease in FSC (gated on the left) were mostly positive for PI (dead cells).

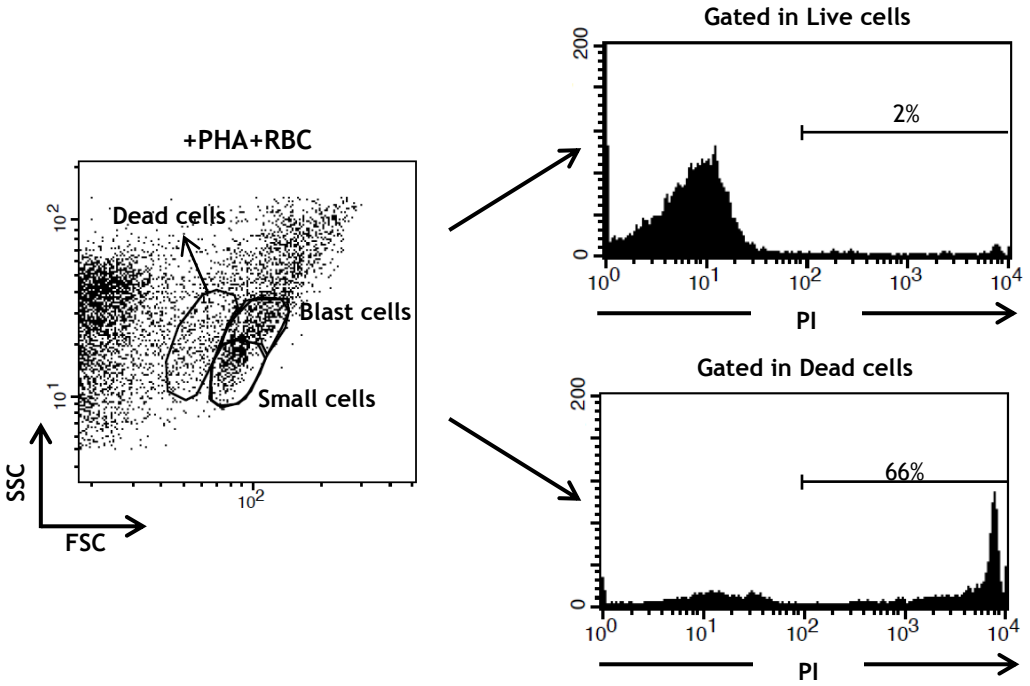

Figure 13. PI labeling of activated T cells. PBMC from a healthy control were activated with 5 µg/mL of PHA in the presence of RBC. After 3 days of culture cells were collected and stained with PI. Cells were analyzed according to FSC/SSC characteristics, (left dot plot) and PI fluorescence determined in the region encompassing both "small cells" and "blast cells" are represented (right histograms).

In terms of proliferation, and as expected from previous studies, the presence of RBC in cultures of stimulated T cells increased proliferation and, consequently, the percentage of dividing cells in all groups under study (see Figure 12E for a representative experiment). When analyzed by groups, the percentage of dividing cells after mitogenic stimulation was higher in HH (32.2±5.8%) and SP (33.2±4.2%) groups when compared to controls (20.8±3.3%), but only in the latter group the difference was statistically significant (p=0.047) (Figure 14A). Similarly, the percentage of surviving cells was slightly higher in HH (28.8±4.1%) and SP (26.1±2.6%) groups in relation to control group (20.8±2.5%) (Figure 14B). The presence of RBC in the PHA-stimulated cultures increased the percentage of dividing cells in all groups (Figure 14C). However, the increase seen in PV group was less evident. Indeed, the percentage of dividing cells in PV patients in the presence of RBC was about two-fold lower than the percentage of dividing cells in the other groups. The percentage of survival of PHA-stimulated cells also increased with the presence of RBC (Figure 14D), an effect that was most evident in the SP group (66.2±3.5%) when compared to controls (49.3±3.7%, p=0.003).

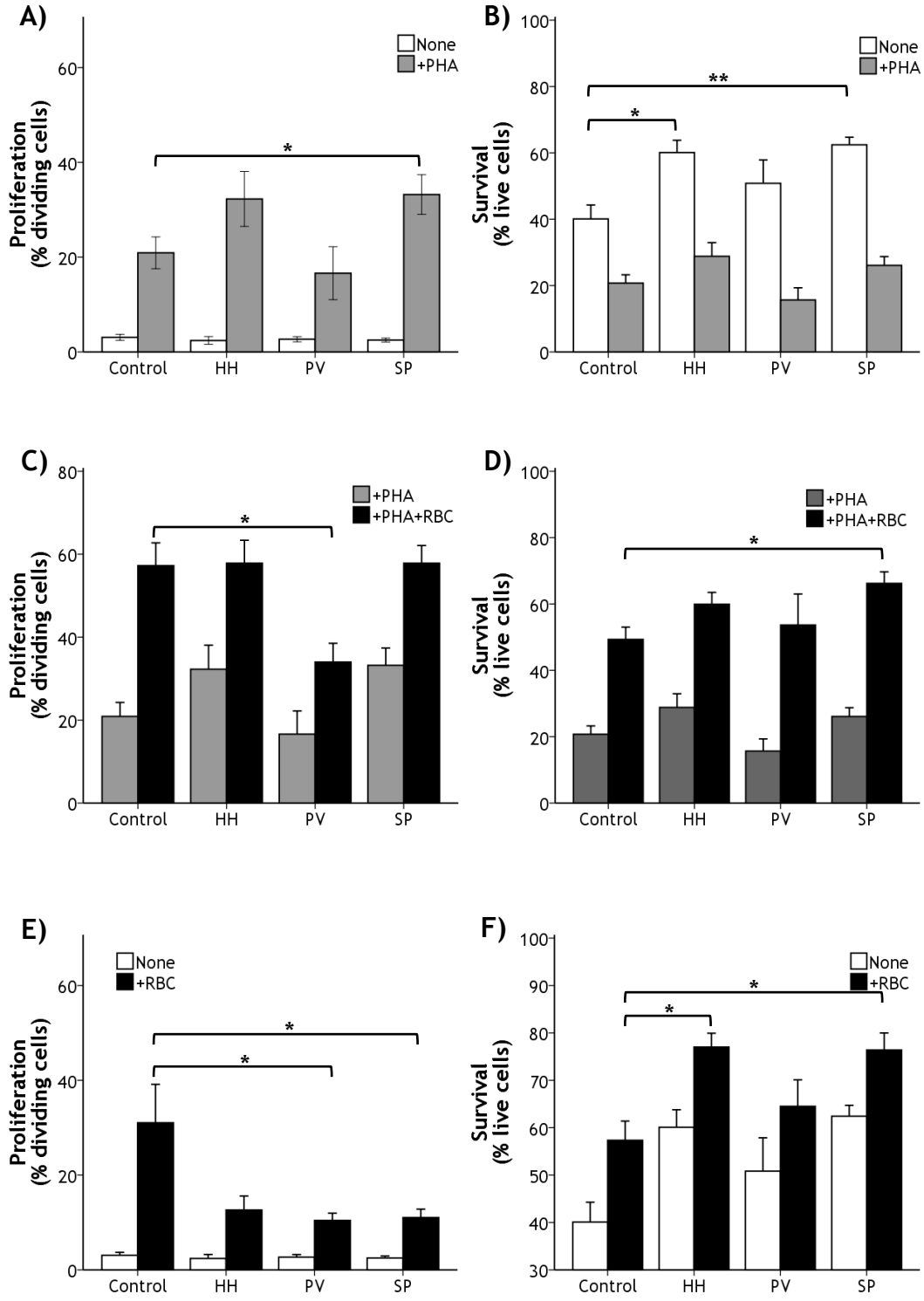

Figure 14. Percentages of proliferation and survival of cultures with PBMC from controls and patients. The graphs show the percentages (mean±SEM) of dividing cells (left graphs) and surviving cells (right graphs) in PBMC from controls (n=12), HH patients (n=12), PV patients (n=6) and SP patients (n=19) cultured under the different culture conditions included in this work. P values are shown (*p<0.05).

Most strikingly, we consistently observed that in unstimulated cultures of PBMC from controls a significant percentage of cells proliferated when cultured in the presence of RBC. These results were also observed in the patients cultures although to a much lesser extent (Figure 14E). The presence of RBC in unstimulated cultures of PBMC also increased cell survival (Figure 14F), an effect that was most evident in PBMC from HH and SP patients. These results were unexpected. Indeed, the dividing cells were exclusively small cells, as determined by FSC/SSC (Figure 15).

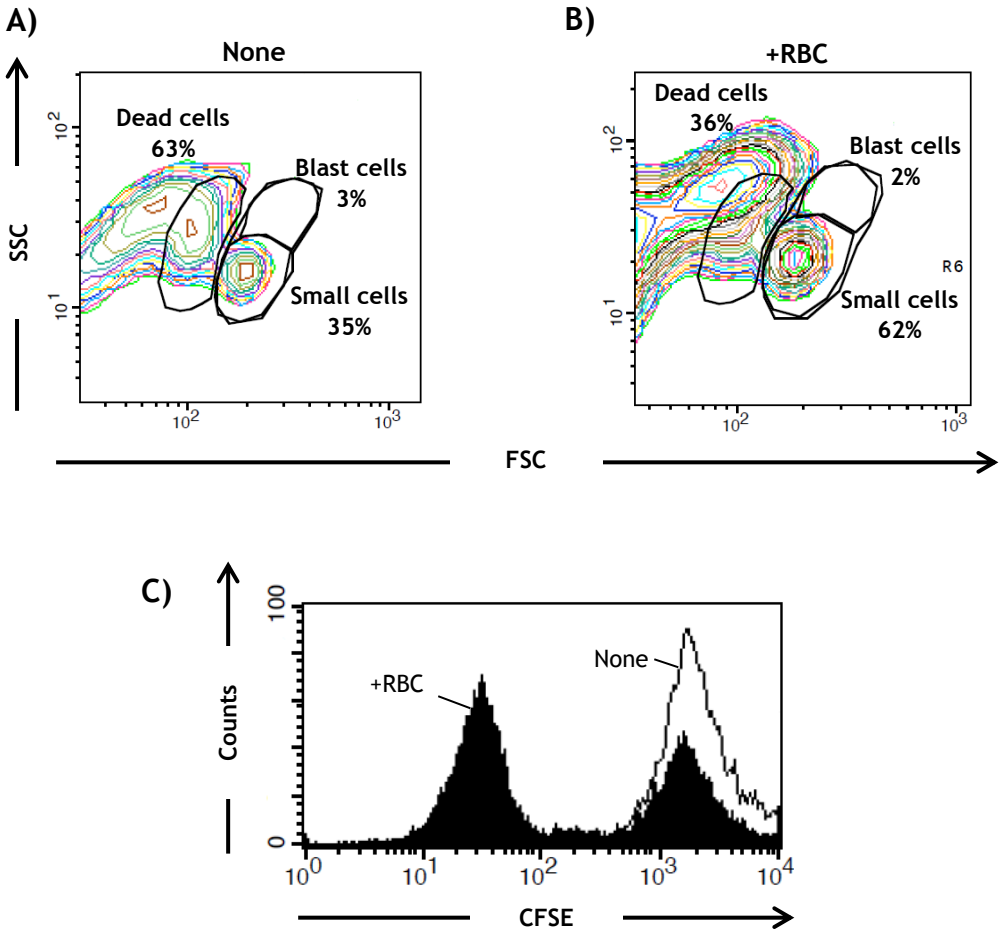

Figure 15. RBC are able to induce cell proliferation in unstimulated cultures of PBMC. Isolated PBMC from a healthy regular blood donor were cultured in the absence (A) and presence of autologous RBC (B), in 6-well plates for 7 days. The dot plots show the FSC/SCC characteristics of 7 day cultured cells under the different culture conditions (A and B). The histogram shows the loss of CFSE fluorescence of cultured cells in the region encompassing both the "small cells" and "blast cells" under the different culture conditions (C).

To make sure whether the dividing cells in cultures of unstimulated PBMC in the presence of RBC were T cells, we labeled cells with CD3 and CD235a (a marker of glycophorin A, present in human RBC membrane) at the end of the culture. As shown in Figure 16 and based on the expression of CD3, proliferating cells were mostly T cells. A small percentage of dividing cells was positive for CD235a.

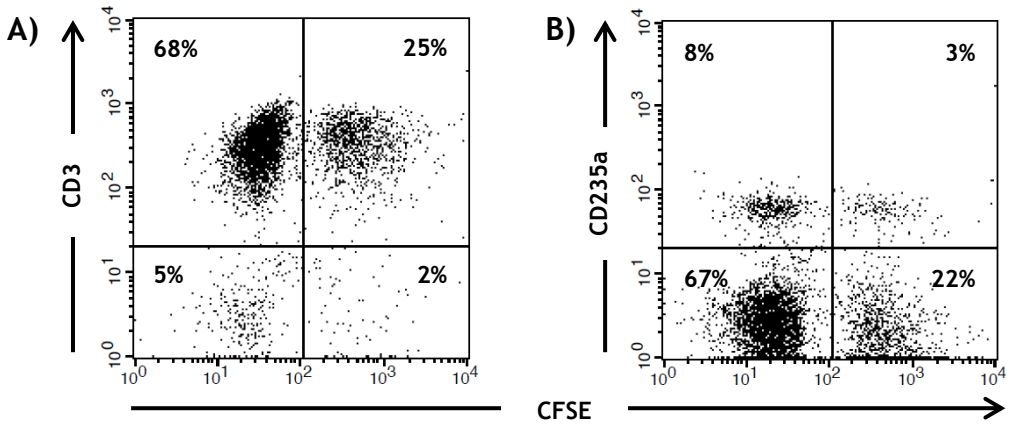

Figure 16. Proliferating cells in cultures of unstimulated PBMC in the presence of RBC are T cells. CFSE labeled PBMC from a healthy donor were cultured in the presence of autologous RBC and without stimulus. At the end of the culture cells were labeled with CD3 and CD235a and acquired in a FACSCalibur. Dot plots show CFSE versus CD3 (A) and CD235a (B) fluorescence. The percentage of dividing T cells (A) ($CD3^+$ upper left quadrant) and glycophorin$^+$ cells (B) ($CD235a^+$, upper left quadrant) are indicated.

In order to evaluate if the effect of RBC isolated from the controls' buffy coats was specific, we performed cross-experiments where RBC from controls were cultured with PBMC from patients and vice versa. The results showed that RBC from control samples were capable to increase the percentage of dividing cells when added to cultures of PBMC from all patient groups (Figure 17A). However, only in the PV group the difference reached statistical significance (p=0.025). No significant differences were observed between the addition of autologous or heterologous RBC to patients cultures in what concerns to cell survival (Figure 17B).

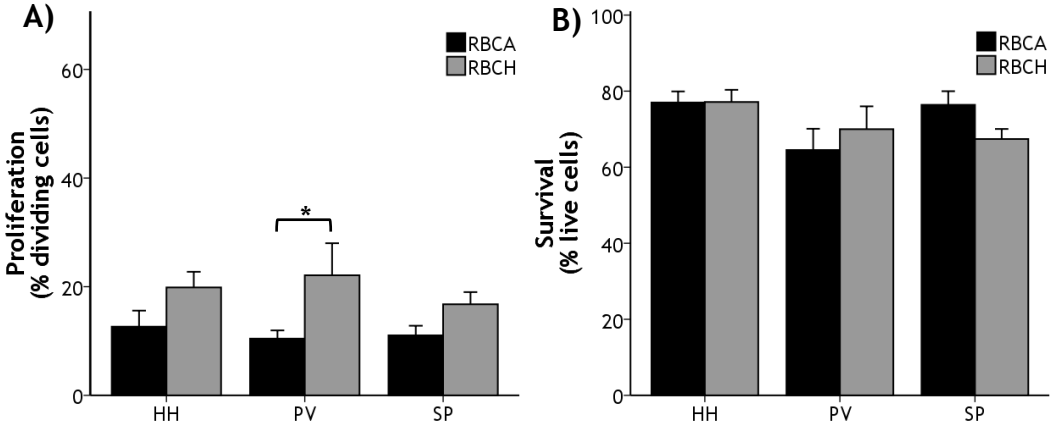

Figure 17. Comparison between the addition of autologous and heterologous RBC to cultures with cells from patients. Isolated PBMC from patients were cultured in the presence of autologous RBC (RBCA) (HH, n=10; PV, n=6; SP, n=18) and heterologous RBC (RBCH) (HH, n=7; PV, n=2; SP n=12) isolated from controls. The graphs show the results of cell proliferation and survival after 6 days of culture, according to the loss of CFSE fluorescence and cells FSC/SSC characteristics, respectively. P values from the comparison between the percentages (mean±SEM) of dividing cells (A) and live cells (B) with the addition of autologous or heterologous RBC to cultures with PBMC from patients are shown (*p<0.05).

The addition of RBC isolated from patients to control cultures was not able to induce the same level of proliferation induced by the autologous RBC (Figure 18A). Indeed, control cells cultured with RBC isolated from SP patients (Figure 18A) exhibited less than three-fold levels of proliferation, when compared to the addition of autologous RBC (p=0.015). In terms of survival, the differences between the addition of autologous or heterologous RBC to the control cultures was not very marked. Only the addition of RBC from HH patients was able to induce a higher percentage of survival when compared to the addition of autologous RBC to control cells (Figure 18B).

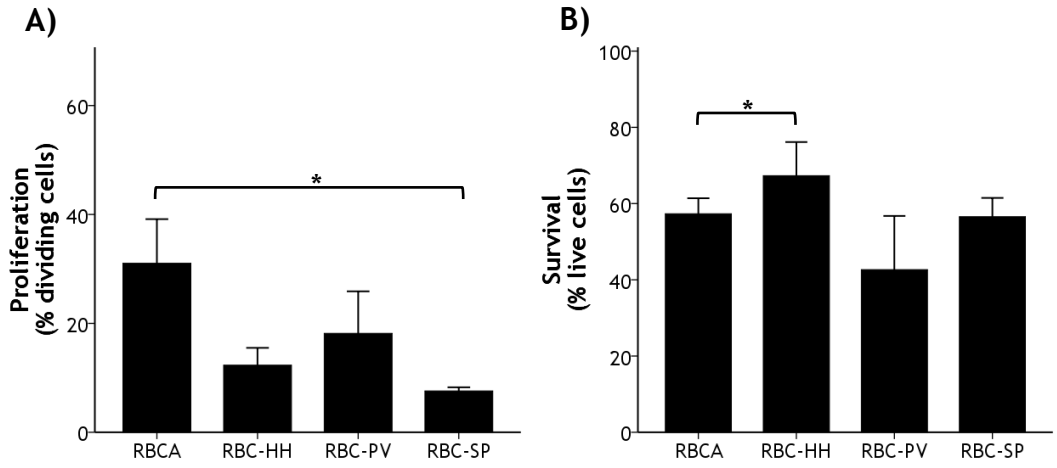

Figure 18. Comparison of the addition of autologous and heterologous RBC to cultures of PBMC from controls. Isolated PBMC from healthy blood donors were cultured in the presence of autologous RBC (RBCA) and heterologous RBC (RBCH) isolated from patients for 6 to 7 days. The graphs show the comparison between the percentages (mean±SEM) of dividing cells (A) and live cells (B) in cultures with PBMC isolated from controls in the presence of autologous (n=12) and the addition heterologous RBC isolated from HH (RBC-HH, n=6), PV (RBC-PV, n=6) and SP patients (RBC-SP, n=9). The percentages of cell proliferation and survival were obtained according to the loss of CFSE-fluorescence and cells FSC/SSC characteristics, respectively. P values are shown (*p<0.05).

V. Discussion

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Previous experimental studies suggest that RBC may influence T cell survival and proliferation in vitro [49,71–73]. The main objective of this work was to ascertain if patients with anomalies in RBC numbers and/or cell mass have phenotypic and functional alterations in lymphocyte populations. In the present study, several alterations in lymphocyte subsets were observed in blood dyscrasias patients, mainly in NK and B cells. The percentage of NK cells ($CD56^+CD3^-$ cells) was increased in all groups of patients when compared to controls, but this increase was only statistically significant in patients with HH and SP. In contrast, the percentage of B cells ($CD19^+$ cells) was markedly decreased in all groups of patients in relation to controls. Other lymphocyte alterations included anomalies in $CD8^+$ T cells. In fact, the percentages of $CD3^+CD8^+$ and $CD8^+CD28^+$ T cells were significantly increased in SH and SP patients, while they were decreased in PV patients. The percentage of NK cells seemed to be positively correlated with the percentage of reticulocytes as well as positively correlated with the age of patients. In contrast, the percentage of reticulocytes was negatively correlated with the percentage of $CD8^+$ T cells. Furthermore, in follow-up studies, the percentage of NK cells apparently decreased with phlebotomy treatment in five out of the seven patients studied. Other main finding of this work was that T cells from PV patients proliferated poorly in response to PHA-stimulus either in the presence or absence of RBC. Unexpectedly, it was consistently observed that RBC were able to stimulate T cells without any mitogenic stimulus. This effect was most striking with RBC obtained from buffy coats from regular blood donors.

The few immunological studies performed in blood dyscrasias patients showed minor alterations in lymphocyte populations. Clinical evidence suggest that the $CD4^+/CD8^+$ T cell ratio is increased in PV patients, mostly due to a decrease in the percentage of $CD8^+$ T cells [74]. In accordance with this study, the percentage of $CD8^+$ T cells in our PV patients was also decreased, although without statistically significance. Yet, we observed a statistically significant decrease in the percentage of $CD8^+CD28^+$ cells in PV patients when compared to controls. Our results also showed a significant increase in the percentage of $CD8^+$ T cells in patients with SP in comparison to controls. To our knowledge, this is the first report describing anomalies in $CD8^+$ T cells in SP patients. Investigations, involving the study of $CD8^+$ T cell populations in HH patients are far more abundant. Although previous studies showed marked decreases in $CD8^+$ T cells in clinically ill HH patients, no significant differences between HH patients and controls were observed in our study, which may be explained by the heterogeneity of $CD8^+$ T cells found within HH patients [39], and the fact that HH subjects included in this study were not distinguished with regard to severity of the disorder, due to the impossibility to access clinical files. Although HH patients showed an increase in $CD4^+CD28^-$ T cells in comparison to controls, the relative percentage of $CD28^+$ and $CD28^-$

lymphocytes within the $CD4^+$ T cell pool did not differed from controls, suggesting that the expression of CD28 remained unaltered within $CD4^+$ T cells.

Perhaps the main lymphocyte alterations found in our work were the alterations observed in the percentage of NK cells and B cells. Previous described alterations in NK cells related with blood dyscrasias include a decrease in NK activity in patients with PV [76] and a decrease in the number of NK cells in C282Y homozygous HH patients [79]. In our study, it was evident that the percentage of NK cells was increased in all groups of patients when compared to controls, mainly in PV patients (more than four-fold, despite a lack of statistical significance perhaps due to the low number of patients studied). Contrary to the report of Fabio et *al*. we showed that the percentage of NK cells was increased in HH patients. The fact that in the study conducted by Fabio et *al*. NK cells were identified by the expression of CD56, CD3 and CD16 cell surface receptors ($CD56^+CD16^+CD3^-$ cells) and that the percentage of NK cells in the control group was about three-fold higher than the percentage of NK cells in our control group [79], may explain the different results obtained. When the percentage of two NK populations, obtained by the combined use of CD3, CD8 and CD56 antibodies ($CD56^+CD3^-$ and $CD3^-CD8^+$), were compared within groups it was possible to observe that the percentage of $CD56^+CD3^-$ was consistently higher than the percentage of $CD3^-CD8^+$ cells, namely in patients, suggesting that a small percentage of NK cells may not express CD8. Former studies have reported the existence of $CD8^-$ NK cells [82], namely in gastrointestinal tract [83].

NK cells are one of the main components of innate immunity and have the ability to kill other cells without any prior stimulation [84]. NK cells may also influence adaptive immune responses thanks to cell receptors as well as the production of cytokines and chemokines [85]. Clinical studies support the notion that the percentage of NK cells vary among elderly subjects, with some studies showing a maintenance of the percentage of NK cell with age [86,87], while others presenting evidence that the amount of NK cells increases with age [88,89]. The number of NK cells in elderly subjects seems to depend on both intrinsic, like killer immunoglobulin receptor (KIR) haplotype [90], and extrinsic, like body mass index, factors [91]. In fact, different NK cell profiles were described among elderly subjects from different countries [92]. In our study, the percentage of NK cells was positively correlated with the age of patients. Taking into account the discrepancies found in the literature regarding an association of NK cells with age, and the fact that our SH patients and PV patients present markedly distinct percentages of NK cells, despite no differing statistically in age, we believe that the increase in the percentage of NK cells seen in PV patients, cannot be attributed per se to the age of patients. Importantly, the positive correlation observed between the percentage of NK cells and the percentage of reticulocytes and the negative correlation between the percentage of reticulocytes and the percentage of $CD8^+$ T cells, may provide clues to explain the increased percentages of NK reported in our study. In this regard, it is important to note that PV has been previously associated with an increase in circulating

reticulocytes [93,94]. On the other hand, the negative correlation found between the percentage of B cells and the percentage of reticulocytes, suggest that lower percentages of B cells are related with higher amounts of reticulocytes. These observations, together with a recent study showing low B cell numbers in patients with alcoholic liver disease, a disorder characterized by increased levels of serum iron, serum ferritin and Tf saturation like in HH [95], suggest that iron overload as well as erythrocytosis may be associated with low B cell numbers as seen in our patients. Whether the decrease in the percentage of B cells in patients with erythrocytosis, mostly in PV patients, may be in part related with the marked increase in the percentage of NK cells remains to ascertained.

Further correlations analyses revealed a positive correlation between the percentage of monocytes and the percentage of $CD8^+$ T cells, as well as a positive correlation between the percentage of monocytes and the percentage of B cells, in the erythrocytosis group. These correlations are intriguing taking into account recent experimental evidence suggesting that macrophages may be involved in the regulation of erythroid development [53]. Indeed, macrophage depletion in mice carrying the *JAK*2V167F mutation reversed some of the key features of PV, including reticulocytosis (increase of reticulocytes), erythrocytosis and elevated hematocrit [95], strongly pointing to this cell type as a regulator of erythrocytosis. The possible involvement of macrophages into the pathogenesis of PV may provide some explanations about the correlations observed between the percentage of monocytes and the percentage of $CD8^+$ T cells and B cells. These correlations may suggest that monocytes (the precursors of macrophages) might positively influence the number of $CD8^+$ T cells and B cells. Furthermore, the need of the presence of macrophages in the bone marrow to the over production of RBC verified in PV pathology, may lead to a decrease of the percentage of circulating monocytes and may explain in part why low percentages of monocytes were associated with low percentages of CD8+ T cells and B cells in our study.

This work also tried to ascertain if therapeutic phlebotomy might influence lymphocyte populations. Due to several restrictions it was only possible to perform follow-up studies during a small period of time, therefore limiting the amount of data that could have been obtained. However, the results observed in five out of the seven patients followed suggest that the percentage of NK cells apparently decrease with the phlebotomy treatment. This may reinforce the positive correlations observed between the percentage of reticulocytes and the percentage of NK cells, which points to reticulocytes as possible modulators of NK numbers. Considering it was impossible to know whether patients followed in this study were only treated with phlebotomy, the possibility that other therapies may have influenced the results cannot be ruled out.

Regarding proliferation experiments, the major findings were observed in cultures with PBMC isolated from patients with PV. Overall, the percentage of proliferating T cells in PV patients was lower than in controls in all culture conditions. However, this decrease was only

statistically significant when T cells were PHA-stimulated and cultured in the presence of autologous RBC. Assuming that the low number of PV patients studied (n=3) has not influenced the results, they might be related with the increase in the percentage of $CD4^+$ Treg cells ($CD4^+CD25^+FoxP3^+$) previously reported in PV patients [7,75] and deservers further investigation. In any case, the possibility that T cells from PV patients are refractory to PHA-stimulation cannot be ruled out.

One major and unforeseen finding in this study was the consistent effect of RBC to induce T cell proliferation in cultures of unstimulated PBMC. Thus, RBC were able to drive T cells into cell proliferation in the absence of mitogenic stimulus with no evidence of an increase in cell size and complexity of proliferating T cells. This effect was most striking when RBC obtained from buffy coats from regular blood donors where added to PBMC cultures. In fact, RBC isolated from controls were able to induce T cell proliferation both in cultures with PBMC from controls and in cultures with PBMC from all groups of patients. CD3 labeling of proliferating cells confirmed that these cells were mostly $CD3^+$ T cells, excluding the possibility that the results were an artefact. In our opinion, two possible hypotheses can be proposed in order to explain these results. The first hypothesis is related with the fact that RBC from controls used in this work were 2-3 days old when they were isolated from the buffy coats, while RBC from patients were freshly collected after phlebotomy. Thus, previous studies have shown that transfusions of RBC stored for extended periods of time are associated with higher morbidity and complications [96]. RBC long-term storage is associated with RBC alterations including a high pro-oxidant state [96], increase of oxygen affinity, progressive depletion of ATP reservoirs and shape abnormalities [97]. Furthermore, recent studies show that stored RBC, but not freshly collected RBC, are associated with T cell suppression [47,98], suggesting that something in blood bank alters RBC properties [98]. Thus, the fact that RBC isolated from controls remain approximately more 2-3 days in blood bags than RBC isolated from patients, suggests that the time of storage and the age of RBC may be important contributors to the effect of RBC observed in our study. The second hypothesis is related with the fact that RBC isolated from controls remain 2-3 days in contact with leukocytes in the form of leukoconcentrates (buffy coats). RBC leukoreduction (removal of the contaminating donor white cells and platelets in red cell concentrates) has been associated with a decrease in mortality after RBC transfusion [99]. Moreover, it is believed that leukocytes release many potentially harmful proinflammatory and prothrombotic mediators during storage, contributing to what is known as “RBC storage lesion” [64]. Experimental evidences also suggest that deleterious effects of aging on banked blood are ameliorated by prestorage leukoreduction [100]. Moreover, the possibilities that the RBC management in blood bank might alter RBC characteristics and that RBC may be exposed to unfavorable conditions during transportation cannot be ruled out as plausible causes of the unexpected RBC effect observed in PBMC cultures reported in this study.

Whether the differences between the storage time of blood samples from patients and controls have influenced the results of proliferation observed remain to be ascertained. In any case the finding that RBC by themselves can drive T cells into cell division is an observation that deserves further investigations.

VI. Conclusions

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In the present work we were able to observe that several lymphocyte populations were altered in patients with blood dyscrasias in comparison to controls. The main findings reported herein include an increase of the percentage of NK cells and a decrease in the percentage of B cells in all groups of patients. Other lymphocyte alterations included anomalies in $CD8^+$ T cells. In fact, the percentages of $CD3^+CD8^+$ and $CD8^+CD28^+$ T cells were significantly increased in SH and SP patients while they were decreased in PV patients when compared to controls. It was also seen that the percentage of reticulocytes seemed to be negatively correlated with the percentage of $CD8^+$ T cells and positively correlated with the percentage of NK cells. NK cells were also positively correlated with the age of patients. Follow-up studies suggested a possible decrease of the percentage of NK cells with phlebotomy treatment. Finally, the results from the proliferation experiments performed with PBMC isolated from patients suggest that T cells from PV patients proliferate less in the presence or absence of RBC in vitro. Unexpectedly, it was consistently observed that RBC themselves were able to induce T cell proliferation. This effect was most striking when RBC obtained from buffy coats from regular blood donors where added to PBMC cultures.

VII. Future perspectives

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Further immunological studies are necessary in order to confirm our results and to elucidate if there are alterations in other lymphocyte populations besides the ones that were included in this work, namely Treg subsets. The study of lymphocyte populations within patients groups according to the stage and severity of the disorders as well as the increase of the sample size is crucial to a better understanding of the lymphocyte alterations reported in our preliminary study. It is essential to perform follow-up studies during a longer period of time to assess if therapeutic phlebotomies influence lymphocyte populations and if this treatment is able to correct some of the lymphocyte anomalies reported in this work. Since the percentages of NK cells and B cells seem to be altered in patients with blood dyscrasias it may also be important to ascertain the expression of different cell surface receptors, like inhibitory and activation markers, within these populations. In the future, it is fundamental to perform phenotypic characterization of T cells after the culture period in order to understand which T cell populations benefit more from the presence of RBC in patients with blood dyscrasias. More importantly, and based on the evidences of the effect of the addition of RBC isolated from controls to cultures reported herein, it is imperative to perform extensive studies addressing the implications of RBC storage in T cell proliferation and survival. Furthermore, it is essential to characterize the population of proliferating cells that seems to benefit more from the addition of RBC.

VIII. References

VIII. References

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