

**BIOCHEMICAL AND FUNCTIONAL ANALYSIS OF THE T
CELL GROWTH AND SURVIVAL BIOACTIVITIES OF
HUMAN RED BLOOD CELLS**

RICARDO FILIPE DA SILVA ANTUNES

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Preceitos legais

De acordo com o disposto no nº 2 do artigo 8º do Decreto-lei nº 388/70, nesta dissertação foram utilizados os resultados dos trabalhos publicados abaixo indicados. No cumprimento do disposto no referido Decreto-lei o autor desta dissertação declara que interveio na concepção e execução do trabalho experimental, na interpretação de resultados e na redacção dos resultados publicados sob o nome **Antunes RF**:

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Summary / Sumário

Summary

A growing body of evidence points to red blood cells (RBC) as immunoregulatory cells. Thus, apart from being carriers of oxygen and carbon dioxide, RBC display other important biological activities towards a diversity of cells, including fibroblasts, endothelial cells and dendritic cells. On the other hand, scattered evidence obtained during the past three decades suggested a specific role for RBC in the modulation of T cell responses. Although, this has been an understudied area of research worldwide, the potential physiological significance of the immunoregulatory role of RBC described *in vitro* motivated this thesis. Indeed, recent *in vitro* studies in our laboratory have shown that RBC are capable of modulating T cell growth and survival by keeping activated T cells alive and into the cell cycle. However the elucidation of the molecular mechanisms underlying these bioactivities remained unclear. In order to expand our understanding on the immunoregulatory role of RBC, the present thesis was carried out on the assumption that secreted factors might mediate the RBC bioactivity towards activated T cells. For this propose, we used an *in vitro* model where *ex vivo* human peripheral blood T cells were activated with different types of stimuli and cultured in the presence of intact RBC, RBC-conditioned medium (RBC-CM) or its particulate and non-particulate fractions obtained after ultracentrifugation, namely the vesicle-containing pellet fraction (RBC-ves) and the vesicle-free supernatant fraction (RBC-sup). In this thesis, we show that the RBC bioactivities act on intracellular pathways initiated by both TCR/CD3-dependent and -independent stimuli and activate the Akt signalling pathway. Analysis of parameters of T survival, growth and proliferation revealed that RBC carry out bioactivities capable of preserving the antioxidant status of T cells, markedly downplaying apoptosis and further driving T cells to cell cycle progression. Importantly, it was shown that RBC bioactivities are sensitive to the immunosuppressant drug cyclosporine A (CsA). Moreover, we have demonstrated that the RBC bioactivities do not require physical contact between RBC and T cells but are mediated by vesicle-free factors that are released upon RBC culture, instead. Due to the growth-like and survival promoting activities the factors mediating the RBC bioactivities were termed EDGSF (erythrocyte derived growth and survival factor). Our findings show that the EDGSF are thermolabile proteins and do not include lipid compounds, and proteomic characterization revealed multiple hemoglobin isoforms together with peroxiredoxin II and globin chains, suggesting that a complex may be formed. Taken together, the results presented in this thesis, shed new insights into the biology of RBC and its immunoregulatory properties towards activated human T cells. By interfering in the balance between T cell death and survival, it is plausible to think that

proteins secreted by RBC may play an unforeseen role in pathological situations characterized by anomalies in the control of T cell growth and survival, namely in leukemia, by sustaining malignant cell growth and survival.

Sumário

Estudos recentes sugerem os eritrócitos (RBC) como células capazes de desempenhar funções imunoreguladoras. Além do seu papel no transporte de oxigénio e dióxido de carbono, os RBC desempenham outras importantes actividades biológicas, que são exercidas sobre vários tipos celulares, em que se incluem fibroblastos, células endoteliais e células dendríticas. Por outro lado, estudos realizados nas últimas três décadas, sugerem um papel específico para os RBC na modulação das respostas dos linfócitos T. Apesar de esta ser uma área de investigação pouco estudada em todo o mundo, a eventual importância fisiológica do papel imunoregulador dos RBC motivou a elaboração desta tese. De facto, estudos recentes *in vitro* realizados no nosso laboratório, demonstraram que os RBC são capazes de modular a sobrevivência e o crescimento de linfócitos T através da manutenção constante da sobrevivência e da progressão no ciclo celular de linfócitos T activados. Contudo, a elucidação dos mecanismos moleculares subjacentes a estas bioactividades permanece desconhecido. Com o objectivo de aprofundar o conhecimento do papel imunoregulador dos RBC, o presente estudo foi realizado de acordo com a hipótese de factores secretados poderem mediar a bioactividade exercida pelos RBC sobre os linfócitos T activados. Com este propósito, foi utilizado um modelo *in vitro*, em que linfócitos T *ex vivo* de sangue humano periférico foram activados com diferentes estímulos e cultivados na presença de RBC intactos, meio condicionado de RBC (RBC-CM) ou as suas fracções vesicular (RBC-ves) e não vesicular (RBC-sup) obtidas após ultracentrifugação. Deste modo, demonstrou-se que as bioactividades dos RBC actuam sobre vias de sinalização intracelulares iniciadas por estímulos quer dependentes, quer independentes dos receptores TCR/CD3 e, especialmente, sobre a via de sinalização Akt. A análise dos parâmetros de sobrevivência, crescimento e proliferação de linfócitos T revelou ainda que os RBC são portadores de bioactividades capazes de preservar o estado antioxidante dos linfócitos T, nomeadamente através da diminuição da apoptose e do aumento da capacidade de progressão no ciclo celular. Foi igualmente demonstrado que as bioactividades dos RBC são sensíveis à droga imunossupressora cyclosporina A (CsA), e que estas bioactividades não requerem contacto físico entre os RBC e os linfócitos T, sendo antes mediadas por factores não vesiculares presentes no RBC-sup. Devido às actividades promotoras de sobrevivência e crescimento, os factores que medeiam as bioactividades dos RBC foram designadas de EDGSF (factor de sobrevivência e crescimento derivado de eritrócitos). Os nossos resultados demonstram que o EDGSF são proteínas termolábeis que não incluem compostos lipídicos e, uma profunda caracterização proteómica, revelou a presença de múltiplas isoformas de hemoglobina juntamente com peroxiredoxina II e cadeias de

globina, sugerindo a existência de um complexo entre ambos. De modo geral, os resultados presentes nesta tese revelam novas evidências sobre a biologia dos RBC e as suas propriedades imunoreguladoras sobre linfócitos T humanos activados. Por interferirem no balanço entre a morte e a sobrevivência dos linfócitos T, é plausível sugerir que proteínas secretadas pelos RBC possam desempenhar um papel inopinado em situações patológicas caracterizadas por anomalias no controlo da sobrevivência e crescimento de linfócitos T, nomeadamente em leucemias, através da sustentação da sobrevivência e do crescimento das células malignas.

Abbreviations

2D DIGE	Two dimensional differential in-gel electrophoresis
AICD	Activation induced cell death
APC	Antigen presenting cell
BSA	Bovine serum albumin
CFSE	5-(and-6)-Carboxyfluorescein diacetate succinimidyl ester
CsA	Cyclosporine A
CypA	Cyclophilin A
EDGSF	Erythrocyte derived growth and survival factor
FACS	Fluorescence activated cell sorter
FBSi	Inactivated fetal bovine serum
FKBP	FK506 binding protein
Hb	Hemoglobin
Ig	Immunoglobulin
IL	Interleukin
mAb	Monoclonal antibody
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MS	Mass spectrometry
mTOR	Mammalian target of rapamycin
NFAT	Nuclear factor of activated T cells
NF-kB	Nuclear factor-kappa B
PBL	Peripheral blood lymphocytes
PBMC	Peripheral blood mononuclear cells
PHA	Phytohemagglutinin
PI3K	Phosphatidylinositol 3-kinase
Prx	Peroxiredoxin
Rapa	Rapamycin
RBC	Red blood cells
RBC-CM	Red blood cells conditioned medium
RBC-ves	Red blood cells vesicles
RBC-sup	Red blood cells supernatant
T-ALL	T cell acute lymphoblastic leukaemia
TCR	T cell receptor
TRIM	Transfusion-induced immunomodulation

Chapter 1

General Introduction

1. INTRODUCTION

Preface

The overall size of the T cell pool is tightly regulated so as to remain relatively constant in normal individuals. Numerous factors are responsible for maintaining T cell homeostasis (i.e. normal T cell numbers), including cytokines and growth factors as well as interactions with cells of the immunological system (e.g. dendritic cells). Signals transmitted by the T cell receptor (TCR), costimulatory receptors, and cytokine/growth factor receptors are integrated in a dynamic manner and result in the activation of a myriad of intracellular signalling molecules that regulate proliferation, cell survival and cell cycle progression. Interactions with so-called non-immunological cells (e.g. fibroblasts, epithelial cells, endothelial cells, etc.) and factors by them secreted are also thought to be important in maintaining T cell homeostasis. In this context, red blood cells (RBC) have been implicated since the early 1970s in the modulation of T cell responses both *in vitro* and *in vivo*, a modulator capacity that has been extended to other cells (e.g. fibroblasts, endothelial cells, dendritic cells). RBC have, therefore, emerged as a novel cell type displaying important biological activities towards a variety of cell types and have begun to be the subject of reinforced attention.

1.1. RBC as carriers of biological activities

Red blood cells constitute the largest cell pool in the blood, with $3\text{--}5 \times 10^{13}$ circulating RBC in the human body. Adult RBC are produced in the bone marrow during erythropoiesis, stimulated by the hormone erythropoietin (EPO) produced in the kidney. During this process, the erythroid progenitor develops and synthesizes large amounts of hemoglobin (Hb). Then, the nucleus of the erythroblast is removed, being the non-nucleated cell designated as reticulocyte. This cell further loses its remaining organelles, acquires the biconcave shape, with 6 to 9 μm in diameter and becomes a mature RBC. As it lacks the ability to synthesize new proteins, its lifetime is limited to 120 days. Old or senescent and damaged RBC are removed from circulation by cells of the reticuloendothelial system, mainly spleen macrophages and liver Kupffer cells [1-3]. RBC plasma membrane like other cellular membranes is comprised by a lipidic bilayer, carbohydrates (glycoproteins) and proteins. The glycoproteins bind to sialic acid molecules that are negatively charged. This negative charge (Z-Potential) avoids RBC aggregation in the circulation. Moreover, glycoproteins and carbohydrates constitute the ABO antigen and other surface antigens [4].

The primary function of RBC is considered to be oxygen (O_2) transport from pulmonary capillaries, at high O_2 tension, to tissue capillaries, where it is exchanged for the metabolically generated carbon dioxide (CO_2). The most abundant intraerythrocytic protein, the Hb, is the carrier of O_2 and CO_2 throughout the body, accounting for more than 90% of the cellular dry weight and approximately 98% of the overall cytoplasmic protein content. Adult Hb contains four globin subunits, normally 2 alpha and 2 beta chains, each surrounding a core of heme-moiety. During oxygenation, Hb undergoes a transition from the low-affinity (“tense”) structure to the high-oxygenated (“relaxed”) structure that has a lower capacity for binding CO_2 [3]. A fraction of Hb is membrane bound in an association with the N-terminal cytoplasmatic domain of AE1/Band3, one of the major integral proteins of the erythrocyte membrane [5]. At the centre of the heme is iron that is essential for gaseous transport. Although the large majority of erythrocyte iron (Fe^{2+}) is co-ordinately bound to the protoporphyrin moiety of heme, RBC also have some “free” cytosolic Fe^{2+} [6].

Besides their fundamental function of O_2/CO_2 transport and its limited biosynthetic repertoire, RBC interact with and transport a range of other substances [protons, chloride ions, organic phosphates and nitric oxide (NO)] that modulate tissue O_2 supply [7]. Importantly, RBC are resistant to oxidant-induced damage, due to a very efficient antioxidant defense system that combats free radical-induced oxidative damage to cellular macromolecules. Indeed, RBC have the capacity to scavenge reactive oxygen and nitrogen species (ROS and RNS) due to the membrane permeability to oxygen and nitrogen radicals and to the presence of high levels of intracellular antioxidant enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GSH-PX), peroxiredoxins and catalase [8-11]. Membrane-bound proteinases, a secondary antioxidant defence mechanisms is also responsible for RBC damage protection, by preferentially degrading the oxidatively damaged proteins [12,13].

Due to its simple machinery RBC are frequently considered inert cells. However, a number of recent studies have provided important data pointing to RBC as a dynamic cell type actively engaged in reciprocal crosstalk with surrounding and neighbouring nucleated cells. Thus, a new scenario has emerged where RBC bioactivities other than O_2/CO_2 transport can be displayed upon interactions with several cell types, influencing biological processes related with proliferation, survival, aggregation or cytokine secretion, among others. A summary of these biological bioactivities is presented in Table I.

Table I. Biological activities displayed by RBC on immune and non-immune cells

Cell Type	Biological effect	Proposed mechanism(s)	References
Fibroblasts	Inhibition of proliferation and stimulation of apoptosis	Unknown	[14]
	Stimulation of IL-8 secretion	Soluble factors (?)	[15]
	Increased secretion of metalloproteinases	Unknown	[16]
Endothelial Cells	Regulation of vascular homeostasis and blood flow	Sequestration and release of nitric oxide	[17]
Platelets	Inhibition of aggregation	Regulation of the redox state by S-nitrosohemoglobin	[18]
Neutrophils	Inhibition of apoptosis	Catalase and glutathione scavenging	[19]
	Activation/Priming	Soluble factors (?)	[20]
Eosinophils	Enhancement of Leukotriene C ₄ release	Unknown	[21]
	Regulation of trans-endothelial migration	Scavenging of RANTES	[22]
Dendritic Cells	Inhibition of IL-12 secretion	CD47 interaction with SIRP α	[23]
B cells	Enhancement of antibodies secretion	LFA-3 – CD2 interaction	[24,25]
NK cells	Enhancement of NK cytotoxicity	Peroxisredoxin	[26]
T cells	Enhancement of cytokine secretion and IL-2R expression	Non-specific interaction between membrane domains on erythrocytes and CD2 antigen on T cells	[27]
	Rolling and adhesion	Induction of mechanical forces	[28]
	Enhancement of proliferation, cell growth and survival	Unknown	[29]

Although the ability of RBC to regulate some of these biological processes has been attributed, in part, to the capacity to scavenge ROS and RNS due to efficient antioxidant system, the variety and complexity of some of these processes suggest alternative mechanisms used by RBC. Indeed, some studies have provided mechanistic insights to explain some of RBC pleiotropy. For instance, under low O₂ pressure RBC inhibit human platelet activation and aggregation. The regulation of the redox state by the NO transported within RBC (S-nitrosohemoglobin) was shown to mediate this effect [18]. RBC also possesses the ability to regulate vascular endothelium homeostasis in part due to the binding and release by hemoglobin of the endocrine vasoregulator NO [17]. Moreover, inhibition of IL-12 production by dendritic cells depends on the expression of CD47 on

RBC and of its ligand SIRPalpha on dendritic cells [23]. However, despite these few exceptions, knowledge on the mechanisms used by RBC is scarce and in some cases unknown or merely speculative. Interestingly, while in some cases the RBC bioactivities are displayed towards quiescent cells [19,28], in other cases cell activation as a result of triggering of cell surface receptors is mandatory [18,29]. Overall, the accumulated data implicate mature circulating RBC as carriers of bioactivities that are displayed as result of interactions or through close proximity with other cells.

1.2. The immunoregulatory role of RBC

Among the more studied bioactivities of RBC are those displayed towards T cells. Indeed, *in vitro* and *in vivo* studies performed during the last four decades point to RBC as potential modulator of T cell functions. Despite the body of knowledge accumulated, and the fact that RBC and T cells are regular partners *in vivo*, either in blood circulation, in the spleen, in the liver or in places of injury/trauma/inflammation, where vasodilatation or rupture of blood vessels occurs (Fig. 1.1), this theme has remained unfortunately unnoticed and/or disregarded. In spite of this overlook, the possibility that under some of the aforementioned *in vivo* situations RBC could embark on intercellular communication with T cells is a possibility worth to be explored.

1.2.1. Evidences *in vivo* – Insights from blood transfusions

Blood transfusion, used to restore normal blood cell counts, is a widely used and often essential therapy for critically-ill patients. It had their origins in the correction of anemia, and it is nowadays used in conditions of acute blood loss as well as in patients with inadequate bone marrow production or increased breakdown of erythrocytes [30]. Review of the available literature shows controversial results, regarding the immunoregulatory effect of blood transfusions, with some studies showing that may be efficacious while others do not. Nevertheless, it is nowadays strongly recognized that they act upon the immune system through the development of the so-called transfusion-induced immunomodulation (TRIM). Clinical evidences shown that TRIM can cause both activation (e.g. increasing T cell activation) and suppression (e.g. decreasing levels of rejection and improving transplantation outcomes) of the immunological system [31,32]. These effects could last for months or even longer and have been consistently observed.

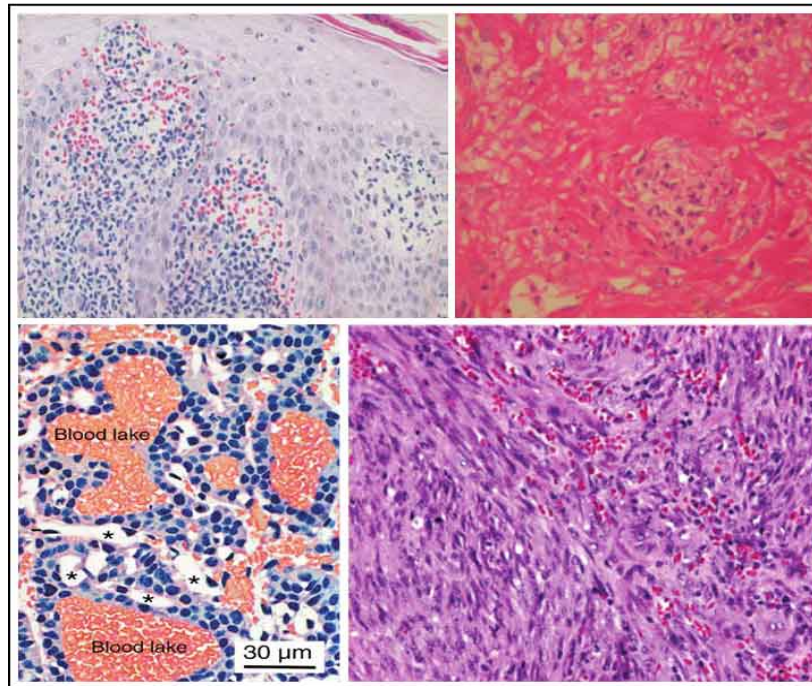


Figure 1.1. RBC extravasion in several pathologies. *Upper left:* Photomicrograph of a purpuric lesion. Perivascular and intraepidermal infiltration of atypical lymphocytes and erythrocytes is visible (HE stain, original magnification x 200) (adapted from [33]). *Lower left:* Histological section of tumor showing blood vessels (asterisks), with blood lakes contain erythrocytes because of lack of connection with the bloodstream (adapted from [34]). *Upper right:* Leukocytoclastic vasculitis characterized by dense erythrocyte extravasation, around small blood vessels (HE stain, original magnification x 400) (adapted from [35]). *Lower right:* Plaque stage Kaposi sarcoma. Large numbers of intracellular and extracellular eosinophilic hyaline globules and red blood cell infiltration in the interstitial spaces (HE stain) (adapted from [36]).

Blood transfusions are associated with a pro-inflammatory response, as suggested by its mechanistic role in multisystem organ failure, transfusion-related acute lung injury or lung inflammation, and transfusion related acute-graft-versus-host-disease [37-40]. Blood transfusions also have immunosuppressive effects, which have been observed since it was first reported by Opelz and colleagues in 1973 when pre-transplantation blood transfusion improved renal-allograft survival [41]. The reported increase in CD8⁺ T cell numbers, along with the decrease in CD4⁺ T cell numbers, observed in multitransfused patients [42] could be related with the immunosuppression induced by RBC transfusions [29]. Indeed, studies carried out by Gafter *et al.* reported the appearance of suppressor CD8⁺ T cells following a single blood transfusion [43]. RBC transfusions were also shown to influence B cell numbers and immunoglobulin secretion in different clinical settings [44,45]. Importantly, blood transfusions have been associated with other immune regulatory effects, namely diminished survival in cancer patients, increased cancer recurrence, decreased severity of autoimmune disease and increased infection risk [46-49].

Despite the evidence accumulated, the immunomodulatory mechanism of the blood transfusion remains insufficiently elucidated [30,31]. The most acknowledge view is that allogenic leukocytes (and leukocyte-derived soluble mediators) present in the RBC preparations are the cell type responsible for the immunomodulatory effect of blood transfusion, namely immunosuppression [50,51]. However clinical trials of leukoreduction have shown conflicting results [52,53]. Thus, the likely possibility that RBC could mediate the TRIM effect could not be ruled out [54,55]. Indeed, experimental and clinical studies point to RBC as the cell type responsible for the immunosuppressive activity of blood transfusions *in vivo* [56]. Recent studies by Baumgartner and colleagues suggest that the transfusion related immunosuppression could be in part mediated by RBC by inducing a population of regulatory T cells [57]. Another study from the same group, points to RBC as capable to potentiate pro-inflammatory cytokine response from peripheral blood mononuclear cells, partially explaining the immune activation seen clinically after blood transfusion [58]. Moreover, erythrocyte chemokine scavenging was shown to be involved in RBC transfusion-related lung inflammation [40]. In other studies, arginine depletion by the RBC enzyme arginase has been suggested as a novel mechanism for transfusion associated immunosuppression [59,60]. Finally, a role for RBC storage conditions and the RBC aging to the side effects of blood transfusions has also been suggested [61,62]. Indeed, several authors have suggested that transfusion of RBC units that have been stored for longer periods of time may not be as beneficial as transfusion of RBC units stored for shorter periods of time in terms of immunomodulation and inflammatory complications, due to time-dependent cellular lesions in the blood components, namely in the RBC.

1.2.2. Evidences *in vitro* (1961-1992) – Early observations

The immunomodulatory activity of RBC *in vitro* was reported even before the earliest clinical observation that blood transfusions had an effect in the regulation of the immune system. A pioneering work published by Carstairs in 1962, preceding the *in vivo* blood transfusions observations, settled the starting point for the experimental *in vitro* verification of the immunomodulatory effect of RBC in activated T cells. While studying transformation of human lymphocytes *in vitro* he noticed that proliferation induced by the T cell mitogen phytohaemagglutinin (PHA) was increased in the presence of autologous RBC [63]. This effect was also noticed by other authors upon experimental verification of a decrease of proliferation after RBC withdrawal from cultures [64,65]. Other studies performed later, started to support the *in vivo* observations that pointed out RBC, or at least the blood

transfusions, as modulators of the immunological system. These included reports about the influence of RBC in cultures of peripheral blood lymphocytes (PBL) in T and B cell activities, such as lymphocyte proliferation, cytokine secretion [27], antibody synthesis [66] and natural killer (NK) cell activity [67].

Despite the preliminary nature of some studies and the lack of conclusive evidence, several authors concluded that the effect of RBC was due to the erythroagglutinating activity resulting from the conditions used to stimulate lymphocytes [68,69]. In contrast, other groups proposed that interactions between LFA-3/CD58 present on RBC and CD2, present on T cells was responsible for the RBC effect on T cells [24,27,70]. These proposed mechanisms were ruled out by subsequent studies described below.

1.2.3. Evidences *in vitro* (1999-2004) – Recent observations

In 1999, a series of comprehensive studies conducted by Arosa's group aiming at characterizing the effect that RBC have on several aspects of T lymphocyte biology demonstrated that this cell type modulate T cell growth, survival and proliferation *in vitro*. Thus, the presence of RBC in cultures of stimulated peripheral blood lymphocytes (PBL) enhanced T cell proliferation and survival by reducing oxidative stress, rescuing dividing T cells from activation-induced cell death (AICD) and favouring T cell division [71,72]. Additional studies concluded that the RBC bioactivities were exerted preferentially on CD8⁺ T cells, were independent of molecular interactions between CD58 and CD2, and were not mediated by monocytes or B cells nor heme or iron [29,72,73]. Importantly, RBC upregulated ferritin and heme oxygenase 1 (HO-1) protein levels and the relative amount of the labile iron pool (LIP) in dividing human T cells [74]. It could be argued that the increased expression of ferritin and HO-1, two cytoprotective proteins, provide a survival advantage to the activated T cells allowing them to divide continuously without significant cell death. Other authors have shown that RBC are capable to enhance cytokine secretion by mitogen activated peripheral mononuclear cells and concluded that the presence of RBC could favour a more physiological environment for activated T cells to respond to stimuli [75]. Although these studies strengthened the immunoregulatory role of RBC, reinforcing the view of RBC as cells capable of exerting immunoregulatory functions [29], the molecular mechanisms underlying the RBC effect remained unsolved. How RBC sustain T cell growth and survival? In other words, how are RBC bioactivities delivered to T cells?

1.3. RBC and the vesiculation process

It is now well documented that nucleated cells release membrane-derived vesicles (also called exosomes) resulting from the fusion of multivesicular endosomes with the plasma membrane. These exosomes have been implicated in the modulation of the function of other cells, including immune cells. Indeed, *in vitro* and *in vivo* studies support the notion of exosomes as an acellular mode of communication, leading to intercellular transfer of molecules [76]. Exosomes could display important regulatory functions in different physiological processes. Thus, follicular dendritic cells were shown to acquire MHC class II molecules by capturing B cell exosomes [77]. Dendritic cell-derived exosomes were also shown to be involved in the indirect or direct activation of T cells [78-80]. Interestingly, exposure of APCs to PS-expressing exosomes and liposome has been implicated in the transient induction of tolerance by down-regulation of the immune response [81,82]. In addition, cancer cells also produce exosomes and may play an important role in disseminating relevant tumour rejection antigens to the immune system [83]. Although regulation of cell function through released vesicles is a rather new concept, it is now acknowledged that vesicle release into the extracellular milieu may represent a novel way of cell-cell communication and hold important physiological implications for the biological function of a variety of cells [84]. In this context, it is worth mentioning that RBC are also capable of releasing vesicles, raising the important issue of whether RBC bioactivities could be mediated, at least in part, by these membranous fragments.

RBC are usually found as biconcave discs. Formation of echinocytes – morphologically altered RBC with numerous and fine spicules throughout the cell membrane – is often overlooked as an artefact of preparation. However, echinocytosis is a common membrane physiological event, both in health and disease and is highly associated with RBC vesiculation, which ultimately leads to the shed of small membrane vesicles (Fig. 1.2) [85,86]. RBC vesiculation is essential during erythroid development, during which reticulocyte membrane remodeling occurs [87,88]. In addition to the vesiculation during erythroid development and the normal physiologic process of aging *in vivo*, the RBC membrane also undergoes vesiculation under a variety of chemical and physical conditions, including blood storage, increased cytoplasmatic Ca^{2+} concentration, ATP depletion, pH lowering and disruption of the membrane lipid-protein organization [89-94]. Interestingly, secretion/release of vesicles by RBC appears to correlate with secretion/release of glycopeptides and peptides resulting from intraerythrocytic proteolysis [95,96].

Early studies conducted in the 1980s described two types of vesicles, differing in size: microvesicles of approximately 150 nm diameter and nanovesicles of approximately 60 nm diameter [89]. More recently, stomatin and sorcin were suggested as marker proteins for micro and nanovesicles, respectively [97]. Vesiculation has been correlated with expression of phosphatidylserine (PS) in the outer-membrane leaflet of the vesicle, indicating that the lipid asymmetry of the two leaflets is lost, at least in part. The expression of PS varies according to the stimulus used to produce vesicles *in vitro*. For instance, powerful stimuli such as Ca^{2+} ionophores lead to high-level of PS expression [98-100]. In any case, treatment of human RBC in different experimental conditions triggers the release of Hb containing, cytoskeletal-free vesicles.

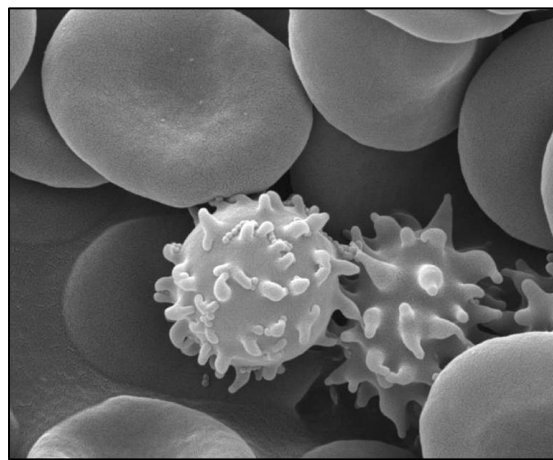


Figure 1.2. RBC echinocyte formation. Red blood cells change shape from biconcave discs to distinctively smaller echinocyte spheres (adapted from [101]).

Anomalies in the vesiculation process have been described in hemolytic anemias and bleeding disorders. The potential consequences of phosphatidylserine exposure in these pathologies include apoptosis, activation of coagulation and worsening of anemia, due to efficient recognition and phagocytic removal of erythrocytes [101-104]. Moreover, RBC generation of microvesicles has been suggested to enhance the activation of the vascular endothelium and lead to vascular inflammation and atherogenesis [105]. *In vivo* vesiculation takes place in a spleen-facilitated manner, and accounts for the loss of approximately 20% of Hb during the lifespan of RBC as well as the loss of the cell surface and an increase in cell density during the RBC life span [106]. Some authors envisage the vesiculation process as a protection strategy of the RBC against the destruction by complement-mediated lysis [107]. In addition, vesiculation seems to protect RBC from immunoglobulin G (IgG) opsonization by sequestering naturally occurring IgG [45,108]. Interestingly, RBC vesicles have been proposed to have immunosuppressive properties and to interfere with inflammatory reactions mediated by the innate immune system. Studies *in vitro* have shown that RBC vesicles (called ectosomes) are taken up by

macrophages and inhibit their activation, by down-regulating Toll-like receptors capacity of activation [109]. The authors speculated that vesicles transfused with RBC blood units may account for some of the immunosuppressive properties attributed to blood transfusions.

1.4. Introduction to the present work and aims of the thesis

Having as starting point the accumulated evidence from *in vitro* studies pointing to RBC as a novel immunoregulatory cell, it was of the foremost importance try to identify the mechanism(s) responsible for the immunoregulatory effect of RBC on activated T cells. This work was initiated having as working hypothesis the possibility that transfer of secreted factors with anti-apoptotic and pro-survival activity between RBC and nearby dividing T lymphocytes could account for the immunoregulatory properties of RBC [73]. Although RBC have largely been regarded as a cytoplasm consisting primarily of hemoglobin encased by a very simple membrane containing only a handful of integral and peripheral membrane proteins, recent proteomic studies have show that RBC contain more than 1500 different proteins [110-112], revealing an unprecedented level of complexity. In addition to the highly abundant antioxidant proteins and hemoglobin, many of the RBC proteins identified are thought to be involved in the regulation of a variety of biological functions, including cellular, physiologic and regulatory processes, including the regulation of signalling cascades important for T cell proliferation and survival [110-113]. The present work addressed the likelihood that the immunoregulatory role of RBC resulted from the release of vesicular and/or non-vesicular factors endowed with T cell growth and survival bioactivities. It also aimed at dissecting the nature and identity of the bioactive factors.

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Chapter 2

**Red blood cells carry out T cell growth and survival
bioactivities that are sensitive to cyclosporine A**

**Adapted from Antunes RF, Brandão C, Carvalho G, Girão C and Arosa FA.
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2.1. ABSTRACT

Red blood cells (RBC) have emerged as a novel regulatory cell type endowed with bioactivities toward activated human T cells. Herein we show that the RBC bioactivities act on intracellular pathways initiated by T cell receptor (TCR)-dependent and -independent stimuli, including IL-2, IL-15, and the mixture of phorbol dibutyrate and ionomycin. The RBC bioactivities preserve the antioxidant status and are capable of rescuing activated T cells from cell death induced by serum deprivation. They are not mediated by glycosylphosphatidylinositol-linked receptors or sialic acids and kinetic studies revealed that they hasten the entrance into the cell cycle. By using cyclosporine A (CsA) and rapamycin (Rapa) we show that the RBC bioactivities are calcineurin-dependent. Thus, treatment of T cells with CsA, but not Rapa, impaired RBC bioactivities and preincubation of RBC with CsA completely abolished their bioactivities. We have demonstrated that RBC carry out bioactivities that are sensitive to CsA.

2.2. INTRODUCTION

Red blood cells (RBC) are emerging as a cell type with the capacity to regulate other cells. A number of studies have shown that besides transporting oxygen and carbon dioxide, RBC are endowed with the capacity to regulate biological processes of non-erythroid cells, including vascular endothelial cell contraction [1], platelet aggregation [2,3], neutrophil apoptosis [4], T cell rolling [5], fibroblast secretion of metalloproteinases [6], B cell responses [7] and IL-12 secretion by dendritic cells [8]. We have reported that human RBC have the capacity to synergize with TCR/CD3-mediated activation signals and enhance T cell growth and proliferation [9]. The RBC-induced T cell proliferation was associated with inhibition of apoptosis and upregulation of cytoprotective proteins and the labile iron pool on dividing T cells [10-12]. Although these studies have unveiled novel regulatory roles for mature circulating RBC, the molecular mechanisms used have remained uncertain. However, recent studies indicate that RBC receptors and released factors may underlie their regulatory role toward non-erythroid cells. Thus, release of nitric oxide by RBC hemoglobin has been proposed to mediate vascular contraction [1]. On the other hand, the RBC-mediated inhibition of IL-12 secretion by dendritic cells depends on interactions between CD47 present on RBC and SIRP α present on dendritic cells [8]. Preliminary studies have shown that interactions between LFA-3/CD58 on RBC and CD2 on T cells might mediate T and B cell responses [7,13].

Most, if not all, studies examining the role that RBC exert on T cell responses have used polyclonal stimuli, including antibodies against CD3/CD28 receptors and the lectin phytohemagglutinin (PHA) in its different forms [9]. Both CD3 ligation and PHA are known to activate intracellular signalling cascades that lead to early tyrosine phosphorylation events, calcium fluxes and activation of downstream kinases, phosphatases and transcription factors that co-ordinately drive T cells into the cell cycle [14]. However, CD3 ligation and PHA are also associated with the triggering of activation-induced cell death (AICD) or apoptosis [15]. In this context, CD28 ligation activates intracellular signals that surpass some of the apoptotic pathways and promote cell cycle progression [16]. Mitogenic combinations that bypass the TCR/CD3 complex, such as phorbol esters and calcium ionophores, also lead to T cell proliferation by inducing calcium fluxes and activation of genes that regulate cell cycle progression [17]. On the other hand, homeostatic cytokines such as IL-2 and IL-15 are known to induce TCR/CD3-independent proliferation and survival of T cells via Jak/Stat dependent signalling pathways [18]. Both cytokines stimulate T cell proliferation and act as survival factors; but IL-15 can inhibit the IL-2-dependent sensitization to AICD [19]. In this context, RBC have consistently been shown to deliver signals that upregulate pathways leading to cell cycle progression while downplaying apoptotic pathways on CD3/PHA-activated T cells [9]. It remains to be elucidated whether the cell growth and survival bioactivities carried out by RBC are also seen when T cells are activated by external signals that bypass the TCR/CD3 complex.

Among the downstream signalling molecules that are activated as a result of these external signals are the hallmark enzymes calcineurin and the mammalian target of rapamycin (mTOR). Calcineurin is a serine/threonine phosphatase that is activated after receptor-mediated signals that lead to an increase in intracellular calcium levels and regulates the activity of nuclear factor of activated T cells (NFAT), a transcription factor that upon translocation into the nucleus upregulates the expression of IL-2 which stimulates growth and differentiation of activated T cells [20,21]. In contrast, mTOR is a serine/threonine kinase involved in the integration of extracellular signals initiated by growth factors and regulates cell growth, proliferation and survival, in part by regulation of the initiation of protein translation [22,23]. Calcineurin and mTOR are the targets of the immunosuppressive drugs cyclosporine A (CsA) and rapamycin (Rapa), respectively. While CsA binds to endogenous cyclophilin A (CypA), Rapa binds to endogenous FK506 binding protein (FKBP)-12 [24]. These complexes bind and inactivate calcineurin and mTOR, respectively, leading to a decrease in T cell proliferation [25].

In the present study, we report that RBC are carriers of bioactivities that act on intracellular pathways regulating inhibition of oxidative stress and apoptosis and entry into the cell cycle. We show that treatment of RBC with CsA but not with Rapa or a phosphatidylinositol 3-kinase (PI3K) inhibitor (LY294002), completely blocked their capacity to increase T cell growth, proliferation and survival of activated T cells. These findings will be discussed in the context of recent findings pointing to CypA as a novel cytokine-like factor mediating cell-to-cell communication [26,27].

2.3. MATERIAL AND METHODS

2.3.1. Reagents and antibodies

Phytohaemagglutinin (PHA-P, from *Phaseolus vulgaris*), antibiotic-antimycotic solution (APS), bovine serum albumin (BSA), propidium iodide (PI), neuraminidase (Neu, from *Vibrio cholerae*), phosphatidylinositol-specific phospholipase C (PI-PLC, from *Bacillus cereus*), ionomycin (Ion, from *Streptomyces conglobatus*), phorbol dibutyrate (PdB) and Triton X-100 were from Sigma-Aldrich (Madrid, Spain). Lymphoprep was from Nycomed (Oslo, Norway). RPMI 1640 GlutaMAX, Hanks Balanced Salt Solution (HBSS) and inactivated Fetal Bovine Serum (FBSi) were obtained from Gibco (Paisley, Scotland). Human rIL-15 and rIL-2 were obtained from R&D Systems (Minneapolis, US). Rapamycin (Rapa) and Cyclosporin A (CsA) and LY294002 were obtained from Calbiochem (Nottingham, UK). 5-(and -6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) and Annexin V-Alexa488 were purchased from Molecular Probes (Amsterdam, The Netherlands). Anti-human glycophorin A PE-conjugated (clone AME-1, CD235a-PE) was from ImmunoTools (Friesoythe, Germany). Anti-human CD3-PE, CD4-APC, CD8-PE-Cy5 and rabbit anti-mouse (RAM)-fluorochrome conjugated antibodies were from DAKO (Glostrup, Denmark). Anti-LFA-3/CD58 antibodies (TS2/9) were kindly provided by Dr. Angelo Cardoso (Melvin and Bren Simon Cancer Center, Indiana University School of Medicine, Indianapolis, Indiana USA). NHS-sulfo-biotin and biotinylated iodoacetamide (BIAM) were from Pierce (Rockford, USA). Protein-A Sepharose CL-4 beads were from Amersham Biosciences (Pittsburgh, USA).

2.3.2. Cells

Fresh peripheral blood mononuclear cells (PBMC) were obtained from buffy coats after centrifugation over Lymphoprep. PBMC were washed twice with HBSS and contaminating RBC lysed in red cell lysis solution (RCLS, 10mM Tris, 150mM NH₄Cl, pH 7.4) for 10 min at 37°C. RBC were collected from the pellet region after Lymphoprep centrifugation,

washed twice with HBSS and diluted 1:10 in RPMI supplemented with 1% APS solution and stored at 4°C until use. RBC purity was assessed by flow cytometry and CD235a labelling which revealed >99.5% CD235a⁺. Partially purified peripheral blood T lymphocytes (PBL) were obtained after culture of PBMC overnight in RPMI supplemented with 1% APS solution and 5% FBSi. The recovered non-adherent cell suspensions were routinely >85% CD3⁺ T cells and are referred to as PBL. T cells were enriched by rosetting with sheep RBC (ProBiologica, Lisboa, Portugal), yielding a population with <1% monocytes or B cells. An Epstein-Barr virus transformed B cell line was kindly provided by Dr. Angelo Cardoso and maintained in RPMI 1640 GlutaMAX supplemented with 10% of FBSi and 1% APS solution.

2.3.3. Cell treatments

CFSE labelling. Ten million PBL/T cells were labelled with CFSE at a final concentration of 5μM for 10 min, with occasional mixing, at 37°C. Then, cells were washed twice with PBS/20% FBSi and resuspended in culture media. Analysis of cells immediately following CFSE labelling indicated a labelling efficiency higher than 99%. Rounds of cell divisions were determined by sequential halving of CFSE-fluorescence intensity.

Rapamycin and cyclosporine treatment. One and a half million PBL (or 15x10⁶ RBC) were incubated for 1 h at 37°C, 5% CO₂ and 99% humidity in culture media alone (see composition below) or supplemented with 10ng/mL of rapamycin or 1μg/mL of cyclosporin, and washed exhaustively prior to their culture in vitro.

Phospholipase C and neuraminidase treatment. Fifteen million RBC were treated with culture media (see composition below) containing 0.1U of phosphatidylinositol-specific phospholipase or 0.1U neuraminidase during 1 h in an orbital rotator at 37°C. As a control, RBC were incubated under identical conditions in control media. Afterwards RBC were centrifuged and extensively washed before addition to cultures of activated PBL and/or T cells.

2.3.4. Culture conditions

PBL or purified T cells (1.5x10⁶) were cultured in 6-well plates or 24-well plates, in a final volume of 5 or 1 mL, respectively, for a maximum of 7 days in an incubator at 37°C, 5% CO₂ and 99% humidity. PBL or T cells were either left unstimulated or stimulated with the following mitogens: (1) 5 μg/mL PHA-P; (2) 5nM PdB + 100nM Ion; (3) 10ng/mL of IL-15; (4) 10ng/mL of IL-2. PBL and/or T cells were cultured in the absence or presence of autologous RBC at a PBL:RBC ratio of 1:10 for up to 7 days. In some experiments heterologous RBC were also used. Culture media was RPMI 1640 GlutaMAX supplemented with 1% APS solution and 1% FBSi. In some experiments PBL and/or T

cells were cultured in the absence of serum in RPMI 1640 GlutaMAX supplemented with 1% APS. For immunosuppression studies, 1.5×10^6 PBL were stimulated with $5 \mu\text{g/mL}$ PHA-P and cultured in the absence or presence of untreated RBC or RBC treated with rapamycin and cyclosporin at a PBL:RBC ratio of 1:10 and cultured in six-well plates as above. At the end of the culture PBL/T cells were harvested, washed and acquired in a FACSCalibur (Becton-Dickinson, Mountain View, CA). For each sample 50,000 events were acquired using FSC/SSC characteristics and analyzed using CellQuest or FlowJo software.

2.3.5. Flow cytometry determinations

Cell stainings were normally performed at 4°C for 30 min in 1X PBS or staining buffer (PBS, 0.2% BSA, 0.1% NaN_3) in 96-well round-bottom plates (Greiner bio-one, Frickenhausen, Germany). In cultures that received RBC, erythrocytes were first lysed with RCLS. Irrelevant mAb were used as negative controls to define background staining. T cell death and survival were determined by two methods: (1) a decrease in cell size according to forward light scatter (FSC)/side light scatter (SSC) parameters; (2) double Annexin V and PI staining using Ca^{2+} -based staining buffer (10nM HEPES/140mM NaCl/2.5mM CaCl_2). T cell activation and division were studied by two methods: (1) determination of cell size and complexity according to FCS/SSC parameters (blasts); (2) CFSE fluorescence loss.

2.3.6. Biotinylation, immunoprecipitation, SDS/PAGE and Westernblot

For the detection of cell surface LFA-3/CD58, 5×10^6 B cell lymphoma cells and 250×10^6 RBC were surface biotinylated as described [10]. Briefly, cells were incubated with 0.5 mg/mL of NHS-sulfo-biotin (Pierce, Rockford, USA) in 1X PBS for 10 min at room temperature followed by four washes in PBS. After washing, labeled cells were lysed in lysis buffer (20mM Tris pH 7.6, 150mM NaCl, 1mM PMSF and 1% Triton X-100) for 30 min on ice. The lysates were centrifuged at $10,000 \times g$ to remove cell debris and precleared for 1 h with protein-A Sepharose beads (Amersham Biosciences). Precleared detergent lysates were immunoprecipitated with TS2/9 antibodies followed by Sepharose beads for 2 h at 4°C . Washed immunoprecipitates were boiled in sample buffer and resolved by SDS/PAGE under non-reducing conditions and resolved proteins transferred to nitrocellulose membranes (Whatman, Pittsburgh, USA). Membranes were blocked with 5% non-fat dry milk in TBS-T and then incubated with ExtrAvidin horseradish peroxidase (Sigma-Aldrich). Biotinylated proteins were visualized after exposure to Kodak Biomax MR1 films (Sigma-Aldrich) using the enhanced chemiluminescence (ECL Super Signal West Pico detection reagent). For the detection of proteins containing free thiols, cell

lysates of activated T cells in the absence or presence of RBC were labeled with BIAM for 30 min at room temperature. The labelling reaction was quenched by the addition of dithiothreitol (DTT). Afterwards, equivalent aliquots of cell lysates, quantified by the BCA protein assay kit (Pierce), were run in a 10% SDS/PAGE, transferred to a membrane and visualized by ECL, as described above. In parallel, a similar aliquot of cell lysates was run and total proteins visualized by Coomassie brilliant blue R250 (Sigma-Aldrich).

2.3.7. PKB and MEK1/2 phosphorylation status determination

For PKB and MEK1/2 phosphorylation studies PBL (50×10^6) were cultured in 75 cm³ flasks, in a final volume of 25 mL, for different periods of time in an incubator at 37°C, 5% CO₂ and 99% humidity. PBL were left unstimulated or stimulated with PHA without or with RBC and used immediately for phosphorylation studies. Culture media was RPMI 1640 GlutaMAX supplemented with 1% APS solution and 1% FBSi. In some experiments PHA-stimulated PBL were harvested, washed with 1X PBS and re-cultured in the absence of stimulus (PHA starvation) for different periods of time. Then, PHA starved PBL were collected, washed with 1X PBS and incubated without or with RBC in a water-bath at 37°C for different periods of time at a concentration of 5×10^6 cells/mL. Afterwards, aliquots of 1mL (corresponding to 5×10^6 cells) were spun down. Pelleted cells were washed 3 times with cold 1X PBS and lysed in lysis buffer in the presence of protease and phosphatase inhibitors (Sigma-Aldrich) for 30 min on ice. PBL stimulated with RBC were harvest, RBC lysed for 9 min with RCLS and cells treated as previously indicated. Cell lysates were centrifuged at 10,000×g for 15 min and the supernatant was collected and quantified by using the BCA protein assay kit, according to manufacturer's instructions. Equal amounts of protein (25ug) were resolved by 10% SDS-PAGE, transferred into nitrocellulose transfer membranes, blocked and then incubated overnight in TBS-T 5% BSA with the following rabbit anti-human antibodies (Cell Signalling Technology, USA): phospho-PKB (Ser473, clone D9E, dilution used 1:500); phospho-MEK1/2 (Ser217/221, clone 41G9, dilution used 1:2000) and total PKB (dilution used 1:1000) and MEK1/2 (dilution used 1:2000). Afterwards, membranes were extensively washed with TBS-T and incubated with horseradish peroxidase–conjugated anti–rabbit IgG (1:1000) and proteins visualized by ECL according to manufacturer's instructions. Subsequently, membranes were reprobed with different antibodies after membrane stripping using 5% acetic acid. Band intensity was analysed by densitometry using the program UNI-SCAN-IT. Relative phospho-PKB was obtained by the ratio of the band intensity of phospho-PKB and total PKB. The same procedure was done to obtain the relative phospho-MEK1/2 values.

2.3.8. Statistical analysis

Statistical analysis was performed using Excel or GraphPad™ Prism 5 software. Student's *t* test was used to evaluate the significance of the differences between group means. Statistical significance was defined as $p < 0.05$.

2.4. RESULTS

2.4.1. RBC have bioactivities that enhance cell growth and division signals initiated by TCR/CD3-independent stimuli

Our previous studies showed that the presence of RBC in close proximity of peripheral blood lymphocytes (PBL) that have been activated *in vitro* with polyclonal stimuli dependent on the expression of a TCR/CD3 complex (e.g. PHA-P, PHA-L, anti-CD3, anti-CD3⁺anti-CD28) enhanced proliferation and survival of the activated T cells ([10-12], Fig. 2.1). To ascertain whether the RBC bioactivities can also influence TCR/CD3-independent signals, PBL were left unstimulated or stimulated with TCR/CD3-dependent (PHA-P) and TCR/CD3-independent (phorbol+calcium ionophore, IL-15 and IL-2) stimuli in the absence or presence of autologous RBC and proliferation determined by CFSE fluorescence loss using flow cytometry. As expected, PHA-stimulation induced an overall increase in cell growth (Fig. 2.1A) that correlated with reasonable levels of T cell proliferation (Fig. 2.1B). However, PHA also induced marked levels of activation-induced cell death (Fig. 2.1A). The presence of RBC, either autologous or heterologous, augmented cell growth and proliferation while markedly inhibiting cell death (Fig. 2.1, A and B, and data not shown). Similar results in cell growth, survival and proliferation were observed when T cells were activated with the combination of PdB and ionomycin, two mitogens that bypass plasma membrane receptors (Fig. 2.1, A and B). RBC had no effect when either mitogen was used alone (see Fig. 2.2). IL-15 also induced an overall increase in cell growth (Fig. 2.1A) that correlated with modest levels of proliferation (Fig. 2.1B). In contrast to PHA, IL-15 induced high levels of survival (Fig. 2.1A). As a result, the presence of RBC did not impact on survival but induced a significant increase in the number of blasts and proliferating cells, instead (Fig. 2.1, A and B). CD3-labelling at the end of the culture showed that the large majority of lymphocytes that divided in response to IL-15 were CD3⁺ T cells and the presence of RBC induced the preferential expansion of CD3⁺CD8⁺ T cells (data not shown). Similar results in cell growth, survival and proliferation were observed when T cells were activated with IL-2 (Fig. 2.1, A and B). Overall, RBC were capable of augmenting the percentage of T cells that entered cell division (Fig. 2.1C) and also the percentage of cell survival (Fig. 2.1D) both after TCR-dependent and -independent stimuli.

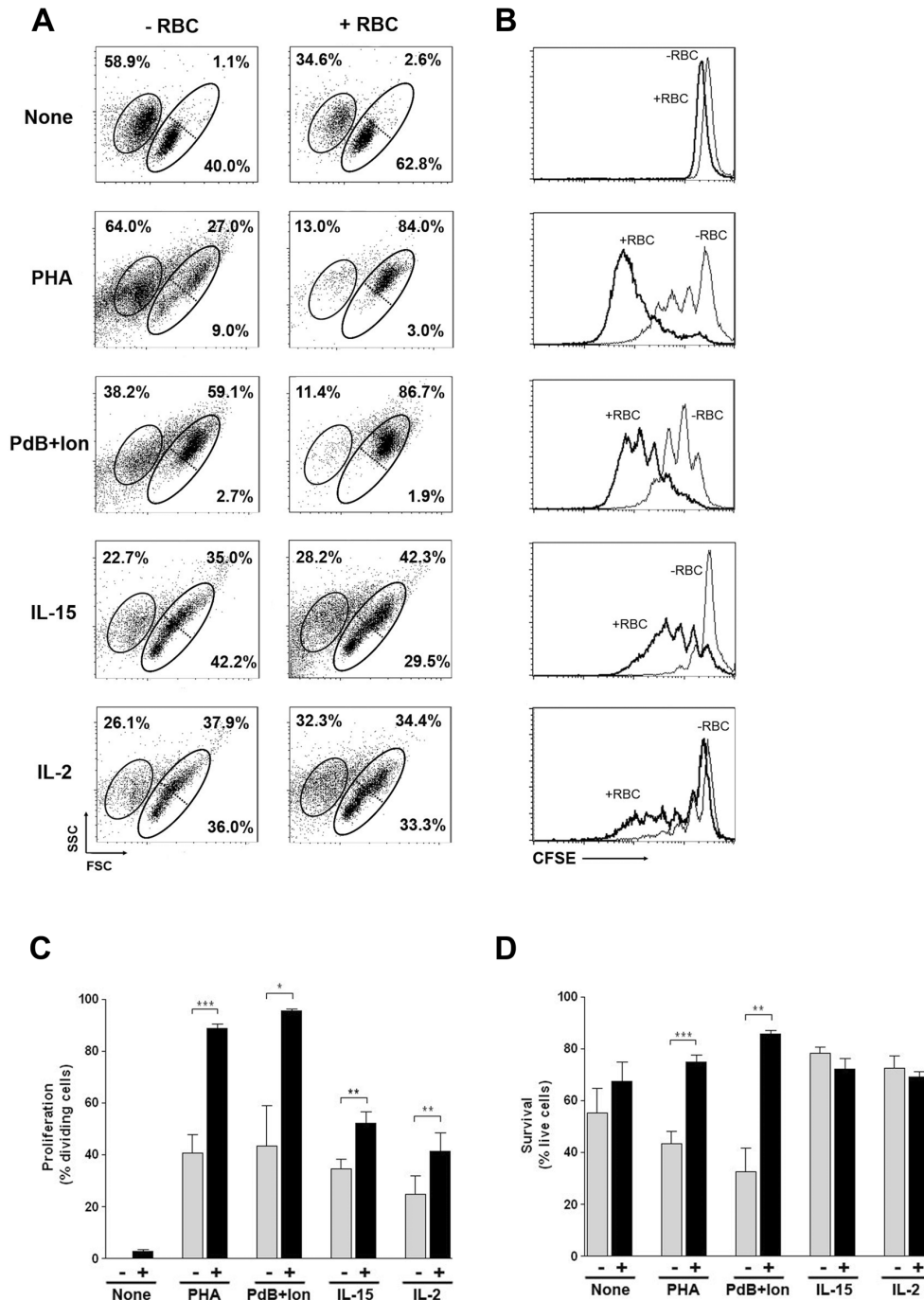


Figure 2.1. RBC bioactivities strengthen proliferation and survival of dividing T cells. PBL (1.5×10^6) were labelled with CFSE and cultured either alone or with autologous or heterologous red blood cells (1:10 ratio) in the absence or presence of different stimuli for 7 days, and then harvested, stained and acquired in a FACSCalibur. **A**, Dot Plots (FCS vs SSC) show the percentage of T cell death (left gated population, as determined by positive PI labelling) and survival (right gated population, as determined by negative PI labelling) of PBL cultured alone (-RBC) or in the presence of autologous RBC (+RBC). Also shown are the percentage of small (lower-right gated population) and blast (upper-right gated population) cells. **B**, Histograms illustrate the corresponding T cell proliferation levels obtained in the different conditions shown in A, in the absence (-RBC, thin line) or presence (+RBC, thick line) of RBC, as determined by CFSE fluorescence loss. **C-D**, Graphs show the percentage of dividing (**C**) and surviving (**D**) T cells (mean $\pm$ SEM, n=4) for the different conditions in the absence (-) or presence (+) of RBC. *P* values are shown: (***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$).

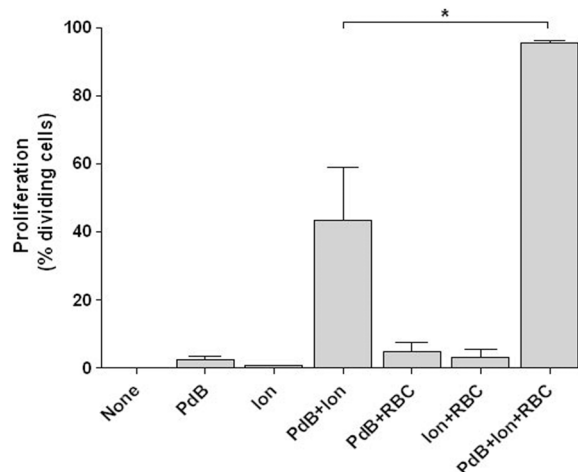


Figure 2.2. RBC enhance proliferation of PBL activated by the combination of PdB and Ionomycin. PBL (1.5×10^6) were labelled with CFSE and cultured either alone or with red blood cells (1:10 ratio) in the absence or presence of the different stimuli for 7 days. Then, cells were harvested, stained and acquired in a FACSCalibur. Graph shows the percentage of dividing PBL (mean $\pm$ SEM, n=4) in the different culture conditions. *P* values are shown (*, $p < 0.05$).

2.4.2. RBC bioactivities are independent of GPI-linked receptors and sialic acids

RBC have been reported to express LFA-3/CD58, the ligand for CD2, and previous studies suggested that the enhancing effect that human RBC have on T cell proliferation was mediated by CD58 present on the plasma membrane of RBC [7]. However, neither removal of GPI-linked receptors from the cell surface of RBC by treatment with PLC nor preincubation of RBC with TS2/9 antibodies (that specifically recognize CD58) abolished the RBC bioactivities toward activated T cells (Fig. 2.3).

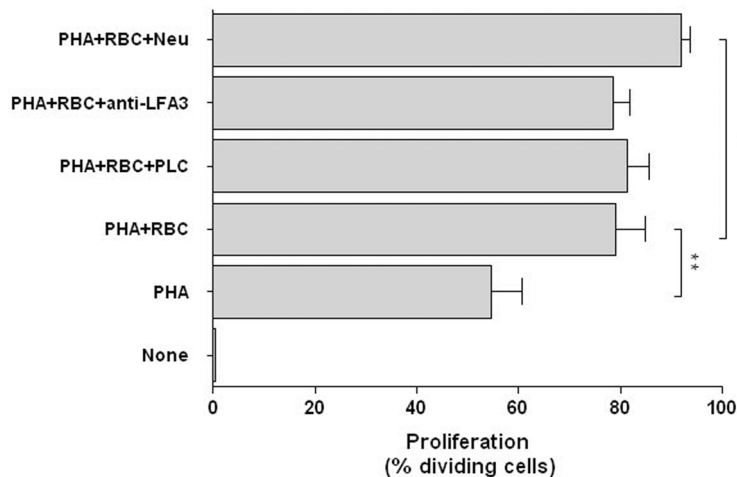


Figure 2.3. RBC bioactivities are independent of major RBC membrane proteins. PBL (1.5×10^6) labelled with CFSE were stimulated with 5 μ g/mL of PHA and cultured for 7 days either alone or in the presence of untreated RBC or RBC treated with PLC and neuraminidase. Afterward, cells were harvested and acquired in a FACSCalibur. The graph shows the percentage of proliferating PBL (mean $\pm$ SEM, n=7) as determined by CFSE fluorescence loss in the different conditions. *P* values are shown: (**, $p < 0.01$; *, $p < 0.05$).

In view of these results, we investigated the expression of LFA-3/CD58 by freshly isolated human RBC. Surprisingly, flow cytometry and immunoprecipitation studies consistently showed that LFA-3/CD58 cannot be detected on the cell surface of human RBC, or at least it is expressed at levels beyond detection, indicating the unlikelihood of mediating the RBC bioactivities (Fig. 2.4, see inset). The lack of detection of LFA-3/CD58 on RBC was not an artifact since the TS2/9 antibodies detected LFA-3/CD58 on the cell surface of an immortalized B cell line (Fig. 2.4, see inset). Next, we examined whether removal of sialic acids from the RBC membranes could influence RBC bioactivities. Sialic acids are the ligands of certain receptors (SIGLEC), some of which are involved in the regulation of activation events in T cells [28]. As shown in Figure 2.3, treatment with neuraminidase to remove sialic acids did not abolish RBC bioactivities; rather, neuraminidase-treated RBC significantly increased the percentage of T cells entering division. Sialic acid removal was confirmed by a complete down-modulation in glycoporphin A expression (data not shown), which is known to represent more than 80% of the total sialic acid amount of RBC membrane glycoproteins [29].

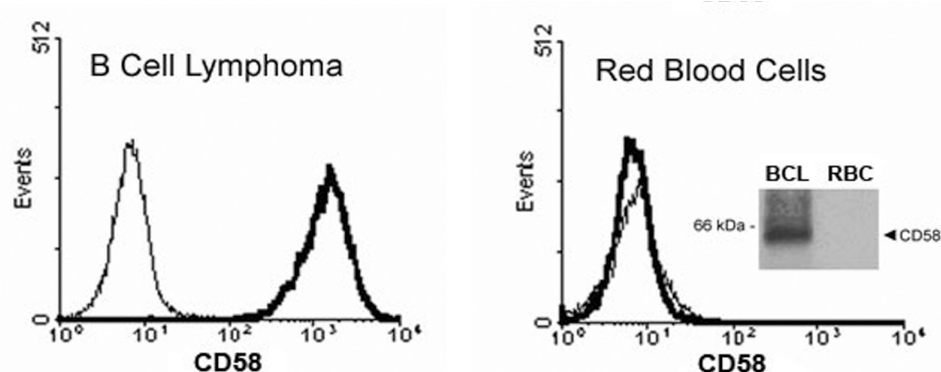


Figure 2.4. Human RBC lack expression of LFA-3/CD58. Red blood cells (RBC) and B cell lymphoma (BCL) cells were cell surface labelled with anti-LFA-3/CD58 antibodies (TS2/9) followed by RAM-FITC conjugated antibodies and acquired in a FACSCalibur. Histograms show CD58 expression (thick line) in BCL cells (left histogram) and RBC (right histogram). Background staining with RAM-FITC (thin line) is shown. *Inset:* BCL cells and RBC were surface biotinylated, lysed and LFA-3/CD58 immunoprecipitated with TS2/9 antibodies as described in “Material and methods”. Aliquots of the immunoprecipitates were resolved in a SDS/PAGE, blotted and visualized by the ECL technique. The band corresponding to LFA-3/CD58 is indicated.

2.4.3. RBC bioactivities rescue T cells from activation-induced cell death: correlation with the preservation of thiol levels

In order to ascertain whether the RBC bioactivities needed accessory cells we purified T cells and their cell growth, proliferation and survival rates were examined upon PHA-activation. As shown in Figure 2.5A, PHA-stimulation of pure T cells induced high levels of cell death without inducing cell growth when compared to PBL preparations, which were

paralleled by very low levels of proliferation (Fig. 2.5B). Of note, the presence of RBC in the cultures completely reversed the incapacity of pure T cells to grow and proliferate (Fig. 2.5). The effect of RBC on reversing the unresponsiveness of pure T cells was of such magnitude that it resulted in more than a 25-fold increase in cell proliferation when compared to T cells alone. Interestingly, examination of the level of cell surface thiols in dividing T cells in the absence or presence of RBC by using the free-cysteine reagent BIAM showed higher levels in cultures with RBC (Fig. 2.5B, inset). In order to examine further the strength of the RBC bioactivities, cultures of PBL preparations in the absence of serum were performed. Removal of serum from the culture medium resulted in a complete absence of T cell proliferation (Fig. 2.6A), mostly because of a strong decrease in cell survival (Fig. 2.6B). However, the presence of RBC in these cultures allowed T cells to proliferate to levels very similar to those observed in cultures with serum (Fig. 2.6A), literally rescuing the activated T cells from cell death (Fig. 2.6B).

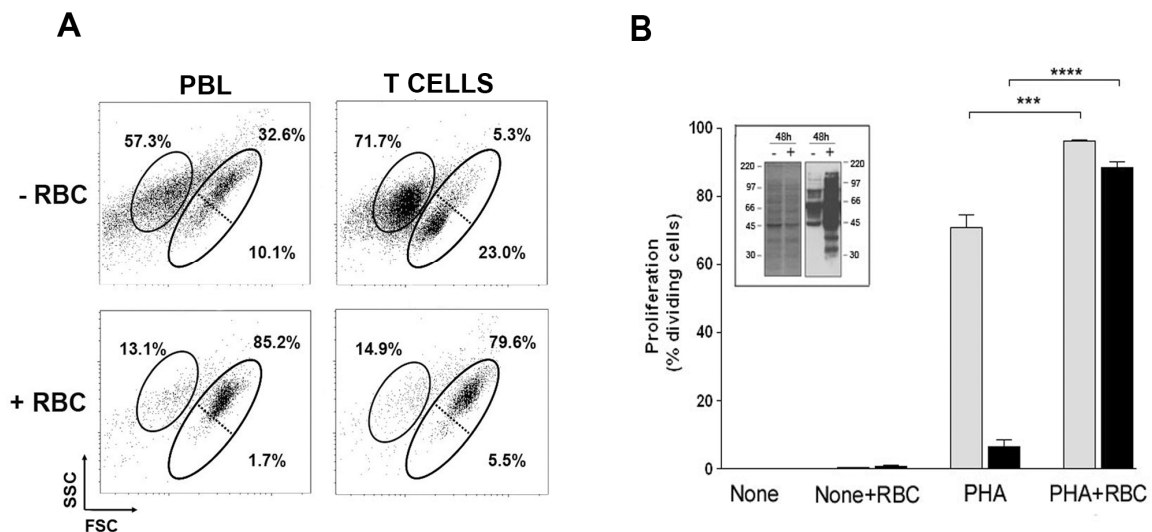


Figure 2.5. RBC bioactivities rescue activated T cells from cell death. PBL or purified T cells (1.5×10^6) were labelled with CFSE, stimulated with $5 \mu\text{g/mL}$ of PHA and cultured in the absence or presence of RBC for 7 days. Then, cells were harvested and acquired in a FACSCalibur. **A**, Dot Plots (FCS vs SSC) show the percentage of T cell death (left gated population, as determined by positive PI labelling) and survival (right gated population, as determined by negative PI labelling) of PHA-activated PBL or T cells cultured alone (-RBC) or in the presence of RBC (+RBC). Also shown are the percentage of small (lower-right gated population) and blast (upper-right gated population) cells. **B**, The graph shows the percentage of proliferating cells (mean $\pm$ SEM, $n=6$) in cultures of PHA-stimulated PBL (grey bars) or PHA-stimulated T cells (black bars) in the absence or presence of RBC. The percentage of proliferating cells in unstimulated (None) cultures are included. *P* values are shown: (****, $p < 0.0001$; ***, $p < 0.001$). **Inset**: Cell lysates of 48-hour cultures of PBL activated with PHA in the absence (-) and presence (+) of RBC were labelled with BIAM as described in "Material and methods", and two aliquots of the cell lysates were run on separate SDS/PAGE under non-reducing conditions. One of the gels was stained with Coomassie blue to visualize the total amount of protein (left graph). The other gel was blotted into membranes and thiols-containing proteins visualized by the ECL technique (right graph). Molecular weight markers are indicated.

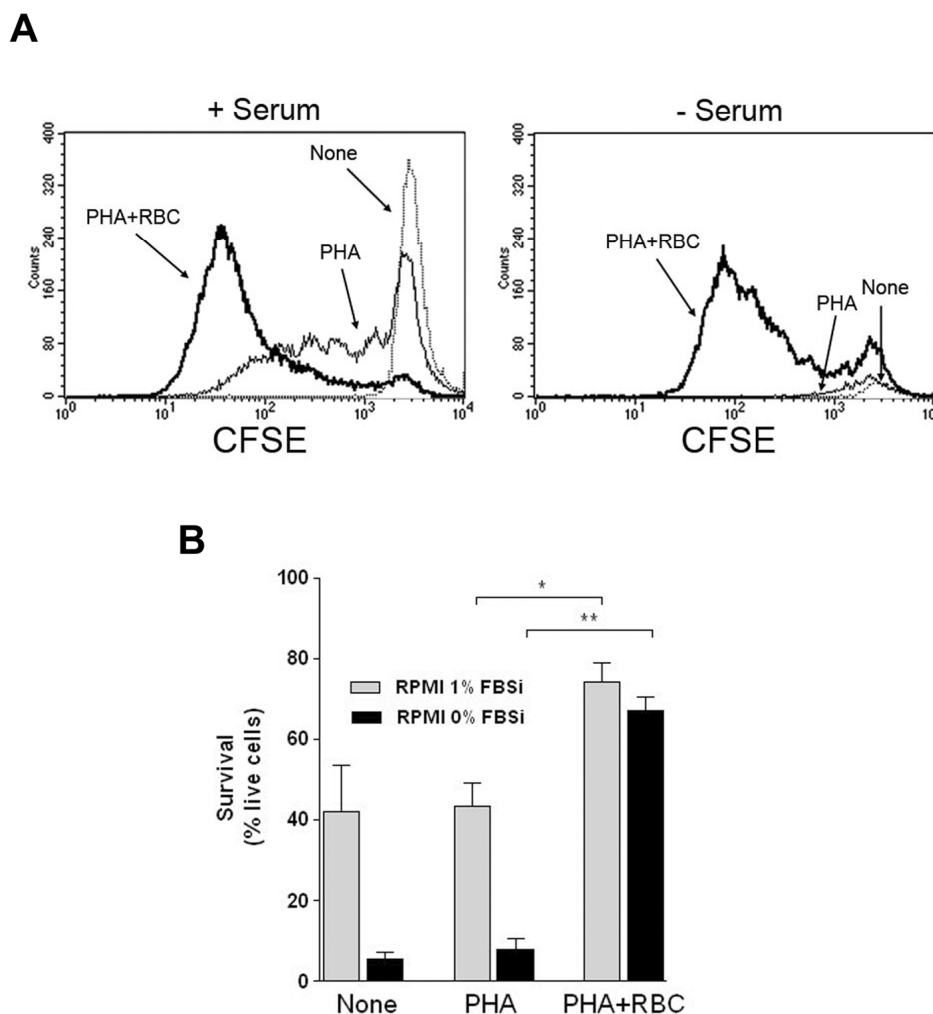


Figure 2.6. RBC bioactivities rescue activated T cells from cell death and enhance proliferation under serum deprivation. PBL or purified T cells (1.5×10^6) were labelled with CFSE, stimulated with $5 \mu\text{g/mL}$ of PHA in culture media with or without serum and cultured for 7 days in the absence or presence of RBC. Then, cells were harvested and acquired in a FACSCalibur. **A**, Histograms show loss of CFSE fluorescence in cultures of PHA-activated PBL with (left histogram) or without (right histogram) serum, in the absence (thin line, PHA) or presence (thick line, PHA+RBC) of RBC. Dotted-line (None) shows CFSE fluorescence in unstimulated PBL. **B**, Graph shows the percentage of live cells (mean $\pm$ SEM, $n=3$) for the different culture conditions in the presence (grey bars) or in the absence (black bars) of serum. *P* values are shown: (**, $p<0.01$; *, $p<0.05$).

Finally, kinetic studies (1-6 days) of cultures of PHA-activated PBL revealed that T cell division is only detectable 3 days after the start of the culture (Fig. 2.7). Thus, the percentage of T cells that divided at least once after 3 days of activation was, on average, about 23% (Fig. 2.7). Noteworthy, the presence of RBC shortened the time necessary to see the same level of T cell division by 24 hours (compare PHA with PHA+RBC in Fig. 2.7). As a result, the percentage of T cells that divided at least once in cultures with RBC 3 days after activation was threefold higher than in cultures without RBC (67% vs. 23%,

Fig. 2.7). This difference was maintained thereafter. In addition, the effect of RBC in inhibiting T cell apoptosis (as measured by Annexin V labeling), was seen soon after the start of the culture and maintained thereafter (data not shown).

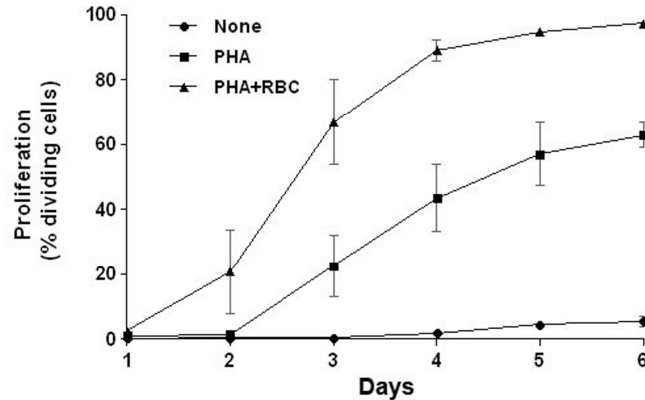
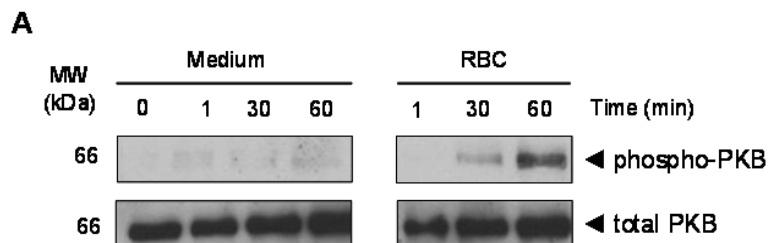


Figure 2.7. Kinetics of the effect of RBC on T cell proliferation. PBL (1.5×10^6) were labelled with CFSE, and either left unstimulated (None) or stimulated with $5 \mu\text{g/mL}$ of PHA and cultured for 6 days in the absence or presence of RBC. Then, cells were harvested at days 1, 2, 3, 4, 5 and 6 and acquired in a FACSCalibur. Graph shows the percentage (mean $\pm$ SEM, $n=2$) of dividing cells for the different culture conditions along the culture period.

Studies examining the phosphorylation status of intracellular signalling molecules regulating cell survival and proliferation, namely analysis of PKB/Akt and MEK 1/2 activation, showed that the PI3K/Akt axis may be a target of the RBC bioactivities (Fig. 2.8). Indeed RBC were capable of inducing the activation of PKB in activated normal human T cells *in vitro* when added to cells that were starved for 48 hours. We could not detect significant changes in PKB phosphorylation when RBC were added directly to activated T cells. In contrast, RBC did not influence significantly the activation of MEK1/2 (data not shown).



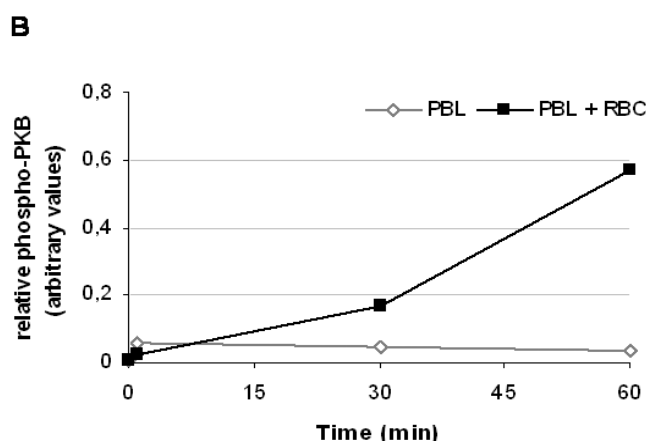


Figure 2.8. RBC activates the Akt/PKB pathway. PBL were stimulated with 5µg/mL of PHA overnight, harvested and washed out to remove PHA. Activated T cells were re-cultured without stimulus (PHA starvation) for an additional 48 hours. Then, cells were harvested and incubated at 37°C in culture medium alone or with RBC (1:10 ratio) for 1, 30 and 60 minutes as indicated. After lysis of RBC, T cells were immediately spun down and lysed in cell lysis buffer containing a protease and phosphatase inhibitor cocktail. Cell lysates were quantified using the BCA protein assay and aliquots corresponding to 25 µg of protein were resolved by 10% SDS-PAGE and transferred to nitrocellulose membranes. **A**, phosphorylation of PKB was determined by using anti-phospho-Akt/PKB (Ser473) followed by rabbit anti-mouse horseradish peroxidase-conjugated antibodies and visualized by ECL. Levels of total Akt/PKB were determined after membrane stripping and re-probing with anti-Akt/PKB polyclonal antibodies. **B**, Levels of phosphorylated and total Akt/PKB were quantified by densitometry with UN-SCAN-IT program. Graph shows the relative phospho-PKB plotted against time. Results show 1 representative experiment of 3 separate experiments.

2.4.4. RBC bioactivities are differently sensitive to the immunosuppressive drugs rapamycin and cyclosporine A

Based on the aforementioned results and in view of the overwhelming effects that RBC exert on T cells that have been activated *in vitro*, we decided to investigate whether these RBC bioactivities could be abolished or inhibited by drugs commonly used to suppress immune responses such as cyclosporine A (CsA) and rapamycin (Rapa). Preliminary dose-inhibition experiments showed that 10ng/mL of Rapa and 1µg/mL of CsA induced more than 50% inhibition on T cell proliferation without decreasing cell death (data not shown, see also Fig. 2.9D), and these were used in all subsequent experiments. As shown in Figure 2.9A (thin lines), pre-treatment of PBL with either drug negatively impacted on the relative extent of T cell proliferation as determined by higher levels of CFSE mean fluorescence intensity. As summarized in Figure 2.9B (grey bars), these differences were statistically significant. In addition, Rapa and CsA treatment also negatively affected the percentage of T cells that divided, without significantly affecting survival (Fig. 2.9, C and D, grey bars). The addition of RBC to the cultures surpassed the immunosuppressive effects of both drugs differently. Thus, even though the addition of RBC to the cultures of Rapa- and CsA-treated PBL reversed the inhibition caused by

these drugs, as determined by loss of CFSE labelling (Fig. 2.9A), the effect was less efficient in CsA-treated PBL (Fig. 2.9B, black bars). The lesser capacity of RBC to surpass the immunosuppressive effect of CsA was also observed at the level of the percentage of dividing T cells (Fig. 2.9C, black bars; see also Fig. 2.9A thick lines) and the level of survival (Fig. 2.9D, black bars).

Then, we wanted to ascertain whether treatment of RBC with these drugs, prior to their addition to the cultures, could have an impact on their bioactivities. As shown in Figure 2.10A, Rapa-treated RBC were as efficient as untreated RBC in augmenting the extent of T cell proliferation. In marked contrast, CsA-treated RBC were completely incapable of augmenting T cell proliferation (Fig. 2.10A). The differential sensitivity of the RBC bioactivities to Rapa and CsA treatment is summarized in Figure 2.10B and is corroborated when the percentages of T cells that divided (Fig. 2.10C) and the percentage of live cells (Fig. 2.10D) were analyzed. Interestingly, the level of survival observed in cultures with Rapa-treated RBC increased significantly when compared with untreated RBC (Fig. 2.10D). The negative impact that the treatment of RBC with CsA has on their bioactivities toward activated T cells is specific since treatment of RBC with Rapa (Fig. 2.10) or the specific PI3K inhibitor LY294002 (data not shown) had negligible effects on the RBC bioactivities.

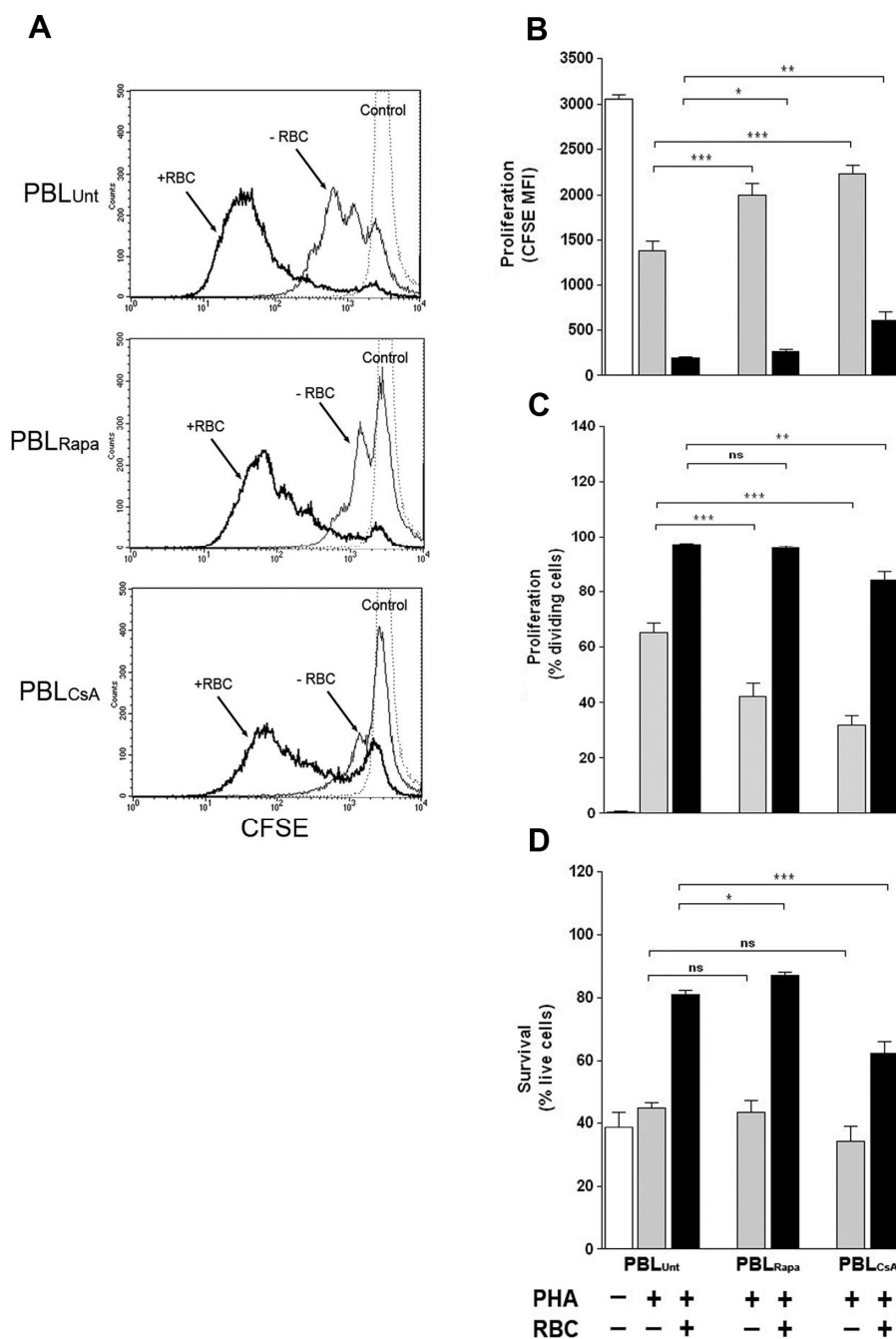


Figure 2.9. RBC bioactivities differently overcome immunosuppression induced by Rapa and CsA. PBL (1.5×10^6) were incubated with culture media alone or containing either Rapa (10ng/mL) or CsA (1ug/mL) for 1 hour at 37°C and extensively washed. Then, PBL were labelled with CFSE and left either unstimulated or stimulated with 5µg/mL of PHA and cultured in the absence or presence of RBC for 7 days. Afterwards, cells were harvested, stained, and acquired in a FACSCalibur. **A**, Histograms show proliferation levels of activated PBL, as determined by CFSE fluorescence loss, of either PBL that were untreated (PBL_{Unt}) and pre-treated with Rapa (PBL_{Rapa}) or CsA (PBL_{CsA}), and cultured in the absence (-RBC, thin line) or presence of RBC (+RBC, thick line). CFSE levels in unstimulated PBL (Control, dotted-line) are also shown. **B-D**, The graphs show the corresponding percentage (mean±SEM, n=9) of proliferating T cells (**B**), the percentage of dividing T cells (**C**) and the percentage of survival (**D**) in the different culture conditions in the absence (grey bars) or presence (black bars) of RBC. Proliferation and survival values in unstimulated PBL are indicated in the first-left bar. *P* values are shown: (***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$, ns=not significant).

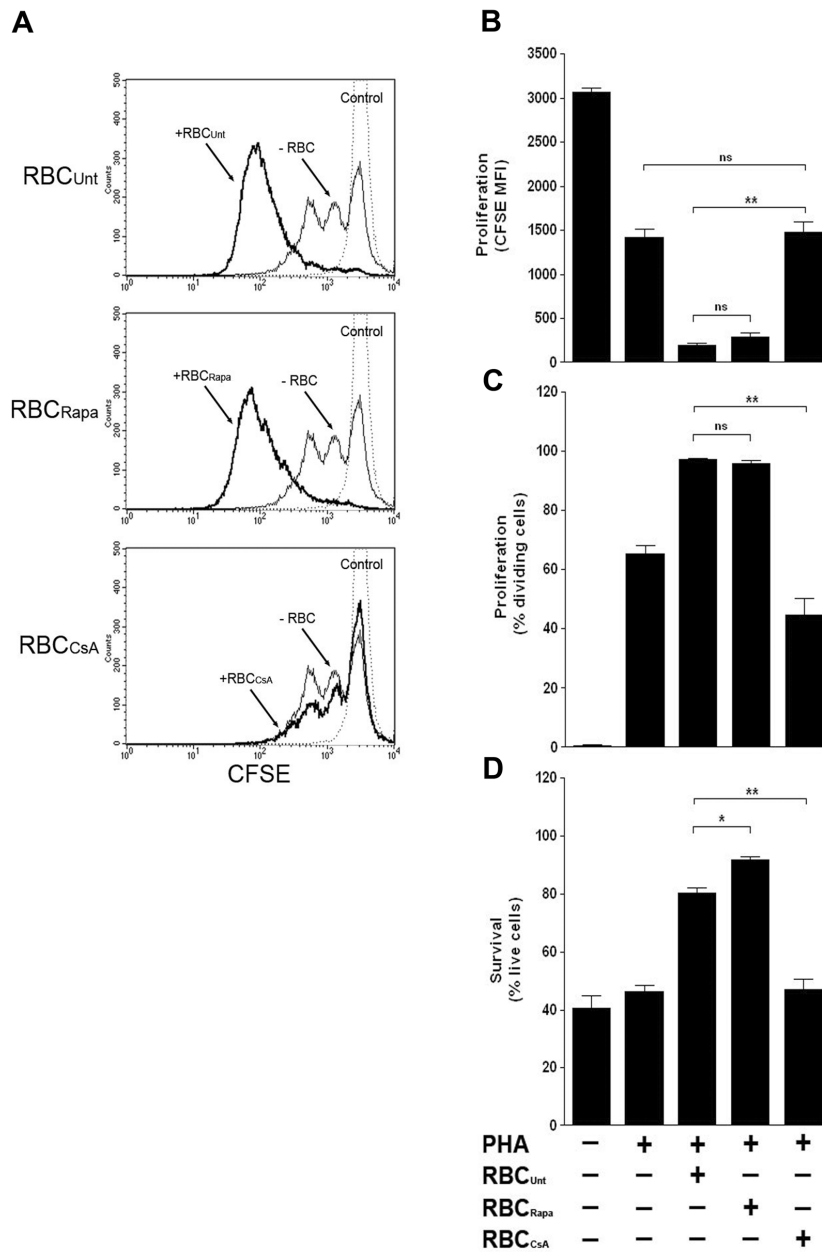


Figure 2.10. RBC bioactivities are abolished by CsA treatment. RBC (15×10^6) were incubated with culture media alone or containing either Rapa (10ng/mL) or CsA (1 μ g/mL) for 1 hour at 37°C and extensively washed. Then, PBL were labelled with CFSE and left either unstimulated or stimulated with 5 μ g/mL of PHA and cultured in the absence or presence of RBC for 7 days. Afterwards, cells were harvested, stained, and acquired in a FACSCalibur. **A**, Histograms show proliferation levels of activated PBL, as determined by CFSE fluorescence loss, in the absence of RBC (-RBC) or in the presence of RBC that were either left untreated (RBC_{Unt}) or treated with Rapa (RBC_{Rapa}) or CsA (RBC_{CsA}). CFSE levels in unstimulated PBL (Control, dotted-line) are also shown. **B-D**, The graphs show the corresponding percentage (mean $\pm$ SEM, n=6) of proliferating T cells (**B**), the percentage of dividing T cells (**C**) and the percentage of survival (**D**) in the indicated different culture conditions. *P* values are shown: (**, $p < 0.01$; *, $p < 0.05$, ns=not significant).

2.5. DISCUSSION

We have previously shown that RBC can enhance cell proliferation and inhibit apoptosis of human T cells that have been activated in vitro with polyclonal stimuli, such as phytohemagglutinin or CD3 triggering either alone or in combination with CD28 [11,12]. By using different approaches we showed that the RBC effect is closely linked with the upregulation of cytoprotective proteins heme oxygenase 1 and ferritin and downregulation of the prooxidant state generated after activation [10]. As a result, we proposed that the RBC is a cell type endowed with bioactivities capable of regulating T cell growth and survival of normal human T cells [9].

In the present study we extend these findings by showing that bioactivities carried out by RBC act on intracellular pathways initiated by TCR-dependent and TCR-independent stimuli, including IL-15 and the mixture of phorbol dibutyrate and a calcium ionophore. IL-15 is a gamma-common cytokine with a wide range of biological effects on cells of the immunological system. Regarding T cells, we have shown the induction of activation and proliferation of CD8⁺ T cells and contribution to T cell homeostasis [18]. The interaction of IL-15 with its receptor triggers activation signals different from the ones triggered by the TCR, namely the activation of the JAK/ STAT family proteins whose combined action leads to the subsequent activation of the MEK/ ERK and PI3K/ Akt pathways, which regulate T cell survival and proliferation [30]. RBC increased IL-15 induced T cell growth and proliferation while leaving survival levels unchanged, suggesting that the RBC bioactivities are able to intersect in a positive manner with the intracellular pathways activated by IL-15, namely pathways related with cell growth and entry into the cell cycle. This assumption is supported by similar results obtained with IL-2, another gamma-common cytokine that activates similar activation pathways. The lack of an effect on survival of RBC on IL-15 stimulated T cells is not surprising since IL-15 is already known to markedly increase T cell survival. The combination of PdB and ionomycin, though bypassing TCR-proximal events, initiates a cascade of downstream events that are very similar to the ones initiated by TCR-mediated signals and include activation of serine/threonine protein kinase C and the serine/threonine phosphatase calcineurin. Of note, the presence of RBC increased cell growth, proliferation and survival of PdB+Ion activated T cells to levels similar to those seen in PHA-activated T cells. These results strongly suggest that RBC bioactivities act on downstream signalling regulators, such as calcineurin, responsible for the initiation of transcription of genes related with the control of proliferation, such as IL-2, and not on isolated early pathways that are activated when PdB and ionomycin are used alone.

We have also demonstrated that the RBC bioactivities are not mediated by RBC receptors, are exerted directly over T cells and are independent of serum factors. Our previous studies suggested that RBC surface receptors proposed to mediate the effect of RBC were not involved [11,12]. In this study we have extended these results by showing that neither GPI-linked receptors nor sialic acids present at the plasma membrane of RBC are involved in the enhancement of T cell growth, survival and proliferation, pointing to a different mechanism of action. It is worth mentioning that in our studies we could not detect expression of LFA-3/CD58 in human RBC. However, the possibility that the levels of LFA-3/CD58 on the surface of human RBC are below the detection limits of these sensitive techniques cannot be ruled out. In any case, these levels of expression would be inappropriate in terms of the delivery of co-stimulatory signals to activated T cells and most likely do not play an important role in the RBC effect. These data together with previous results indicating that the RBC effect could be observed even when RBC are added to cultures of activated T cells 1-2 days after the initial stimulation suggest a lack of involvement of RBC co-stimulatory signals during the early stages of T cell activation [11]. Importantly, RBC were also capable of driving unresponsive pure T cells activated with PHA to increase cell size and proliferate, reinforcing the notion that RBC bioactivities act directly on the activated T cells without the need of accessory cells (i.e. monocytes, B cells or NK cells), ruling out erythrophagocytosis mechanisms, attributed to monocytes [31]. Moreover, we have shown that this effect is associated with the preservation of the antioxidant state of the activated T cells, namely the level of cell surface thiols. These results are in agreement with our previous results showing that RBC are capable of reducing the intracellular production of oxygen and nitrogen radicals in activated T cells [12]. The possible role of NF- κ B, a transcription factor regulated by oxidative stress, in mediating the RBC effect remains to be determined. NF- κ B is known to block apoptotic pathways that in turn will favor cell division [32]. The pro-survival bioactivities of RBC are of such magnitude that they were able to rescue activated T cells from cell death induced by serum deprivation and allow the activated T cells to enter multiple cycles of cell division. Besides stressing the importance of the bioactivities carried out by RBC, these results indicate that the RBC bioactivities do not need any serum factor to augment survival and proliferation of activated T cells. Based on the kinetic studies, we concluded that the RBC bioactivities are not exerted during the immediate hours following T cell activation but later on, perhaps after 18-24 hours when key cell cycle regulators have already been activated. As a result there is a hastened entry of activated T cells into the cell cycle in cultures with RBC in comparison with cultures without, namely at days 2 and 3 after activation, and our results point to the PI3K/Akt axis as a putative target of the RBC bioactivities. To our knowledge these are the first studies addressing the study of the

activation of signalling cascades in T cells induced by RBC bioactivities. This signalling pathway is likely a candidate of the effect of the cell growth and survival RBC bioactivities. Indeed, Akt is thought to be an upstream regulator of NF- κ B, reinforcing the possible involvement of this transcription factor on the RBC effect [33].

From these studies we can conclude that two main bioactivities can be distinguished in RBC: (1) survival bioactivities responsible for inhibiting pathways related with apoptosis and (2) cell growth bioactivities responsible for potentiating pathways related with progression of T cells into the cell cycle. The combined action of these bioactivities is capable of rescuing activated T cells from cell death induced by serum deprivation. In this context, by using the immunosuppressive drugs rapamycin and cyclosporine A, we have provided evidence that the RBC bioactivities appear to use the calcineurin and mTOR pathways differently. Thus, we have shown that the RBC bioactivities were able to revert very efficiently the immunosuppressive effect caused by Rapa, but not by CsA when PBL were pre-treated with these drugs. While Rapa is known to bind to a family of proteins known as FK506-binding proteins (FKBP), more specifically to FKBP12, CsA binds to a family of proteins called cyclophilins, namely to cyclophilin A [22,24]. While the Rapa-FKBP complex binds and inhibits the kinase mTOR, the CsA-CypA complex binds and inhibits the phosphatase calcineurin [24]. The capacity of RBC to efficiently counteract the inhibition caused by Rapa but not by CsA has, in our opinion, several interpretations. First, the peptidyl-prolyl-isomerases FKBP12 and CypA are part of the RBC proteome and compete for the binding of the drugs present within the pre-incubated T cells releasing the inhibitory effect and allowing the RBC bioactivities to exert their function. Although FKBP12 and CypA are present in RBC [34,35], we think this hypothesis unlikely. Second, different amounts of CsA-CypA and Rapa-FKBP12 complexes within the T cells used in our assays might underlie the differences observed. This hypothesis is not unlikely if we consider early studies indicating differences in the amount of CypA among lymphocyte populations [36]. Third, the RBC bioactivities are capable by themselves to overcome efficiently the inhibition caused by Rapa but not by CsA, through the activation of downstream signalling pathways capable of driving T cells into the cell cycle. These pathways may be independent of mTOR activity but not from upstream pathways, such as those resulting from calcineurin activity, therefore justifying the differences observed. In this context, a recent study has shown that the inhibitory capacity of Rapa in leukemia cells can be overcome by IL-7-mediated signalling, most likely through the alternative activation of the p70 S6 kinase [37]. The RBC bioactivities may act in a similar manner. Nevertheless, the two latter hypotheses are not mutually excluded.

Considering that the Rapa and CsA ligands are also present in RBC [34,35], we wanted to ascertain whether treatment of RBC with these drugs had any effect on their bioactivities. Most interestingly, pretreatment of RBC with CsA, but not Rapa, completely blocked the bioactivities of RBC. Similar results were obtained when the drugs were added directly to cultures, mimicking an *in vivo* drug administration. To our knowledge, these are novel results regarding the biology of RBC and they unveil a novel relationship between RBC and the immunosuppressive drug CsA. The fact that the bioactivities carried by RBC are also exerted with heterologous RBC raises important issues in the context of blood transfusions administered to patients undergoing immunosuppression [9]. In this context, the complete block of the RBC bioactivities described herein by CsA and not Rapa raises interesting questions. First, both drug ligands are present in the RBC but only CsA interferes with the RBC bioactivities that regulate T cell growth, survival and proliferation, pointing to a specific inhibitory effect. Indeed, pretreatment of RBC with LY294002, an inhibitor of PI3K lacking an endogenous RBC ligand had no effect on the RBC bioactivities. All these results suggest the possible involvement of CypA, the CsA ligand, as being responsible for the RBC bioactivities. In this context, recent studies point to CypA as a novel growth factor involved in cell-cell communication that is secreted under inflammatory conditions by several types of leucocytes and endothelial cells [26,27]. Unfortunately, we found that recombinant human CypA *per se* is incapable of augmenting T cell growth, survival and proliferation as intact RBC do (unpublished results). The possibility that CypA involvement could be related to its isomerase activity within the RBC or to the regulation of the secretion of bioactive factors present within the RBC remains to be elucidated.

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Chapter 3

**Red blood cells release factors with growth and survival
bioactivities for normal and leukemic T cells**

**Adapted from Antunes RF, Brandão C, Maia M and Arosa FA
(Submitted, under revision)**

3.1. ABSTRACT

Human red blood cells (RBC) are emerging as a cell type capable to regulate biological processes of neighboring cells. Hereby, we show that human RBC conditioned media (RBC-CM) contains bioactive factors that favor proliferation of normal and leukemic Jurkat T cells and therefore is called EDGSF (erythrocyte derived growth and survival factors). Flow cytometry and electron microscopy in parallel with bioactivity assays revealed that the EDGSF are present in the vesicle-free supernatant (RBC-sup), which contains up to 20 different proteins. The EDGSF are thermo sensitive and do not contain lipids. Native PAGE followed by passive elution and mass spectrometry (MS) identification reduced the potential EDGSF to hemoglobin (Hb) and peroxiredoxin II (PrxII). 2D DIGE of the EDGSF revealed the presence of multiple Hb oxy-deoxy states and PrxII isoforms differing in their isoelectric point akin to the presence of beta globin chains. Our results show that RBC release protein factors with the capacity to sustain T cell growth and survival. These factors may play an unforeseen role in sustaining malignant cell growth and survival.

3.2. INTRODUCTION

Red blood cells (RBC) are emerging as a pleiotropic cell with the capacity to engage in reciprocal crosstalk with nearby cells during their life time. The mounting evidence indicates that in addition to transporting oxygen and carbon dioxide, RBC also regulate vascular contractility, neutrophil apoptosis, T cell growth and survival, proteinase release by fibroblasts and IL-12 release by dendritic cells [1-5]. Recent studies have provided mechanistic insights to explain some of RBC pleiotropy. Thus, while inhibition of IL-12 release by dendritic cells is explained by the interaction of SIRP α with CD47 present on RBC [5], vascular contractility is due to the action of nitric oxide release by hemoglobin [2]. The few studies investigating the mechanisms used by RBC to inhibit T cell apoptosis and favor proliferation have shown that they are linked with upregulation of cytoprotective proteins and downregulation of oxidative stress [6,7].

One reasonable mechanism for activated T cells to increase their cell cycle progression, while lowering activation-induced cell death to marginal levels, in response to the presence of RBC would be by means of interactions with RBC receptors or with RBC released factors that will trigger signals on T cells involved in cell cycle and survival pathways. Although RBC were proposed to enhance T and B cell responses through CD58-CD2 interactions [8], we have shown that molecular interactions between GPI-

linked receptors present on RBC and their ligands present on T cells are not involved [9,10]. On the other hand, it has been reported that RBC release vesicles containing erythrocyte proteins upon changes of the environment, such as pH lowering or ATP and calcium depletion [11-15]. Although RBC derived vesicles are cleared from the circulation, during their life-time they are putative carriers of bioactivities for other cells [16,17]. In view of studies showing that dendritic cell derived vesicles, or exosomes, can activate T cells [18,19], the possibility that vesicles, or other factors, released by RBC may participate in the enhancement of cell cycle and survival signaling pathways on activated T cells has never been addressed.

In our previous studies we observed that stimulation of human T cells in vitro in the presence of RBC resulted in a remarkable increase in proliferation regardless of the addition of RBC being done at the start of the culture or 24 hours later, suggesting that the growth and survival effects of RBC are exerted on pathways that have been turned-on [3]. In this context, we have hypothesized that RBC may release factors that promote survival and proliferation of activated, but not quiescent T cells. By studying RBC conditioned medium we demonstrate that RBC release particulate (i.e. vesicles) as well as soluble factors into the extracellular milieu. We show that the erythrocyte-derived soluble factors, but not the vesicles, hold bioactivities capable to increase T cell growth, proliferation and survival of either activated normal T cells or leukemia T cells. These bioactivities are termed EDGSF for erythrocyte derived growth and survival factor and include up to 20 different proteins. Proteomic analysis narrowed the potential EDGSF to hemoglobin and peroxiredoxin. Overall, these results suggest that intracellular RBC proteins are potentially physiologic mediators of the response of T cells to activation that under malignant conditions may play an unforeseen role in sustaining leukemia cells.

3.3. MATERIAL AND METHODS

3.3.1. Reagents and antibodies

Phytohaemagglutinin (PHA-P, from *Phaseolus vulgaris*), antibiotic-antimycotic solution (APS), bovine serum albumin (BSA), propidium iodide (PI), ionomycin (Ion, from *Streptomyces conglobatus*), phorbol dibutyrate (PdB), human hemoglobin, calcium ionophore A23187 and Sudan black B were from Sigma-Aldrich (Madrid, Spain). Lymphoprep was from Nycomed (Oslo, Norway). RPMI 1640 GlutaMAX, Hanks Balanced Salt Solution (HBSS) and inactivated Fetal Bovine Serum (FBSi) were obtained from

Gibco (Paisley, Scotland). Human rIL-2, rIL-7 and rIL-15 were obtained from R&D Systems (Minneapolis, US). 5-(and -6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) and Annexin V-Alexa488 were purchased from Molecular Probes (Amsterdam, The Netherlands). Anti-human glycophorin A PE-conjugated (clone AME-1, CD235a-PE) was from ImmunoTools (Friesoythe, Germany). Anti-human CD3-PE, CD4-APC, CD8-PE-Cy5 and rabbit anti-mouse (RAM)-fluorochrome conjugated antibodies were from DAKO (Glostrup, Denmark). Anti-human hemoglobin (clone GTX77484) was from GeneTex Inc. (Irvine, US). Prx II was obtained from Lab Frontier (Seoul, South Korea). Cleanascite™ was from Biotech Support Group (North Brunswick, US). Molecular weight rainbow markers were from Amersham Biosciences (Uppsala, Sweden).

3.3.2. Cells

Fresh peripheral blood mononuclear cells (PBMC) were obtained from buffy coats after centrifugation over Lymphoprep. PBMC were washed twice with HBSS and contaminating RBC lysed in red cell lysis solution (RCLS, 10mM Tris, 150mM NH₄Cl, pH 7.4) for 10 min at 37°C. RBC were collected from the pellet region after Lymphoprep centrifugation, washed twice with HBSS and diluted 1:10 in RPMI supplemented with 1% APS solution and stored at 4°C until use. RBC purity was assessed by flow cytometry and CD235a labelling which revealed >99.5% CD235a⁺. Partially purified peripheral blood T lymphocytes (PBL) were obtained after culture of PBMC overnight in RPMI supplemented with 1% APS solution and 5% FBSi. The recovered non-adherent cell suspensions were routinely >85% CD3⁺ T cells and are referred to as PBL. Additionally, the leukemic T cell line Jurkat (clone E 6.1) was used and maintained in two different culture conditions. A high-serum medium (RPMI supplemented with 1% APS solution and 10% FBSi) and a low-serum medium (RPMI supplemented with 1% APS solution and 2.5% FBSi). In both conditions culture medium was changed every 3 days. To remove dead cells in the serum deficient cultures, Jurkat T cells were centrifuged over Lymphoprep (800xg, 12 min), live cells collected from the interface and after washing twice with 1x PBS placed again in culture.

3.3.3. Production and isolation of RBC-CM, RBC-sup and RBC-ves

To obtain RBC conditioned media, RBC (60 x 10⁶/ml) were cultured in 6-well plates in an incubator at 37°C, 5% CO₂ and 99% humidity for 48 hours (thereafter designated by RBC-CM) in RPMI media supplemented with 1% APS solution. RBC-CM was obtained after two sequential centrifugations at 1.700xg for 10 min to remove RBC. The absence of RBC as well as the presence of vesicles in the RBC-CM was confirmed by flow cytometry after staining with CD235a, a monoclonal antibody against glycophorin A. Vesicles

revealed 99% CD235a⁺. Annexin V staining of RBC using Ca²⁺-based staining buffer (see below) was performed to evaluate the RBC apoptotic state in culture. Alternatively, and as a control, RBC-CM was produced after treating RBC cultures with 5μM of A23187, a Ca²⁺ ionophore, in RPMI media supplemented with 1mM CaCl₂. A23187 treatment was stopped by addition of 5mM EDTA. RBC-derived vesicles (RBC-ves) were isolated from RBC-CM, previously depleted of RBC, after ultracentrifugation at 100.000xg for 2 h, at 4°C in a Sorvall Ultra Pro80 centrifuge with a T1270 angular rotor. After centrifugation the non-vesicular supernatant (RBC-sup) and the pellet containing the vesicles (RBC-ves) were collected, quantitated using the BCA protein assay kit (Pierce, Rockford, IL, US) and either used immediately for bioactivity assays *in vitro* or frozen at -70°C for further studies. On average the RBC-sup and the RBC-ves produced by 60x10⁶ RBC contained approximately 20μg/mL and 1μg/mL of protein, respectively, which agrees with the amount of protein in the bulk RBC-CM (that ranges between 18 and 22μg/mL). Absence of vesicles in the RBC-sup was confirmed by flow cytometry.

3.3.4. CFSE labeling

Ten million PBL were labeled with CFSE at a final concentration of 5μM for 10 min at 37°C, with occasional mixing. Then, cells were washed twice with PBS/20% FBSi and resuspended in culture media. Analysis of cells immediately after CFSE labeling indicated a labeling efficiency higher than 99%.

3.3.5. RBC-sup treatments

RBC-sup was routinely concentrated using Vivaspın 20 ultrafiltration spin columns with a 3 kDa molecular weight cut-off (MWCO) (Sartorius, Goettingen, Germany) and quantitated. In the *in vitro* assays the concentrated RBC-sup was diluted to 1X to maintain the original concentration of the RBC-CM. For thermostability studies, 100ul of the concentrated RBC-sup was boiled at 56°C, 72°C or 96°C for 30 min in a heat block. The RBC-sup was centrifuged at 11.000xg prior to the *in vitro* bioactivity assays. For lipid depletion, Cleanascite™ was added to the RBC-sup in a ratio 1:4 and the mixture incubated first in a rotator at room temperature for 10 min, followed by a further incubation at 4°C for 30 min, following manufacturer's instructions. Then, the mixture was centrifuged to remove the resin and the RBC-sup collected and concentrated as indicated above before the *in vitro* bioactivity assays. Differential concentration of RBC-sup proteins was done by using Vivaspın 20 ultrafiltration spin columns with 30 and 50 MWCO (Sartorius). Recovery of RBC-sup from the upper and lower fraction after centrifugation allowed obtaining four different fractions that were subsequently assayed for bioactivity *in vitro*.

3.3.6. Culture conditions

PBL (1.5×10^6) and Jurkat T cells (0.5×10^6) were cultured in 6-well plates, in a final volume of 5mL for up to 7 days in an incubator at 37°C, 5% CO₂ and 99% humidity. PBL were either left unstimulated or stimulated with PHA-P (5µg/mL) and either IL-2, IL-7 or IL-15, all at 10ng/mL. Jurkat T cells were left unstimulated. PBL and Jurkat T cells were cultured in the absence or presence of autologous RBC at a PBL:RBC ratio of 1:10 and 1:100 respectively, or in the presence of 1X RBC-CM, RBC-ves or RBC-sup. Culture media was RPMI 1640 GlutaMAX supplemented with 1% APS solution and 1% FBSi. In some experiments PBL were cultured in the absence of serum in RPMI 1640 GlutaMAX supplemented with 1% APS. At the end of the culture PBL were harvested, washed and acquired in a FACSCalibur (Becton-Dickinson, Mountain View, CA). For each sample 50.000 events were acquired using FSC/SSC characteristics and analysed using CellQuest or FlowJo software.

3.3.7. Flow cytometry determinations

Cell stainings were normally performed at 4°C for 30 min in 1X PBS or staining buffer (PBS, 0.2% BSA, 0.1% NaN₃) in 96-well round-bottom plates (Greiner bio-one, Frickenhausen, Germany). In cultures that received RBC, erythrocytes were first lysed with RCLS. Irrelevant mAb were used as negative controls to define background staining. T cell death and survival were determined by two methods: 1) a decrease in cell size according to forward light scatter (FSC)/side light scatter (SSC) parameters; 2) double Annexin V and PI staining, for 15 min at room temperature in the dark using Ca²⁺-based staining buffer (10mM HEPES/140mM NaCl/2.5mM CaCl₂). T cell activation and proliferation were studied by two methods: 1) determination of blast transformation according to FCS/SSC parameters; 2) CFSE fluorescence loss. Rounds of cell divisions were determined by sequential halving of CFSE-fluorescence intensity

3.3.8. Transmission electron microscopy

RBC-derived vesicles were fixed in a solution of 1.25% glutaraldehyde/ 4% paraformaldehyde and post-fixed in osmium 1% in sodium cacodilate. After washing with H₂O, the pellets were fixed in uranyl acetate 1%, and placed overnight in glutaraldehyde 2.5%. After dehydration, the samples were embedded in EPON. Thin sections were mounted on grids, stained with lead citrate and post-stained with uranyl acetate. Images were acquired with a Zeiss 902A (West Germany).

3.3.9. SDS/PAGE, native PAGE and passive elution

The RBC-sup was precipitated with 10% Trichloroacetic acid (TCA) for 30 min on ice, centrifuged at 11.000xg for 15 min and then washed in cold acetone overnight at -20°C. Samples were centrifuged again at 11.000xg for 15 min and resuspended in sample buffer (25% Tris pH 6.8, 10% SDS, 0.5% bromophenol blue, 10% glycerol), heated at 96°C for 10 min and quantitated by the BCA protein assay kit (Pierce). Aliquots corresponding to 20-25µg of protein were resolved in 15% SDS/PAGE under reducing conditions. Gels were stained with Coomassie brilliant blue R250 (Sigma-Aldrich) to visualize protein bands. In some experiments, gels were also stained using silver staining. For native-PAGE studies aliquots of the precipitated RBC-sup were diluted in sample buffer (0.5M Tris pH 8.0, 0.1M DTT, 50% saccharose/10x bromophenol blue) and resolved by 6% native PAGE in a 4°C room. For visualization of proteins, gels were stained with Coomassie blue solution or silver staining. For bioactivity assays, native gel fractions were excised, washed with 1x PBS, and placed in eppendorfs containing 1ml of 1x PBS. Proteins were allowed to passively elute out of the gel slices for 24 hours at 4°C. Afterwards, gel slices were removed and eluted proteins concentrated using Vivaspın 500 spin columns with 3 MWCO (Sartorius). Concentrated proteins were washed several times on Vivaspın 500 spin columns using RPMI media supplemented with 1% APS and stored at -70°C.

3.3.10. 2D DIGE and mass spectrometry analysis

Two dimensional differential in-gel electrophoresis (2D DIGE) was performed by Applied Biomix (Hayward, CA, USA). Briefly, aliquots of concentrated RBC-sup proteins were covalently linked to CyDye and run on first dimension isoelectric focusing, followed by a second dimension SDS/PAGE. Image analysis was performed using DeCyder software, and spots of interest were picked and analyzed by mass spectrometry (MALDI/TOF/TOF). Further protein identification was performed after 1D electrophoresis. Briefly, aliquots of concentrated RBC-sup proteins were run on 15% SDS/PAGE followed by Coomassie brilliant blue R250 (Sigma-Aldrich) and protein bands excised. Protein identification (MALDI/TOF/TOF) was performed by Alphalyse (Odense, Denmark).

3.3.11. Hemoglobin immunodepletion

For Hemoglobin (Hb) depletion, concentrated RBC-sup was subjected to sequential immunoprecipitations using the Seize[®] Primary Immunoprecipitation Kit (Pierce). Briefly, Handee[™] Spin Cup Columns were equilibrated with AminoLink[®] Plus Coupling Gel, centrifuged and washed with Coupling Buffer (0.1M Na₂PO₄, 0.15M NaCl, pH 7.2). 400µg of affinity purified hemoglobin antibody was added to the spin columns and mixed together

with 5 μ L of reducing agent (0.5M sodium cyanoborohydride) for 4 hours in an orbital shaker. After centrifugation, quenching buffer (1M Tris-HCl, pH 7.4) and 4 μ L of reducing agent were added to gel spin columns, incubated 30 min with gentle end-over-end mixing. Then the gel was washed with Binding Buffer (Dulbecco's 1X PBS) and the concentrated RBC-sup added to the spin columns and incubated for 3 hours in an orbital shaker for immunoprecipitation. Afterwards, RBC-sup depleted from hemoglobin was recovered from the flow-through centrifugation and immunoprecipitated 2 additional times after Hb elution. For Hb elution ImmunoPure[®] Elution Buffer was added to the gel-bound complex, after washed with IP Buffer (0.025M Tris, 0.15M NaCl, pH 7.2), and then mixed and centrifuged. The hemoglobin was eluted by performing this last step 3 times. Recovered RBC-sup depleted from hemoglobin was subsequently assayed for *in vitro* bioactivity and simultaneously resolved in 15% SDS/PAGE and visualized by silver staining.

3.3.12. Statistical analysis

Statistical analysis was performed using Excel or GraphPad[™] Prism 5 software. Student's t test was used to evaluate the significance of the differences between group means. Statistical significance was defined as $p < 0.05$.

3.4. RESULTS

3.4.1. RBC bioactivities are present in conditioned medium

We have previously shown that RBC sustain the growth and survival of peripheral blood T cells activated *in vitro* without the need of accessory cells or physical contact [6,7,10]. Considering that RBC release factors into the extracellular milieu during maturation or upon environmental changes in the culture conditions [11-15], we wanted to ascertain whether the bioactivities carried by RBC are present in conditioned media. To that end, we placed RBC in culture from 90 min to 48 hours to produce RBC conditioned media (RBC-CM) and tested its bioactivity towards activated PBL after complete removal of RBC by two rounds of low-speed centrifugation. As illustrated in Figure 3.1A, RBC-CM closely reproduced the proliferation (left histograms) and cell growth and survival (middle histograms) bioactivities of intact RBC towards PHA-activated PBL. Importantly, CD3 labeling at the end of the culture showed that only T cells benefited from the RBC-CM bioactivities (right dot-plots), with preferential expansion of CD3⁺CD8⁺ T cells (data not shown). Overall, RBC-CM induced a statistically significant increase in both proliferation (Fig. 3.1B) and survival (Fig. 3.1C). In agreement with our recent studies (Chapter 2),

RBC-CM also induced an increase in proliferation when pure T cells were used and when TCR-independent stimuli, such as the gamma-common cytokines IL-2, IL-7 and IL-15 were used (Fig. 3.1D, E; [10] and data not shown). Contrary to PHA, all three cytokines induced high levels of survival. As result, the presence of RBC-CM did not impact further on survival (data not shown).

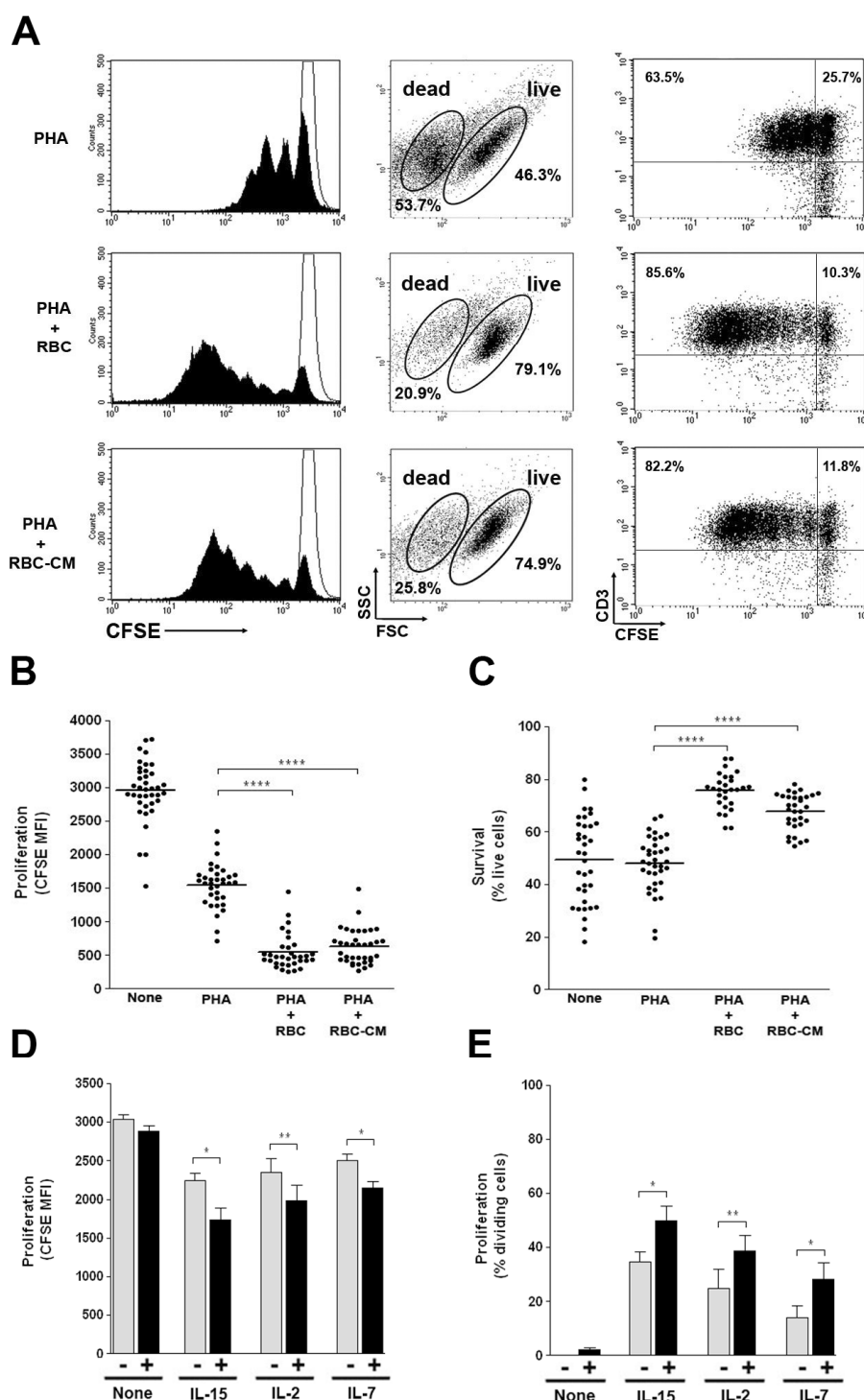


Figure 3.1. RBC bioactivities are present in RBC-CM. PBL (1.5×10^6) were labeled with CFSE and cultured either alone or with RBC (1:10 ratio) or RBC-CM in the absence or presence of PHA

for 7 days, and then harvested, stained, and acquired in a FACSCalibur. **A**, Filled histograms illustrate the T cell proliferation levels obtained in the different conditions, as determined by CFSE fluorescence loss. Empty histograms show CFSE fluorescence in unstimulated PBL. Middle dot-plots (FCS vs SSC) show the percentage of T cell death (left gated population, as determined by positive PI labeling) and survival (right gated population, as determined by negative PI labeling) in the different culture conditions. Right dot-plots (CFSE vs CD3) show CFSE fluorescence loss versus CD3 staining in the different culture conditions. The percentage of CD3⁺ T cells that divided (upper left quadrant) and the percentage that did not enter division (upper right quadrant) are indicated. **B-C**, Graphs show the percentage of proliferating (**B**) and surviving (**C**) T cells (mean $\pm$ SEM, n=30) in the indicated culture conditions. **D-E**, Graphs show the level of proliferation, as determined by CFSE fluorescence loss (**D**), and the percentage of dividing (**E**) T cells (mean $\pm$ SEM, n=5) for the different conditions in the absence (-) or presence (+) of RBC-CM. *P* values are shown: (****, $p < 0.0001$; **, $p < 0.01$; *, $p < 0.05$).

In order to exclude the possibility that the bioactivities present in the RBC-CM were dependent upon the presence of serum factors, cultures of activated PBL with and without serum were performed (Fig. 3.2). Importantly, the cell growth and survival bioactivity present on the RBC-CM was capable to rescue activated T cells from apoptosis induced by serum-deprivation, and allow T cells to proliferate to levels seen in cultures with serum (Fig. 3.2).

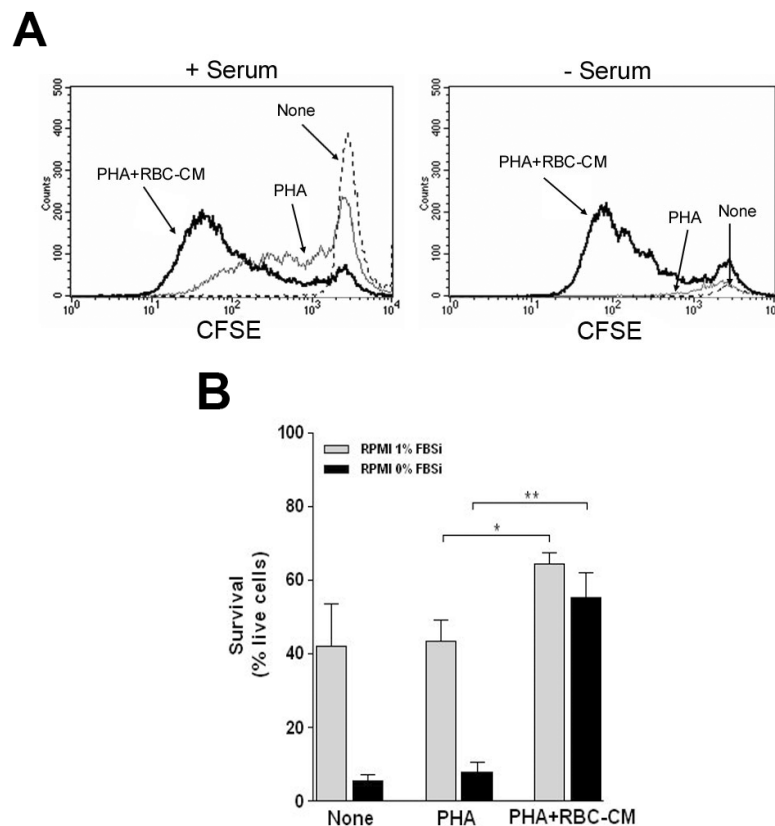


Figure 3.2. RBC-CM bioactivities are independent of serum factors. PBL (1.5×10^6) were labeled with CFSE, stimulated with $5 \mu\text{g/mL}$ of PHA in culture media with or without serum and cultured for 7 days in the absence or presence of RBC-CM. Then, cells were harvested and acquired in a FACSCalibur. **A**, Histograms show CFSE fluorescence loss in cultures of PHA-activated PBL with (left histogram) or without (right histogram) serum, in the absence (grey line) or

presence (black line) of RBC-CM. CFSE fluorescence in unstimulated PBL (dotted-line) is shown. **B**, Graph shows the percentage of live cells (mean $\pm$ SEM, n=3) for the different culture conditions in the presence (grey bars) or in the absence (black bars) of serum. *P* values are shown: (**, $p<0.01$; *, $p<0.05$).

Next, we wanted to determine whether RBC-CM bioactivities were also exerted on Jurkat cells, a human leukemia T cell line that constitutively produces IL-2. Since most *in vitro* cultures are performed in the presence of high amounts of serum (10% or more) which drive cells to proliferate and divide to elevated levels, Jurkat T cells were maintained in low-serum medium (2.5% FBS), besides normal culture conditions in high-serum medium (10% FBS). In these former conditions, Jurkat T cells showed not only a decrease in cell survival, but also impaired proliferation and cell division when compared with cells maintained in high-serum medium (Figure 3.3). Interestingly, when Jurkat T cells were grown in this high-serum medium (10% FBS) and then cultured in the presence of RBC-CM or intact RBC for six days, they proliferated better than Jurkat T cells cultured alone (Fig. 3.3A, left histogram). The growth and survival bioactivities present in RBC-CM were even more evident when Jurkat T cells were expanded in low-serum medium (2.5% FBS) prior to the six-day culture period (Fig. 3.3A, right histogram). Overall, the increase in Jurkat T cell proliferation levels in the presence of RBC-CM was statistically significant (Fig. 3.3C). Determination of survival levels in cultures of Jurkat T cells grown in low-serum medium (2.5% FBS) prior to the six-day culture by using Annexin V and propidium iodide showed also a marked improvement when RBC-CM was present (Fig. 3.3B), even though they were slightly lower than those induced by intact RBC (Fig. 3.3B). Overall, the increase in the level of Jurkat T cell survival in the presence of RBC-CM was statistically significant (Fig. 3.3D), indicating that the RBC-CM contains the bioactivities carried by intact RBC.

The same above mentioned results were even observed earlier, as for the third day of culture, in which significant differences between the RBC and RBC-CM efficiency were not observed (data not shown). The capacity of the RBC-CM bioactivities to rescue Jurkat T cells from apoptosis is even more striking when maintained under highly restrictive conditions (1% FBS) or even when cultured in the absence of serum (data not shown), though they appear to be exerted with different efficiencies towards normal and leukemic T cells.

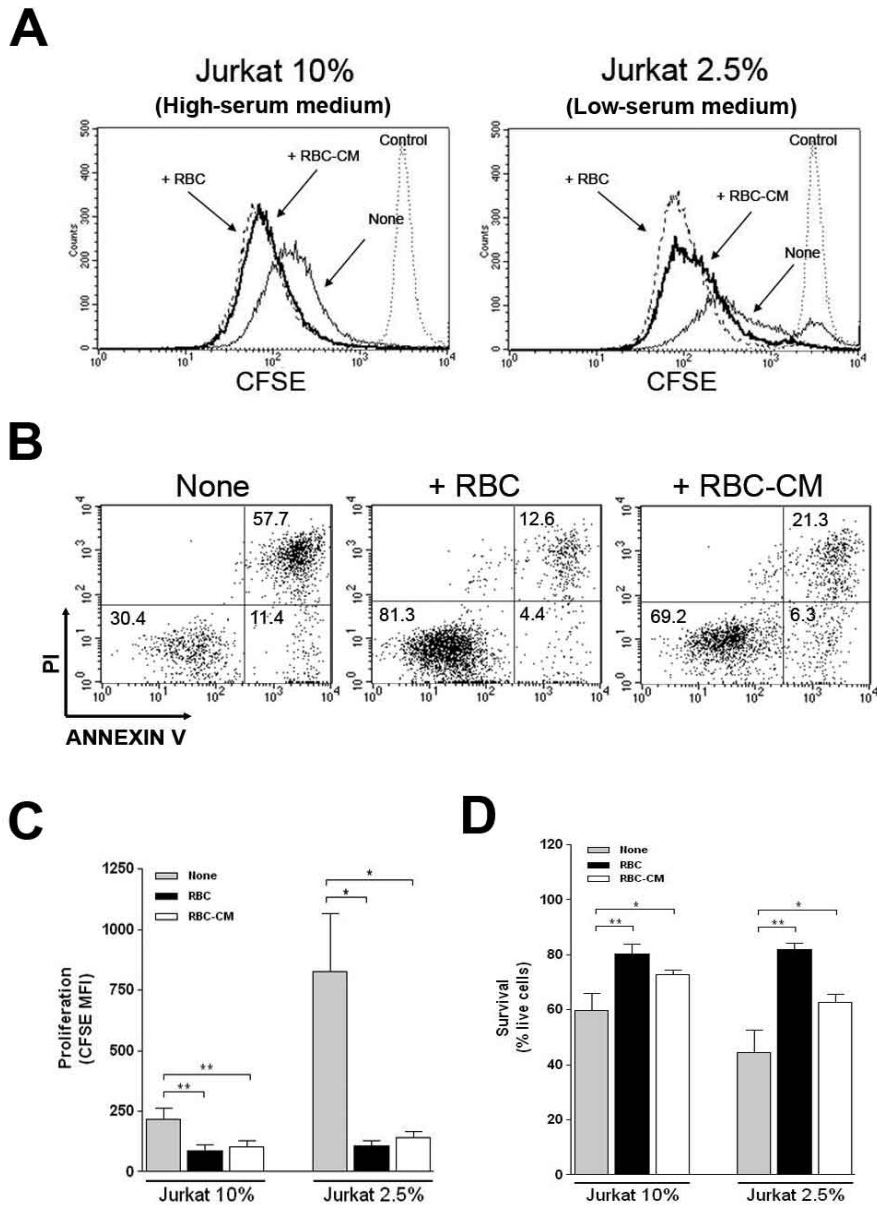


Figure 3.3. RBC bioactivities enhance proliferation and inhibit cell death of leukemic T cells. Jurkat T cells (0.5×10^6), grown in high-serum (10% FBS) or low-serum (2.5% FBS) medium, were labeled with CFSE and cultured in the absence or presence of RBC or RBC-CM for 6 days, and then harvested, stained and acquired in a FACSCalibur. **A**, Histograms show loss of CFSE fluorescence for the two Jurkat T cell lines cultured in the absence (grey) or presence of RBC-CM (black) or RBC (dashed). Control CFSE fluorescence at day 0 is shown (dotted histogram). One representative out of 6 different experiments is shown. **C**, Graph shows the level of Jurkat T cell proliferation (mean $\pm$ SEM, $n=6$), as determined by CFSE fluorescence loss in the indicated culture conditions. **B**, Dot-plots show Annexin V versus PI staining in Jurkat T cells grown in low-serum conditions and then cultured for six days in the indicated culture conditions. The percentage of live cells (lower left quadrant), early apoptotic (lower right quadrant) and late apoptotic (upper right quadrant) are indicated. One representative out of 6 different experiments is shown. **D**, Graph shows the percentage of Jurkat T cell survival (mean $\pm$ SEM, $n=6$) as determined by Annexin V and PI negative labeling in the indicated culture conditions. *P* values are shown: (**, $p<0.01$; *, $p<0.05$).

3.4.2. RBC conditioned medium contains bona-fide vesicles

Recent reports have shown that vesicles secreted by professional antigen presenting cells, such as dendritic cells, may work as activation devices for T cells, either via APC or acting directly on T cells [18,19]. Thus, we wanted to ascertain whether the bioactivities present in RBC-CM could be mediated by RBC-derived vesicles. To that end, short-term (1-3 hour) and long-term (1-2 day) cultures of RBC were examined for the presence of vesicles. As a control, we also analyzed RBC cultures after treatment with the calcium ionophore A23187. Both cultures were stained with anti-glycophorin antibodies and directly analyzed by flow cytometry after gating on CD235a positive events, a known specific marker of RBC. As shown in Figure 3.4A, RBC cultures revealed the presence of intact RBC together with smaller fragments that based in previous studies resembled RBC-derived vesicles [23]. Interestingly, untreated RBC displayed low levels of phosphatidylserine expression in comparison with ionophore-treated RBC, even after a 3 day culture period (Fig. 3.4A; data not shown). Among the different RBC-CM samples studied, it was frequently observed the existence of two populations of vesicles that can be discriminated by size into nanovesicles and microvesicles (Fig. 3.4B). Size calculations done according to the FSC values of the three populations shown in Figure 3.4B and the reported size of RBC (8 μ m) gave sizes that ranged between 100-400nm and 25-75nm for microvesicles and nanovesicles, respectively, which are in agreement with previous reports [12,13]. To confirm that the small CD235a positive events shown in Figure 3.4A and 3.4B were bona-fide RBC-derived vesicles; RBC-CM was depleted of RBC by two rounds of centrifugation to obtain RBC-CM containing only vesicles (Fig. 3.4C, upper dot-plots). Then, RBC-CM was subjected to ultracentrifugation to obtain two fractions: a pellet of vesicles and a non-vesicular supernatant. Analysis of the vesicular fraction from untreated RBC cultures by electron microscopy revealed the presence of mainly round-shaped vesicles whose diameter varied between 75-300 nm, contrasting with ionophore-treated RBC cultures which contained mainly tubular vesicles (Fig. 3.4C, lower graphs). Furthermore, by using an amphiphilic membrane dye, we observed by fluorescence microscopy the incorporation of the lipid dye in vesicles preparations, indicating the existence of a lipid membrane, corroborating the observations of the vesicle bilayer structure also identified by electron microscopy (data not shown).

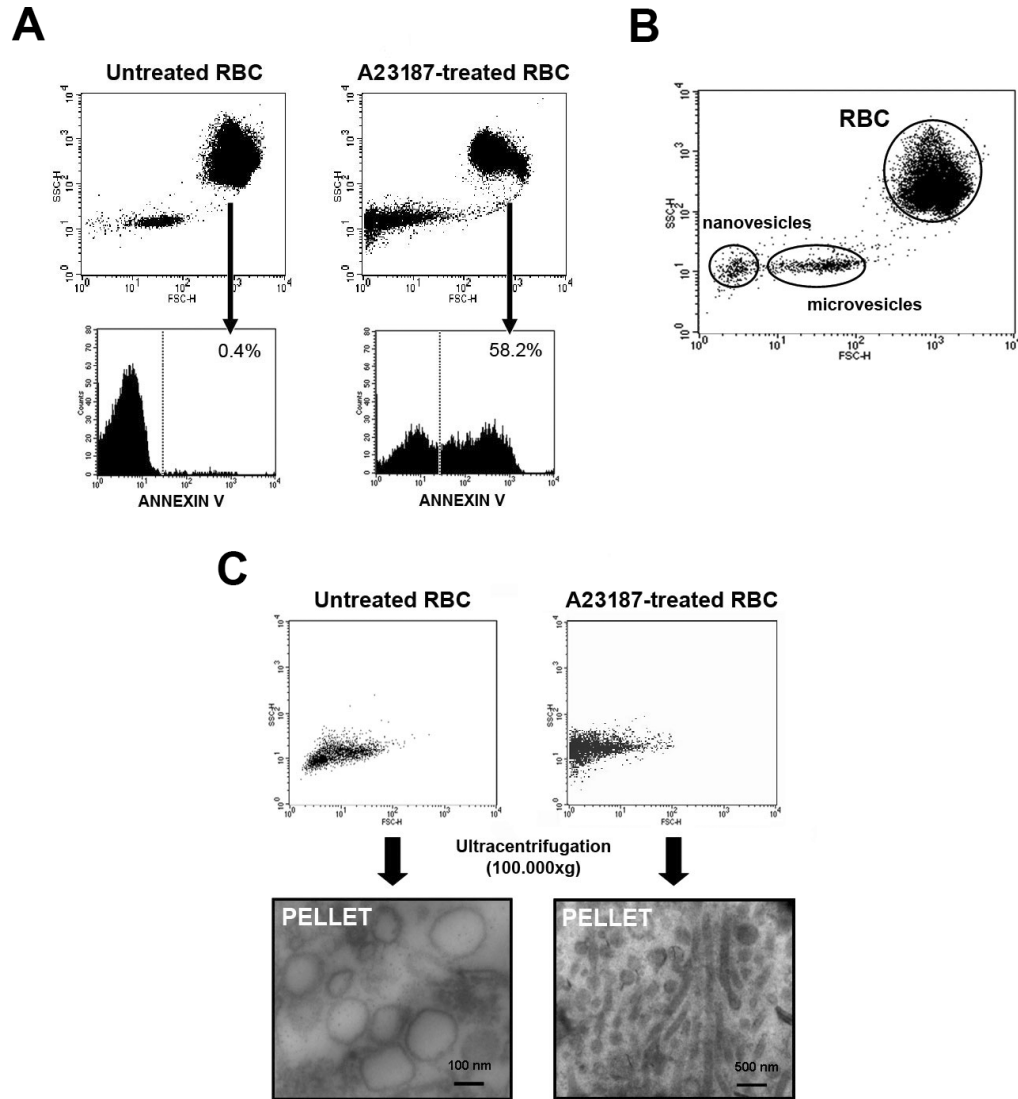


Figure 3.4. RBC spontaneously release vesicles upon in vitro culture. **A**, RBC were cultured in RPMI for 90 min in the absence or in the presence of the calcium ionophore A23187. Two aliquots were harvested and stained with anti-glycophorin A antibodies or Annexin V. Samples were acquired in a FACSCalibur. Dot-plots show RBC (large population) and RBC-derived vesicles (small population) in cultures of untreated or A23187-treated RBC gated in glycophorin A positive events according to FSC and SSC characteristics. Histograms show Annexin V expression in untreated and A23187-treated RBC. **B**, RBC were cultured in RPMI for 90 min in the absence of A23187 and an aliquot stained with anti-glycophorin A antibodies and acquired in a FACSCalibur. Dot-plot shows a representative experiment where micro and nanovesicles can be observed. **C**, Cultures of untreated and A23187-treated RBC were subjected to two rounds of centrifugation at $1.700\times g$ to obtain RBC-CM. An aliquot of the RBC-CM was collected and acquired in a FACSCalibur. Upper dot-plots shows RBC-derived vesicles in each culture condition after gating in glycophorin A positive events. The remaining RBC-CM was subjected to ultracentrifugation at $100.000\times g$ to obtain a pellet fraction containing vesicles as determined by electron microscopy. The RBC-sup obtained after the ultracentrifugation step was free of vesicles (data not shown). The results are representative of 1 out of 6 different experiments performed.

3.4.3. RBC-CM bioactivities are proteinaceous factors present in the non-vesicular fraction

To ascertain whether the vesicles spontaneously released by RBC hold the bioactivities present in the RBC-CM, human PBL were activated with PHA in the absence or presence of RBC-ves or the RBC-sup obtained after the ultracentrifugation step, as indicated in materials and methods. Parameters of activation, growth, proliferation and survival were monitored by flow cytometry as described above. Surprisingly, the results revealed that the RBC-CM bioactivities are confined to the RBC-sup, with the vesicular fraction having no effect on the parameters under study (Fig. 3.5A). The use of higher amounts of RBC-ves, up-to 20 fold higher, gave similar results (data not shown). As summarized in Figure 3.5B, the bioactivities carried out by RBC are spontaneously released into the extracellular milieu and present in the non-vesicular fraction, or RBC-sup, of the RBC-CM. Indeed, the bioactivity of the RBC-sup was dose-dependent and was present even after 90 min of RBC culture (data not shown).

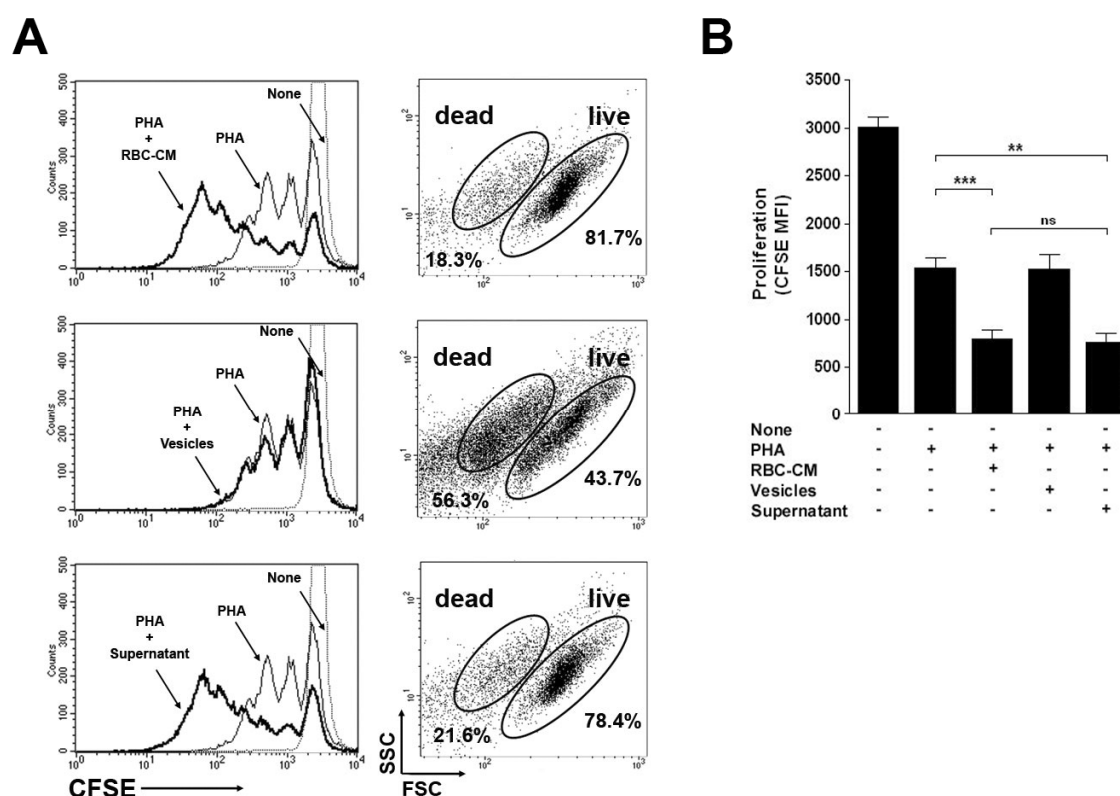


Figure 3.5. RBC bioactivities are secreted soluble factors. PBL (1.5×10^6) were labeled with CFSE, stimulated with $5 \mu\text{g/mL}$ of PHA and cultured in the absence or presence of 1X RBC-CM, RBC-ves or RBC-sup for 7 days, as indicated in “Material and methods”. Afterwards, cells were harvested, stained, and acquired in a FACSCalibur. **A**, Histograms show proliferation levels of activated PBL in each condition, as determined by CFSE fluorescence halving. CFSE levels in unstimulated PBL (none, dotted-line) are also shown. Dot-plots (FCS vs SSC) show the percentage of T cell death (left gated population, as determined by positive PI labeling) and survival (right gated population, as determined by negative PI labeling) of PHA-activated PBL in the presence of

RBC-CM (upper dot-plot), RBC-ves (middle dot-plot) or RBC-sup (lower dot-plot). **B**, Graph shows the level of T cell proliferation (mean $\pm$ SEM, n=10), as determined by CFSE fluorescence loss in the indicated culture conditions. *P* values are shown: (***, $p<0.001$; **, $p<0.01$; ns=not significant).

Experiments studying the thermo stability of the RBC-sup showed a temperature-dependent loss of bioactivity with significant loss above 56°C (Fig. 3.6). Although we could not detect lipids in the RBC-sup by Sudan black staining, we wanted to exclude the possibility that a lipid compound was responsible for the bioactivity. As shown in Figure 3.6, treatment of the RBC-sup with a lipid-depletion resin did not alter significantly its bioactivity. These results pointed to proteinaceous factors as likely mediators of the bioactivity present in the RBC-sup, which we named EDGSF (for erythrocyte derived growth and survival factors).

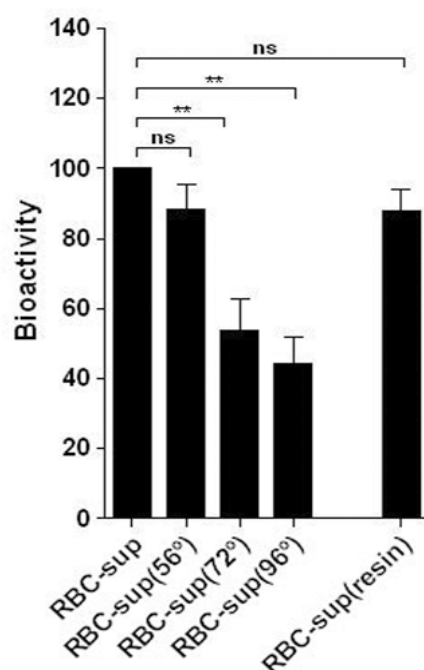


Figure 3.6. RBC bioactivities are thermo labile and not mediated by lipids. PBL (1.5×10^6) were labeled with CFSE, stimulated with 5 μ g/mL of PHA and cultured for 7 days in the presence of untreated RBC-sup or RBC-sup that was previously treated at 56°C, 72°C or 96°C for 30 min, or with a resin for lipid removal as described in “Material and methods”. Afterwards, cells were harvested, stained, and acquired in a FACSCalibur. Graph shows the bioactivity (CFSE fluorescence loss, mean $\pm$ SEM, n=6) observed with the different treated RBC-sup. Bioactivity of the untreated RBC-sup was normalized to 100%. *P* values are shown: (**, $p<0.01$; ns=not significant).

3.4.4. Identification of the erythrocyte-derived cell growth and survival factors

To examine the protein content of the supernatant, the RBC-CM was harvested, centrifuged twice to remove RBC and subjected to ultracentrifugation to obtain the non-vesicular fraction or RBC-sup. Then, the RBC-sup was precipitated and proteins analyzed either by 1D or 2D electrophoresis. As shown in Figure 3.7A, about 20 different protein bands were detected by Coomassie blue staining. Silver staining did not reveal additional protein bands. Although 2D analysis of the same RBC-sup revealed additional protein spots at 30 and 15 kDa (Fig. 3.7B), they represent the same protein but with different

isoelectric points (see below). Identification of the protein bands shown in the 1D gel revealed the presence in the RBC-sup of mainly erythrocyte proteins (Table II).

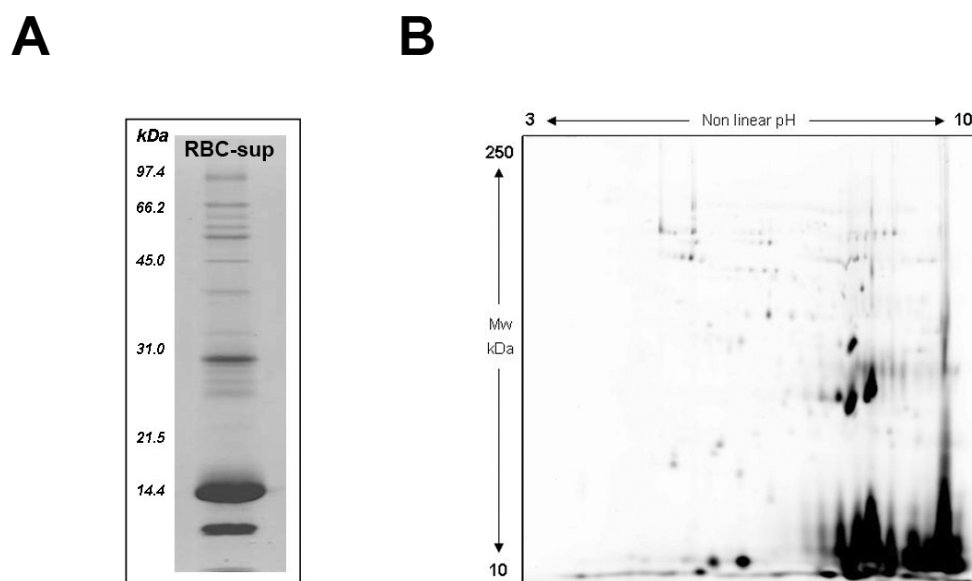


Figure 3.7. Profile of proteins secreted by RBC. RBC-sup was obtained and concentrated as indicated in “Materials and methods”. **A**, Graph shows the protein profile of a representative RBC-sup sample after separation in a 15% SDS/PAGE and visualization by Coomassie blue R250 staining. Molecular weight markers are indicated. **B**, Graph shows the protein profile of a representative RBC-sup sample after two dimensional differential in-gel electrophoresis (2D DIGE), as indicated in “Material and methods”.

In order to reduce the number of possible EDGSF candidates among the proteins listed in Table II, we undertook several proteomic approaches in combination with *in vitro* studies assessing the cell growth and survival bioactivities towards dividing T cells. Thus, the RBC supernatant was subjected to: (a) size exclusion chromatography; (b) protein concentration using conical tubes of different cut-offs; (c) native PAGE followed by passive elution. The use of these techniques, namely the native PAGE followed by passive elution (electroelution turned out to be inefficient), allowed us to narrow the EDGSF bioactivity to proteins eluting from a specific fraction (fraction 7), while other fractions had little or no effect, and ranging between 10-50kDa, as confirmed by SDS/PAGE analysis (Fig. 3.8). Identification of proteins bands of fraction 7 revealed the presence of large amounts of hemoglobin (Hb, bands at 15 and 30kDa) together with small amounts of peroxiredoxin II (Prx, band at 21kDa, see Table III). The presence of Hb and Prx II were confirmed by western blot (data not shown) and by 2D DIGE followed by mass spectrometry identification of proteins present on fraction 7 (see Table III). In agreements with results shown in Figure 3.7B, 2D DIGE confirmed the presence of

multiple isoelectric forms of Hb and Prx in the bioactive fraction of the RBC-sup (Fig. 3.9 and data not shown).

Table II. List of proteins present in the RBC-sup*

Name	Molecular weight	Source	Accession number
Actinin alpha 1 isoform b	105502	RBC	gi 94982457
Lactoferrin	78346	Neutrophils	gi 23268459
Transketolase	67751	RBC	gi 37267
L-plastin	63839	Leucocytes	gi 190030
Glucose phosphate isomerase	63107	RBC	gi 18201905
Catalase	59719	RBC	gi 4557014
Enolase	47111	RBC	gi 62896593
Serine/Cysteine proteinase inhibitor	42743	Neutrophils	gi 62898301
Lactate dehydrogenase A	36665	RBC	gi 5031857
Glyceraldehyde 3-phosphate dehydrogenase	36031	RBC	gi 31645
Carbonic anhydrase II	29031	RBC	gi 999651
Carbonic anhydrase I	28852	RBC	gi 4502517
Triosephosphate isomerase	26653	RBC	gi 4507645
Myeloblastin	24245	Neutrophils	gi 1633228
Peroxiredoxin II isoform a/TSA	21878	RBC	gi 32189392
Lipocalin	20535	Neutrophils	gi 4261868
Alpha 2 globin variant	15271	RBC	gi 62898345
Chain C, Deoxyhemoglobin	15234	RBC	gi 1827905
Hemoglobin alpha chain	15095	RBC	gi 1222413
Beta globin	11469	RBC	gi 62823011
Hemoglobin alpha 1 globin chain	10703	RBC	gi 13195586
S-100 calcium binding protein A8	10828	Various	gi 21614544

*Proteins present in the soluble fraction of RBC-CM were separated by 1D SDS-PAGE and visualized with Coomassie blue (see Fig. 3.7A). Twenty two protein bands were cut-off and sent for mass spectrometry identification by MALDI/TOFF/TOFF. All proteins were identified with confidence interval >95%.

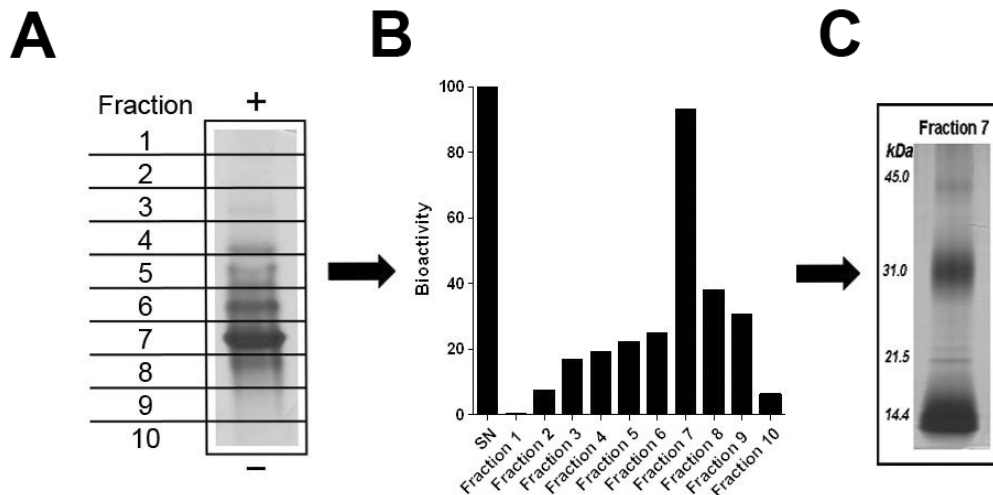


Figure 3.8. RBC bioactivities migrate together with hemoglobin in native PAGE. RBC-sup was obtained and concentrated by using Vivaspin columns. **A**, graph shows the protein profile of an aliquot of the concentrated RBC-sup after run in a native PAGE. Gel slices (fractions 1-10) were excised and proteins obtained by passive elution, as indicated in "Material and methods". **B**, Graph shows the *in vitro* bioactivity (percentage of dividing cells) of the different eluted fractions using CFSE-labeled PBL (1.5×10^6) stimulated with $5 \mu\text{g/mL}$ of PHA. Bioactivity of the unseparated RBC-sup was normalized to 100%. **C**, Graph shows the protein SDS/PAGE profile of the bioactive fraction 7 after TCA precipitation, separation in a 15% SDS/PAGE and silver staining. Molecular weight markers are indicated.

Table III. List of proteins present in RBC-sup bioactive fraction*

Name	Molecular weight	Source	Accession number
Peroxiredoxin 2 isoform a	21878	RBC	gi 32189392
Peroxiredoxin 1	18963	RBC	gi 55959887
Beta globin	15988	RBC	gi 62823011
Deoxy T-State hemoglobin	15857	RBC	gi 229752
Oxy T-State Hemoglobin	15811	RBC	gi 1942686
Hemoglobin	11496	RBC	gi 109893891
Beta globin chain	9463	RBC	gi 194719364

*Proteins present in the bioactive fraction described in Fig. 3.8 were concentrated and separated by 2D DIGE. Spots of interest were picked-up and sent for mass spectrometry identification by MALDI/TOFF/TOFF. All proteins were identified with confidence interval >95%.

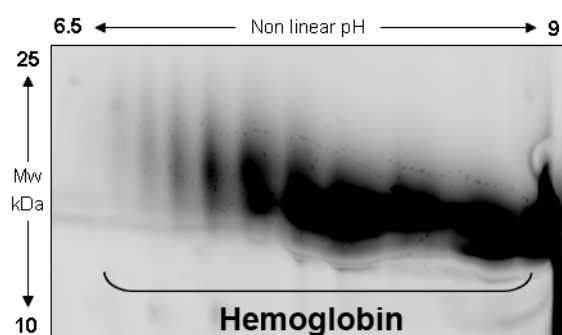


Figure 3.9. EDGSF contain multiple Hb isoforms. The bioactive fraction of the RBC-sup was obtained as indicated in the legend of Fig. 3.8. Graph shows the 2D DIGE gel of a representative fraction, where only the hemoglobin containing region of the gel is shown.

3.5. DISCUSSION

The main finding of the present study is that upon *in vitro* culture human RBC spontaneously release protein factors that enhance T cell growth and survival of normal and malignant activated T cells. Thus, we have shown that RBC-CM generated from cultures of RBC reproduces the effectiveness of intact RBC in modulating proliferation, cell growth and survival of activated T cells. Remarkably, RBC-CM displayed its cell growth and survival bioactivities towards Jurkat T cells, a leukemic T cell line in constant cell division. This was most evident when Jurkat T cells were grown in low-serum conditions. The fact that the presence of RBC-CM inhibited apoptosis and induced additional cycles of cell division in Jurkat T cells is in line with our previous studies showing that the bioactivity of RBC is only exerted on T cells that have been driven into the cell cycle, regardless of the stimuli [6,9,10]. The RBC bioactivities appear to be T cell specific since no significant outcome was observed when freshly isolated B or NK cells were used (unpublished observations). However, other bioactivities, besides cell growth,

proliferation and survival were not considered, so this result does not rule out the RBC involvement in other biological processes of B or NK cells activity as reported elsewhere [20,21]. Importantly, the growth and survival bioactivities were capable to rescue normal activated T cells from apoptosis when cultured in the absence of serum allowing them to proliferate to levels very similar to the ones seen in cultures with serum. These results ruled out the possibility of serum factors mediating the RBC bioactivities and demonstrated that RBC-CM holds the bioactivities carried by intact RBC.

By using sequential centrifugation we demonstrated the presence of bona-fide RBC vesicles in the RBC-CM. Indeed, flow cytometry and electron microscopy studies showed that RBC release vesicles whose size and morphology are in accordance with previous studies [12-16]. Importantly, RBC were capable of continuous vesiculation whenever the culture media was replaced. Unlike reports showing that RBC vesiculation is induced by changes in ATP or H^+/Ca^{2+} concentrations [12,13], in our study we showed that RBC vesiculation was spontaneous and not linked with phosphatidylserine externalization. This contrast with previous studies showing that senescent, damaged and apoptotic erythrocytes express phosphatidylserine at the outer leaflet of the plasma membrane [22-24]. Thus, our results suggest that RBC vesiculation is a constitutive process that takes place *in vitro* and that could take place and be hastened under physiological situations such as aging, damage or pathological conditions, such as in hemoglobinopathies [25]. Importantly, the cell growth and survival bioactivities released by RBC were not vesicles but soluble non-vesicular factors/proteins, instead. Our data suggest that the bioactive soluble factors present in the RBC-sup are spontaneously released in parallel with the vesicles, and are not the result of RBC lysis or vesicle disruption during the purification process. Indeed, the protein content in the RBC-CM was largely the contribution of the soluble proteins and not proteins within the vesicles, which represent on average 5% of the total. Indeed, the RBC-CM and the RBC-sup protein profile are undistinguishable. We concluded that contrary to what has been reported for RBC-derived ectosomes [26] and for vesicles derived from professional antigen presenting cells, or exosomes [18,19], RBC vesicles are immunologically inert under the conditions used in this study. Moreover, these data unequivocally indicate that the previously described T cell growth and survival activities carried by RBC [6,7,9,10] are soluble factors.

Biochemical studies of the non-vesicular RBC fraction revealed that the RBC bioactivities are proteinaceous, thermolabile and are not mediated by lipid compounds. By using 1D SDS-PAGE and tandem mass spectrometry we identified up to 22 different proteins specifically released by ex vivo RBC upon culture *in vitro*. By using native PAGE in

combination with 2D DIGE and mass spectrometry we narrowed the RBC released bioactive factors to several proteins that we termed EDGSF for erythrocyte-derived growth and survival factors. These proteins include peroxiredoxins (Prx), with Prx II being more abundant, hemoglobin (Hb) in oxy and deoxy states, and β -globin. EDGSF release was observed with all *ex vivo* RBC samples studied and as early as 90 min upon RBC culture.

Peroxiredoxins are thiol-specific enzymes present in RBC that exert their protective effect through their peroxidase activity. Still, recent studies have ascribed a range of other cellular roles to mammalian Prx, including the modulation of signal transduction pathways related with survival and proliferation that use the c-Abl and NF- κ B pathways [27,28]. In this context, the putative involvement of Prx on the enhancement of T cell growth and survival described here, if any, could be related with these functions and not necessarily with their peroxidase activity. Nevertheless, preliminary studies using Prx II in recombinant form suggest that this protein is not *per se* the EDGSF (unpublished results).

In our previous studies we showed that commercially available hemoglobin, heme or PPIX are toxic for activated T cells and could not reproduce the effect seen with intact RBC [9]. However, these results do not exclude Hb as an EDGSF candidate. Thus, Hb was the major protein present in our bioactive samples and 2D DIGE experiments of the bioactive fraction revealed that the released Hb exists in multiple forms. Whether one of these forms holds the EDGSF remains to be elucidated. In any case, release of hemoglobin by RBC into plasma is a physiological phenomenon that occurs as a result of intravascular hemolysis during erythrocyte senescence [29]. Although free hemoglobin is toxic for cells and is cleared from circulation by macrophages after binding to haptoglobin, it has been implicated in the augmentation of monocyte and macrophage activation induced by lipoteichoic acid, most likely through activation of Toll-like receptors by heme [30,31]. Interestingly, the heme group of Hb, but not the porphyrin ring was reported to be immunostimulatory for T cells when accessory cells were present [32-34]. In this context, it is important to mention that accelerated intravascular hemolysis occurs in a number of pathological conditions, including sickle cell anemia, thalassemias, hereditary spherocytosis, paroxysmal nocturnal hemoglobinuria and also during malaria infection [29]. To our knowledge, studies addressing the features of Hb released in those conditions are lacking. Therefore the possibility that a particular Hb conformational state either alone or complexed to another protein may underlie the bioactivity of the EDGSF is a likely possibility that remains to be elucidated. Indeed, our results indicate that Hb and Prx migrate together on native gels, raising the likelihood that they may form a complex. Indeed, formation of a complex between Hb and Prx has been reported in erythrocyte

hemolysates [35, 36] and SDS/PAGE analysis of the immunodepleted Hb after elution revealed the presence of co-precipitated proteins, including PrxII (unpublished results).

Finally, β -globin was also present in the bioactive fraction of the RBC-sup, placing the protein part of Hb, and not the whole molecule, as a putative candidate holding the biological activity of the EDGSF. Although we are not aware of any evidence pointing to β -globins as molecules possessing cell growth and survival bioactivities, there are reports showing that specific fragments resulting from intraerythrocytic proteolysis of the globin chains are endowed with an array of biological activities [37]. Among these fragments, the pentapeptide neokyotorphin (NKT) has been reported to induce modest increases in the proliferation of transformed murine fibroblasts [38]. In our hands, neokyotorphin did not contain any growth or survival activities for activated T cells (data not shown). Whether other β -globin fragments hold the EDGSF bioactivity remains to be ascertained.

Overall, the present studies have unveiled that RBC release novel factor(s) involved in the regulation of growth, proliferation and survival of dividing T cells, placing RBC as a novel regulatory cell. In the context of recent proteomic studies showing that some of the RBC released proteins identified in this study are present in plasma of cancer patients [39, 40], it is tempting to speculate that the EDGSF might play an unforeseen role in sustaining cell growth and survival of leukemia cells, and other tumour cells, under pathological situations.

3.6. REFERENCES

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Chapter 4

General Discussion

Because the primary function of RBC is transport O₂ from pulmonary capillaries to tissue capillaries where it is exchanged for CO₂, the capacity of RBC to regulate and/or influence biological processes of other cells has been generally disregarded. This overlook has been particularly impressing in relation to the alleged capacity of RBC to modulate T cell proliferation *in vitro*. Following Carstairs's pioneering report demonstrating that the presence of RBC enhanced proliferation of PHA-stimulated peripheral blood mononuclear cells, the series of studies carried out between 1970s-1990s, showing that RBC enhance T cell proliferation, B cell immunoglobulin secretion and NK cytotoxicity *in vitro* were considered an artifact resulting from the culture conditions and devoid of any physiological relevance. The following few studies carried out in the 1990s "re-discovered" the RBC effect on T cell proliferation (reviewed in [1]).

The lack of interest of the scientific community on the *in vitro* immunoregulatory effect of RBC contrasted with the intensive clinical investigations on the effect that RBC transfusions have on the immunological system *in vivo* [1]. Thus, since the description by Gerhard Opelz in 1973 that blood transfusion is associated with immunosuppression [2], numerous clinical and experimental studies have established a cause-effect correlation between transfused RBC, activation of T and B cells, and subsequent triggering of immunomodulatory effects. Although residual leukocytes present in the transfused RBC have long been implicated in the immunomodulatory effects, clinical trials of leukoreduction do not support this view [3-5]. Indeed, recent data strongly points to biochemical and morphologic alterations underwent by RBC themselves during storage, rather than residual leukocytes and platelets, as the cause of the immunomodulation associated with RBC transfusion [6,7].

Besides unveiling a novel aspect of RBC biology *in vivo*, these clinical studies have contributed to reinforce the aforementioned *in vitro* observations. The strong similarity between the RBC-induced effects on T cell activation *in vitro* [8,9] and *in vivo* [10], on immunoglobulin secretion by B cells *in vitro* [11,12] and *in vivo* [13], or on NK activity *in vitro* [14] and *in vivo* [15,16] are just more than simple coincidences and have to be taken as an strong evidence of the immunoregulatory role of RBC. Indeed, our previous studies preceding this thesis concluded that the capacity of either autologous or heterologous RBC to regulate growth and proliferation of T cells activated *in vitro* was not a simple phenomenological effect and was the result of a summing up of protective effects on the activated T cells (reviewed by [1]). However, these studies did not ascertain the molecular mechanism(s) used by RBC to sustain T cell growth and proliferation.

The work presented in this thesis dissertation represents the first attempt to elucidate and characterize the molecular mechanism(s) accountable for the immunoregulatory role of RBC *in vitro* using as experimental model *ex vivo* human peripheral blood T cells induced to enter into the cell cycle by means of mitogenic activation. In this work the bioactivities of intact RBC, RBC conditioned medium (RBC-CM) and the two main fractions obtained after ultracentrifugation of the RBC-CM, that is the vesicle-containing pellet fraction and the vesicle-free supernatant fraction, were studied. We have demonstrated that human RBC carry out bioactivities capable of keeping activated normal T cells and leukemic Jurkat T cells alive and into the cell cycle by markedly downplaying apoptosis. Importantly, we have demonstrated that these bioactivities are mediated by soluble factors released by RBC into the extracellular milieu, which include hemoglobin, peroxiredoxin II and globin chains.

The main findings of this work that we would like to discuss are the following:

- RBC bioactivities act on intracellular pathways initiated by TCR/CD3-dependent and -independent stimuli and activate the Akt pathway.
- RBC bioactivities preserve the antioxidant status and rescue activated T cells from serum deprivation-induced cell death.
- RBC bioactivities appear to be sensitive to immunosuppressant drug cyclosporine A.
- RBC bioactivities are not mediated by glycosylphosphatidylinositol-linked receptors or sialic acids.
- RBC release vesicles devoid of bioactivity and bioactive vesicle-free factors into the extracellular milieu.
- Proteomic characterization of the RBC bioactive secreted factors revealed multiple hemoglobin isoforms.
- We will conclude discussing the likelihood of the RBC bioactivities playing an unforeseen role in maintaining tumour cell growth *in vivo*.

RBC bioactivities act on intracellular pathways initiated by TCR/CD3-dependent and -independent stimuli and activate the Akt pathway.

In vivo, T lymphocytes can be activated and induced to enter cell proliferation both after TCR/CD3-dependent activation signals initiated by cross-linking of the TCR/CD3 complex upon recognition of MHC-peptide complexes [17] or after TCR/CD3-independent activation signals initiated after interaction with homeostatic cytokines such as IL-2 and IL-15 [18,19]. In either case and under appropriate costimulatory conditions (e.g. CD28 engagement in the case of MHC-peptide -mediated activation) a complex intracellular activation program is initiated that will ultimately drive T lymphocytes into the cell cycle [20].

In this work we have shown that the RBC bioactivities positively interfere with activation signals that do not use the TCR/CD3 complex, including direct activators of the protein kinase C (i.e. PdB and ionomycin) and JAK/STAT (i.e. IL-2 and IL-15) pathways (Chapter 2). Our previous data showed that RBC bioactivities enhanced activation and proliferation signals initiated after engagement of the TCR/CD3 complex either using the lectin phytohaemagglutinin (PHA), known to activate T cells after binding to the TCR/CD3 complex [21-23] or anti-CD3 antibodies alone or in combination with anti-CD28 (reviewed by [1]). Taken together, these results have clearly demonstrated that the capacity of RBC to regulate T cell growth and survival is independent of the stimuli used to activate and drive T cells into the cell cycle. These data is a strong argument against remarks by some authors minimizing and lessening the relevance of earlier findings that the presence of RBC in cultures of PHA-stimulated T cells markedly enhance T cell proliferation and survival [24-26]. In our view, these data reinforce and broadens the possible *in vivo* relevance of the immunoregulatory effect of RBC, where primed T cells could be more responsive to activation signals delivered by pro-inflammatory and homeostatic cytokines in the context of a blood transfusion or even in non-transfused (pre)malignant scenario. Further supporting the latter assumption, RBC were able to augment the proliferation of Jurkat T cells, a leukemic cell line in constant cell division, raising the intriguing possibility that RBC could under certain circumstances play a role in sustaining growth and progression of leukemic cells, as discussed later.

As a result of these studies, we also obtained preliminary evidence that the RBC bioactivities likely pass through the PI3K/Akt axis. Thus, data presented in this thesis represent the first evidence that the bioactivities carried by RBC deliver intracellular signals that up-regulate the activity of Akt, a serine/threonine kinase acting on multiple

targets which plays an important role in controlling cell growth and apoptosis (reviewed by [27]). Among its targets, it is worth mentioning mTOR, a kinase playing a central role in integrating environmental signals critical to regulating protein synthesis and survival of T cells [28,29], pro-survival Bcl-2 family members [30], and NF- κ B, an ubiquitous transcription factor that controls the expression of genes involved in immune responses, apoptosis, and the cell cycle [31]. Although the data suggest that all could be downstream mediators of RBC bioactivities, further studies evaluating their involvement are needed. In any case, these findings highlight the importance of a better understanding of how RBC bioactivities are used by T cells that have entered into the cell cycle either as part of a non-dangerous activation/expansion/contraction process or as a result of malignant transformation where constitutive activation of Akt is reported [32].

RBC bioactivities preserve the antioxidant status and rescue activated T cells from serum deprivation-induced cell death.

Reactive oxygen and nitrogen species (ROS/RNS) are the result of the metabolic activity of activated/dividing T cells. Eventhough these reactive species participate in the activation of intracellular signaling pathways, namely the NF- κ B pathway [33,34] they are also potentially toxic to the host T cell [35]. In this regard, the level of cell surface thiols have been used to measure the pro-oxidant state of T cells, with activated and apoptotic T cells displaying low thiol levels [36]. Due to their ability to be reversibly oxidized, thiols are recognized as key components in the protection against oxidation-induced T cell apoptosis [36,37]. It was therefore important to show that RBC had the capacity to preserve the level of surface thiols on activated T cells (Chapter 2). Together with our previous results showing that RBC counteracted ROS/RNS production induced by T cell activation, and up-regulated cytoprotective proteins such as heme oxygenase 1 and ferritin [24,26], these results reinforce the notion that the RBC bioactivities are associated with the activation of intracellular pathways related not only with the entrance into the cell cycle but also with the regulation of oxidative stress, thus contributing to an overall inhibition of activation-induced cell death and favouring T cell division.

Most importantly, the RBC bioactivities described in this thesis were capable of rescuing dividing T cells (both normal activated T cells and leukemic Jurkat T cells) from serum deprivation-induced cell death (Chapter 3). Serum is commonly added to culture media as a source of nutrients, including amino acids, glucose and other unidentified growth factors essential for the growth and survival of a variety of cell types, including T cells [38]. Accordingly, serum withdrawal is associated with inhibition of cell proliferation and cell

death in a variety of cell types, including activated T cells and cell lines [38-40]. These results suggest that RBC contain a previously unnoticed growth factor similar to, or mimicking the action of, a factor(s) present in the serum and playing a key role in T cell growth and survival.

RBC bioactivities appear to be sensitive to cyclosporine A.

Cyclosporine A (CsA) and Rapamycin (Rapa) are drugs widely used to suppress T cell activation in a variety of clinical settings. While CsA binds to endogenous cyclophilin A (CypA), Rapa binds to endogenous FK506 binding protein (FKBP)-12 [41]. These complexes bind and inactivate calcineurin and mTOR, respectively, leading to a decrease in T cell proliferation [42]. Considering recent studies showing that both CypA and FKBP-12 are part of the RBC proteome [43] we investigated whether these two drugs interfered with the RBC bioactivities. Interestingly, we have shown that RBC bioactivities are capable to overcome efficiently the inhibition caused by Rapa, but not by CsA, in activated T cells, suggesting that they act on intracellular pathways that depend on calcineurin, but not mTOR, activity. These results suggest that the cell growth and survival bioactivities of RBC are dependent, at least in part, on the activation of the NF-AT transcription factor and subsequent activation of IL-2 gene expression, two downstream events following calcineurin activation [44].

On the other hand, we have shown that treatment of RBC with CsA, but not with Rapa or with a PI3K inhibitor, thus mimicking an *in vivo* drug administration, completely abolished the cell growth and survival RBC bioactivities towards activated T cells. Although the drug ligand CypA is present in RBC [43,45] and has been reported as a secreted growth factor [46], we have no evidence or whatsoever for CypA being part of the RBC bioactivities either intracellularly or as a secreted factor (Chapter 3 - see below). However, it is important to mention that CypA is a peptidyl-prolyl-isomerase which may play an important role in protein folding, trafficking and assembly [47]. Therefore, the possibility that CsA binding to the endogenous erythroid CypA is somehow inhibiting the RBC bioactivities cannot be ruled out. In this regard, studies conducted in cell lines infected by HIV-1 indicate that CypA is a component of HIV-1 virions and that treatment of infected cells with CsA, that binds to CypA and inhibits its incorporation into virions, inhibits HIV-1 infection [48]. Other recent reports suggest that cyclophilins may be involved in cell surface externalization of receptors, such as the insulin receptor and CD147 [49,50]. Whether endogenous CypA in erythrocytes is involved in the release of bioactive factors and this process is inhibited by CsA binding remains to be elucidated. In any case, our *in vitro* findings could be relevant in an *in vivo* clinical context where patients are undergoing

immunosuppression. This could be of particular interest in organ transplantation were blood transfusions associated with CsA and/or Rapa administration are frequently used [51-54].

RBC bioactivities are not mediated by glycosylphosphatidylinositol-linked receptors or sialic acids.

One important issue arising from this work was the demonstration that RBC surface receptors and/or molecules are not part of the RBC bioactivities. Thus, the capacity of RBC to regulate cell growth and survival of activated T cells was not mediated by interactions between LFA-3/CD58 on RBC and its ligand CD2 on T cells, as proposed by several authors [8,12,55], by other glycosylphosphatidylinositol-linked receptors, or by sialic acids present in the RBC surface. In contrast, recent studies have shown that the capacity of RBC to regulate IL-12 production by dendritic cells depends on the interaction between CD47 present on RBC and its ligand SIRPalpha present on dendritic cells [56]. Indeed, the RBC bioactivities characterized in this work turned out to be independent of RBC surface proteins, but mediated by secreted factors (Chapter 3 - see below), indicating that the (immune)regulatory capacity of RBC can be displayed through cell-cell contact and through secreted factors, or a combination of both.

RBC release vesicles devoid of bioactivity and bioactive vesicle-free factors into the extracellular milieu.

Vesiculation is a spontaneous and widespread process of many hematopoietic cells, which results in the secretion of exosomes endowed with the capacity to regulate the function of the immune system, including a role in the activation of T cells [57-59]. RBC vesiculation has also been reported by several authors and occurs upon biochemical and biophysical changes in the extracellular milieu where RBC are present [60-62]. In view of these data and the fact that RBC cell surface receptors were not involved in the RBC bioactivities, we reasoned that the RBC bioactivities could be present in the culture media as a result of RBC vesiculation. Examination of the bioactivity of the RBC conditioned media (RBC-CM) generated after *in vitro* culture of RBC revealed that it contained all the cell growth and survival bioactivities of intact RBC. In-depth analysis of the bioactive RBC-CM by flow cytometry, transmission electron microscopy and fluorescence microscopy revealed the presence of bone fide RBC-derived vesicles (RBC-ves), indicating that RBC are indeed capable to vesiculate *in vitro* either spontaneously or upon appropriate stimuli (Chapter 3). Importantly, bone fide RBC-derived vesicles were also detected in plasma samples from healthy donors (see Fig. 5.1, Addendum), supporting the assumption that the spontaneous RBC vesiculation we observe *in vitro* is a physiological process that has

an *in vivo* counterpart. The presence of RBC vesicles in plasma has been reported by other authors [63,64].

To our surprise, and contrarily to what was initially hypothesized based on the exosomes data, RBC-ves turned out to be inert and lacking any significant bioactivity, at least in the conditions used in our *in vitro* assays, ruling out RBC-ves as mediators of RBC bioactivities. Unexpectedly, the vesicle-free fraction (RBC-sup) was responsible for all the bioactivity present in the RBC-CM. Although these results clearly demonstrate that the reported RBC bioactivities are released in the form of soluble factors into the extracellular milieu *in vitro*, they do not ascertain whether RBC vesiculation is involved and/or essential for the release of these bioactive factors or whether they are also released *in vivo*. Importantly, and despite this concern, we have shown that the candidate(s) factor(s) is(are) thermolabile proteins and do not include lipid compounds. Due to the growth and survival promoting activities of the RBC released factor(s), they were termed EDGSF (for erythrocyte derived growth and survival factor) and were the focus of our subsequent studies.

Proteomic characterization of the RBC bioactive secreted factors revealed multiple hemoglobin isoforms.

Having characterized the biochemical nature of the RBC bioactivities, we decided to take a proteomic approach in order to identify the EDGSF. These studies led to the characterization of a list of more than twenty putative EDGSF candidates. The proteins present in the bioactive supernatant included RBC-derived proteins and several myeloid-derived proteins, most likely proteins brought down with RBC during the purification process. However, further purification procedures eliminated the possibility of myeloid-derived protein(s) being the EDGSF, and reduced the putative EDGSF candidates to peroxiredoxin II (PrxII), hemoglobin (Hb) in oxy and deoxy states, and β -globin (Chapter 3).

Hb derivatives and PrxII have been shown to be involved in the regulation of cell death and proliferation [65-68]. However, when commercially available forms of PrxII and Hb were tested for bioactivity in our *in vitro* assays, neither protein, either alone or in combination, could reproduce the effect of RBC bioactivities (see Fig. 5.2, Addendum). Although discouraging, these results do not exclude PrxII or Hb as putative EDGSF candidates. First, Hb immunodepletion from the bioactive RBC-sup completely abolished its bioactivities (see Fig. 5.3, Addendum). Second, Hb was found in multiple forms in the RBC-sup and the possibility that a particular conformational state may underlie the RBC

bioactivities remains an open question. In support of this assumption is a recent study showing that a specific conformational state of the heme group derived from the degradation of hemoglobin by the malaria parasite, and called beta-hematin or hemozoin, possesses immunostimulatory properties and is capable to specifically activate the immune response [69]. Third, SDS/PAGE and Westernblot analysis of the immunodepleted Hb after elution revealed the presence of co-precipitated proteins, including PrxII (see Fig. 5.4, Addendum), suggesting that Hb and PrxII may form a heterodimeric complex endowed with the RBC bioactivities.

Do RBC bioactivities play an unforeseen role in sustaining tumour cell growth *in vivo*?

Based on the results arising from this thesis work and broadly speaking, we can envisage RBC as a novel regulatory cell whose released bioactivities could play a role as mediators of T cell immunomodulation *in vivo*. Indeed, we hypothesize that T cells could use the RBC bioactivities in their own benefit to support and assure their own growth and survival. By exerting this role, RBC could participate and support the establishment of efficient adaptive immune responses, specifically in the efficiency of T cell responses against internal and external pathogens. It is important to note, that besides their natural presence in organs and in the blood stream, RBC are present at sites of injury, trauma and inflammation, including tumoral environments. Accordingly, it is conceivable to think that in these sites, encounters between RBC, and/or their secreted bioactive factors, and activated T cells may take place. Indeed, tissue RBC extravasation has been described in several inflammatory situations such as pyogenic granulomas, small-vessel vasculitis, inflammatory granular tissue and in neoplastic environments such as in angiosarcomas, multiple myeloma and adult T-cell leukemia/lymphoma [70-74]. Moreover, during haematological malignancies (e.g. in cases of T cell leukaemia) or autoimmune disorders, the RBC bioactivities may turn out to be harmful to the host. Leakiness of blood vessels in tumors, and in particular RBC extravasation, was suggested to contribute to disease progression [75].

In line with the results shown in this thesis, it is important to note that the Akt signaling pathway, which activity appears to be up-regulated by the RBC bioactivities, is constitutively activated in myeloid leukemia, multiple myeloma, T cell granular lymphocytic leukemia and in adult T cell leukemia cells [76-79], raising the interesting possibility that RBC bioactivities could be in part involved. In this regard, in addition to constitutive cell-autonomous mechanism of cell growth deregulation seen in cases of T cell acute lymphoblastic leukaemia (T-ALL), also microenvironmental signals (e.g. unknown growth

factors present in the leukaemia milieu) are believed to contribute to T-ALL survival and expansion through the activation of several key 'pro-oncogenic' intracellular pathways, including PI3K/Akt, MAPK and JAK/STAT signalling pathways [80,81]. In addition, the downstream target of PI3K/Akt, NF- κ B, was shown to be constitutively activated in some primary T-ALL [82] while calcineurin activation and subsequent NFAT signaling was reported to be critical for the maintenance of the T-ALL phenotype *in vivo*, as well as to the pathogenesis of other aggressive lymphoid malignancies [83,84]. In this context, and in line with our results, it is plausible to think that RBC bioactivities by intersecting with key intracellular pathways related with leukemic T cell growth and survival could play an unforeseen role in sustaining their malignant state *in vivo* and support their expansion (see Fig. 4.1). The final identification of the EDGSF may allow development of specific EDGSF inhibitors in order to lessen tumour progression and allow anti-tumour response of normal T lymphocytes.

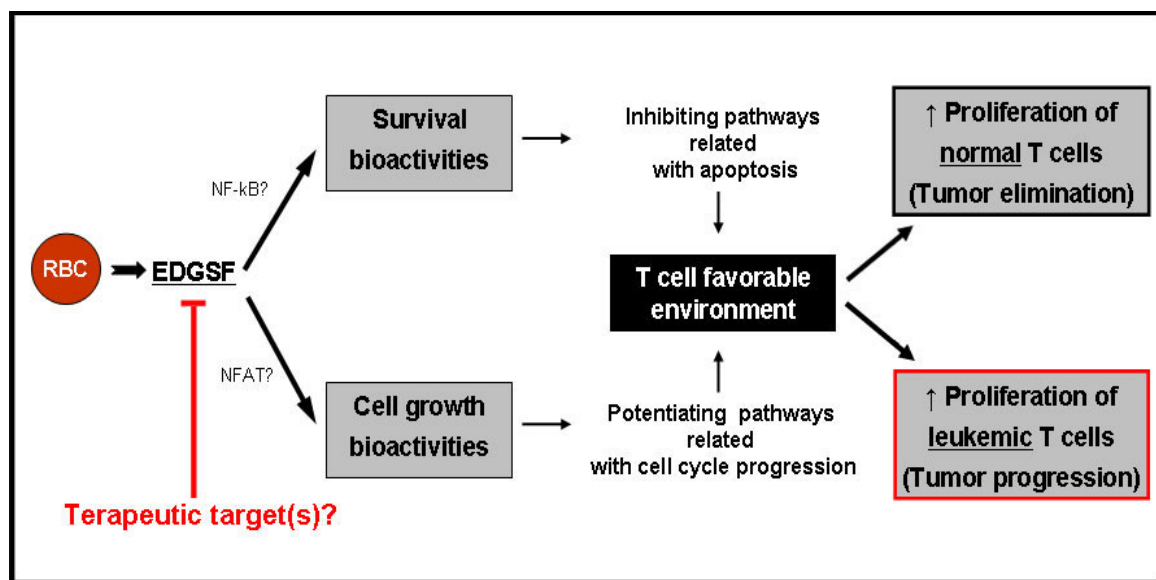


Figure 4.1. RBC release bioactive factors which induce the activation of pathways that promote T cell growth and survival. T cell activation induces NF- κ B and NFAT activity. While the first pathway is involved in redox-regulated anti-apoptotic processes, the second is involved in the regulation of IL-2 transcription and subsequent T cell progression into cell cycle division. RBC secreted EDGDF bioactivities would therefore regulate in a positive manner downstream pathways involved in the transcription of genes that control cell growth and proliferation. EDGSF bioactivities would increase NF- κ B activation and hence regulate in a positive manner downstream pathways involved in the inhibition of apoptotic genes. EDGSF bioactivities are also accountable to act on downstream signalling regulators, such as calcineurin, responsible for the NFAT activation, a transcription factor related with the control of proliferation. The overall result is an increased T cell proliferation and survival. In leukemia patients, leukemic T cells will expand indefinitely, thus outnumbering normal T cells and contributing to the worsening and malignance progression. In this scenario EDGSF bioactivities could become a potential target of therapeutic intervention.

Final Remarks

The present study represents the first comprehensive attempt to start elucidating in detail the molecular mechanism(s) responsible for the bioactivity that RBC exert on activated normal and malignant human T cells *in vitro*, which may have implications in *in vivo* pathological situations. Results presented in this thesis have highlighted the importance of RBC as immunoregulatory cells and have contributed to change the conceptual, yet erroneous view of RBC as inert cells. Indeed, from a broader point of view, RBC could even be considered homeostatic cells, due to their ability of regulating cells of both the adaptive (e.g. T cells and B cells) and innate (e.g. neutrophils, dendritic cells, fibroblasts, endothelial cells) immunity. This assumption can be considered a challenge to the more restrict and dogmatic view of the immune system and may seem outlandish at first. However, the novel data presented in this thesis, together with previous data published on this topic, strongly argues in favour of this novel view and suggest that RBC can have an emergent and important role in the orchestration of the complex immune-cell responses. Deeper understanding of these processes is mandatory and could provide new therapeutic approaches, especially given the diverse immunological interactions that RBC can influence. RBC are indeed red, but far from dead.

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Addendum

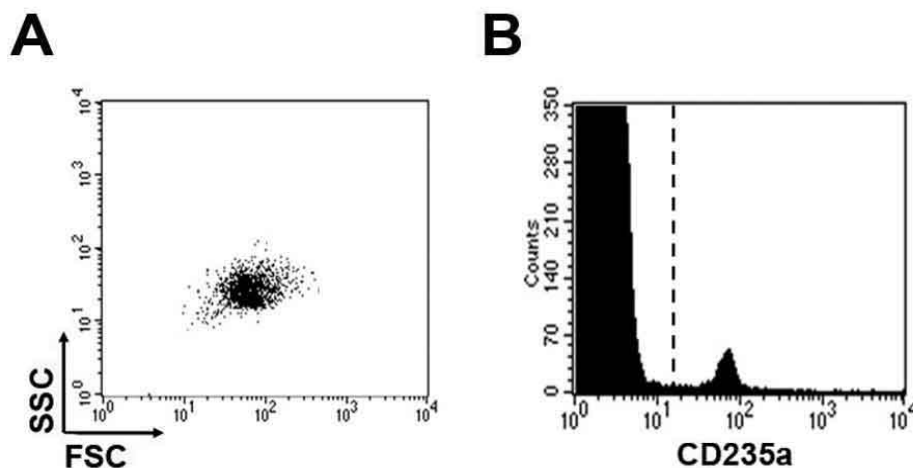


Figure 5.1. RBC-derived vesicles are present in Human blood plasma. Plasma samples from Human blood of healthy donors were obtained from buffy coats after centrifugation. Aliquots were harvested and stained with an anti-glycophorin A antibody (CD235a), and acquired in a FACSCalibur. **A**, Dot-plot shows RBC-derived vesicles (RBC-ves) gated in glycophorin A positive events, according to FSC and SSC characteristics. **B**, Histogram shows CD235a expression of total events. The results are representative of 1 out of 3 different experiments performed.

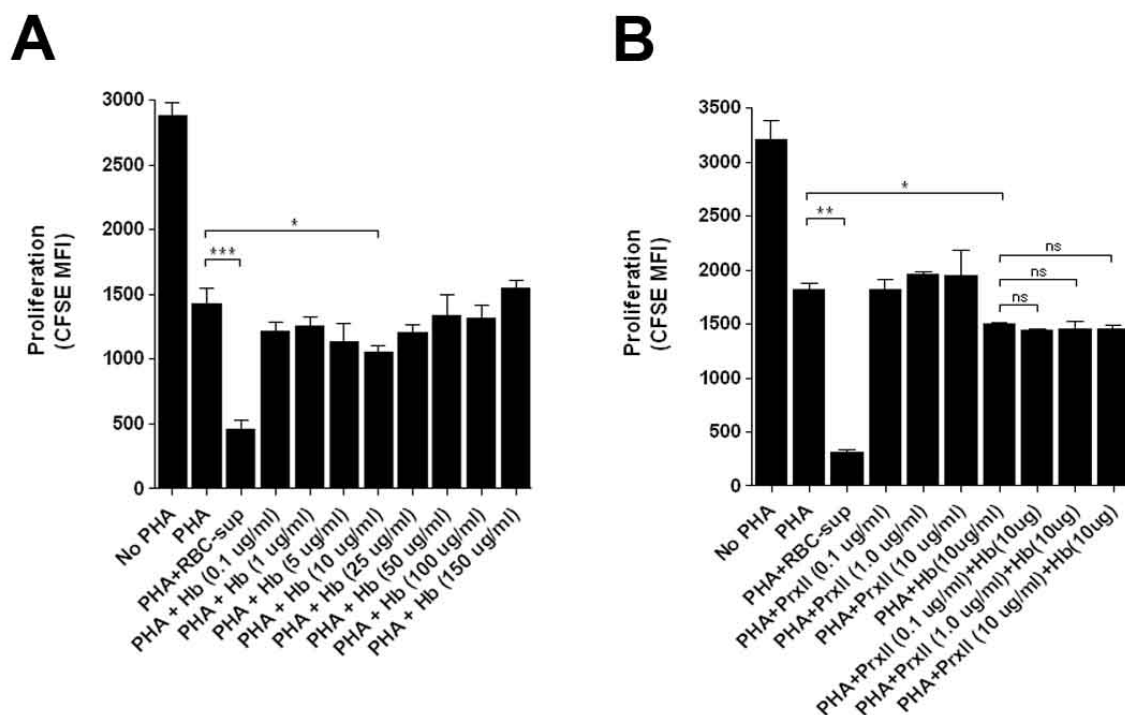


Figure 5.2. EDGSF bioactivities are independent of PrxII and Hb. PBL (1.5×10^6) were labelled with CFSE left unstimulated or stimulated with PHA, and cultured either alone or with RBC-sup, commercial available PrxII (Lab Frontier, Seoul, South Korea) and Hb (Sigma-Aldrich, Madrid, Spain) or both, for 7 days and then harvested, stained, and acquired in a FACSCalibur. Graphs shows the CFSE mean fluorescence intensity of proliferating cells in the presence of Hb (mean $\pm$ SEM, n=7) (**A**) or in the presence of Hb and PrxII (mean $\pm$ SEM, n=3) (**B**) in a different range of concentrations. P values are shown: (***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$; ns not significant).

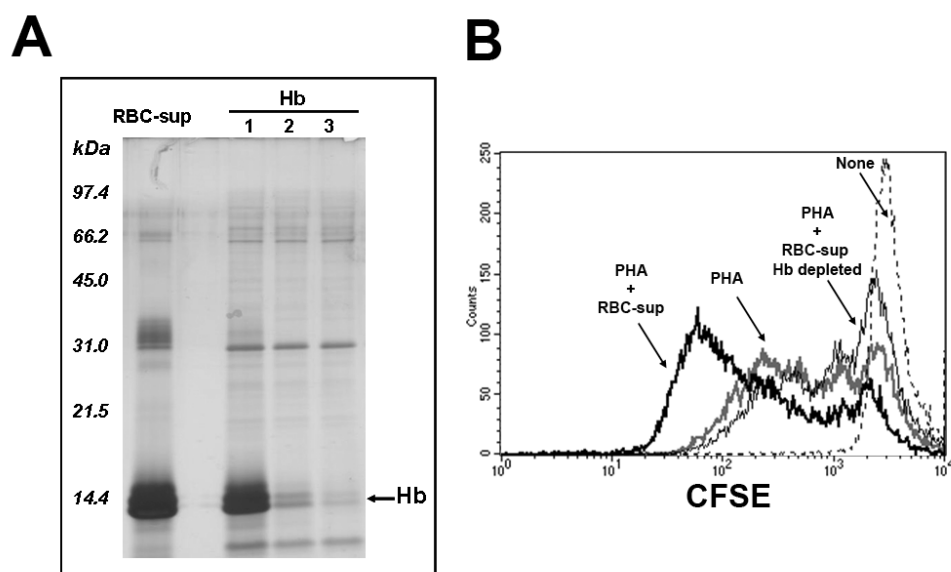


Figure 5.3. EDGSF bioactivities are lost after Hb depletion. The RBC-sup was immunodepleted with a high affinity hemoglobin antibody and its bioactivity assayed *in vitro*. **A**, Hb was depleted from the RBC-sup with 3 rounds of depletion using the GTX77484 mAb as described in “Material and methods” (Chapter 3). Graph shows the protein profile of the RBC-sup following the three sequential immunoprecipitation cycles after TCA precipitation and separation in a 15% SDS/PAGE and silver staining. Molecular weight markers and Hb are indicated. **B**, PBL (1.5×10^6) were labeled with CFSE, left unstimulated or stimulated with $5 \mu\text{g}/\text{mL}$ of PHA in culture media and cultured for 7 days in the absence or presence of RBC-sup or RBC-sup Hb depleted. Then, cells were harvested and acquired in a FACSCalibur. Histograms show loss of CFSE fluorescence in cultures of activated PBL cultured in the absence (grey) or presence of RBC-sup (thick black) or Hb depleted RBC-sup (thin black). Dotted histogram shows CFSE fluorescence in unstimulated PBL. One representative out of 3 different experiments is shown.

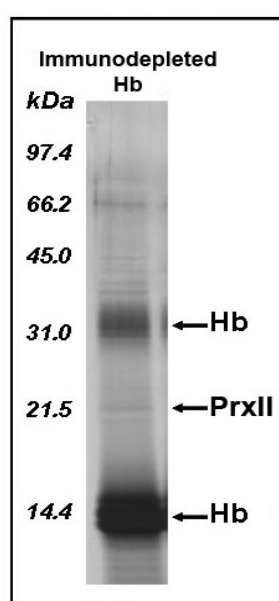


Figure 5.4. Immunodepleted Hb contains co-precipitated proteins. The RBC-sup was immunodepleted with a high affinity hemoglobin antibody as described in “Material and methods” (Chapter 3), and the eluted Hb resolved by electrophoresis. Graph shows the protein profile of the immunodepleted Hb following three sequential cycles of elution after TCA precipitation and separation in a 15% SDS/PAGE and silver staining. Molecular weight markers are shown, and Hb and PrxII are indicated after Westernblot confirmation. One representative out of 3 different experiments is shown.

