Graça Susete Costa de Carvalho Marques

Licenciada em Biologia Celular e Molecular

Establishing a Cell Biology Platform: Isolation and Preservation of Human Blood Products

Dissertação para obtenção do Grau de Mestre em
Genética Molecular e Biomedicina

Orientadora:	Doutora Zélia Maria Cordeiro da Silva, Investigadora, Faculdade de Ciências Médicas da Universidade Nova de Lisboa
Co-orientadora:	Doutora Paula Alexandra Quintela Videira, Professora Auxiliar Convidada, Faculdade de Ciências Médicas da Universidade Nova de Lisboa

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Presidente:	Prof. Doutora Ilda Maria Barros dos Santos Gomes Sanches
Arguente:	Doutora Ana Catarina Maurício Brito Ataíde Montes
Vogal:	Doutora Zélia Maria Cordeiro da Silva

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

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ABSTRACT

The use of human primary cells provide researchers in different areas with irrefutable more biologically relevant data than using cell lines or animal blood cells. The work was performed in the scope of the Cell Biology Services @ CEDOC, aiming to provide viable and trustful human primary cells and products. We had three main objectives: protocol optimizations for blood cell isolation, culture and cryopreservation; cost estimation and divulgation of the services.

We have reviewed standard protocols and compared different strategies for blood cell isolation. The impact of those methodologies was evaluated regarding cell yield and purity, cell functional characteristics and cost. We also developed a method for serum isolation from human plasma in blood buffy coats. The resultant sera were sterile and suitable to be used in leukocyte cultures.

Different protocols for T cells isolation were compared: positive *versus* negative immunomagnetic selection and isolation using nylon wool fiber columns. Positive selection provided the highest isolation yield (32.35%), while negatively selected cells had the highest purity (92.81%). Although nylon wool fiber column was the fastest and cheapest method, unlike the immunomagnetic methods, it did not allow complete separation of T from B lymphocytes.

Positive selection of monocytes was compared using two widely used commercial kits. Miltenyi's kit provided the highest isolation yield (25.92%), recovery rate (86.70%) and purity (95.01%). Monocytes isolated with StemCell kit presented a higher cell complexity, and when differentiated into dendritic cells (DCs), showed a more mature phenotype. Differences between both kits are probably caused by the nature of the magnetic beads, suggesting caution when choosing one or other kit, as it may have an impact on DCs' function.

Overall, although dealing with apparently straight forward methodologies, our results show that testing commercial products and optimizing protocols is very important and contribute for a better quality of products and services.

Key words: sera, cell isolation, cryopreservation, cell culture, PBMCs (peripheral blood mononuclear cells).

RESUMO

O uso de células humanas primárias fornece a investigadores de diferentes áreas, dados irrefutavelmente mais relevantes do que usando linhas celulares ou células de sangue de animais. Este trabalho foi realizado no âmbito dos Serviços de Biologia Celular @ CEDOC, com o objectivo de fornecer células primárias e produtos humanos viáveis e de confiança. Tínhamos três objectivos principais: optimização de protocolos para o isolamento cultura e criopreservação de células do sangue; estimativas de custo e divulgação dos serviços.

Revimos protocolos padrão e comparámos diferentes estratégias para o isolamento de células do sangue. O impacto dessas metodologias foi avaliado tendo em conta o rendimento e pureza celular, características funcionais celulares e o custo. Também desenvolvemos um método para o isolamento de soro a partir de plasma em *buffy coats* de sangue. O soro resultante era estéril e adequado para uso em culturas leucocitárias.

Foram comparados diferentes protocolos para o isolamento de células T: selecção imunomagnética positiva *versus* negativa e isolamento usando colunas de fibra de lã de *nylon*. A selecção positiva forneceu o rendimento celular mais alto (32,35%), enquanto as células seleccionadas negativamente tinham a maior pureza (92,81%). Apesar de a fibra de lã de *nylon* ser o método mais rápido e barato, ao contrário dos métodos imunomagnéticos, não permitiu uma separação completa de linfócitos T e B.

A selecção positiva de monócitos foi comparada usando *kits* comerciais. O *kit* da Miltenyi forneceu o maior rendimento de isolamento (25,92%), taxa de recuperação (86,70%) e pureza (95,01%). Os monócitos isolados com o *kit* da StemCell apresentavam uma maior complexidade celular e quando diferenciados em células dendríticas, mostravam um fenótipo mais maturo. As diferenças entre os dois *kits* são provavelmente causadas pela natureza das esferas magnéticas, sugerindo cautela aquando da escolha entre um *kit* ou outro, pois este pode ter impacto na função das células dendríticas.

No geral, apesar de lidarmos com metodologias aparentemente lineares, os nossos resultados mostram que testar produtos comerciais e optimizar protocolos é muito importante e contribui para uma melhor qualidade de produtos e serviços.

Palavras-chave: soro, isolamento de células, criopreservação, cultura de células, PBMCs (células mononucleares do sangue periférico).

The work developed until the present date has originated:

- **One poster:**

Graça S. Marques; Inês Iria; Zélia Silva; Paula A. Videira. 2013. Cell Biology Services at CEDOC/FCM. XXXVIII Jornadas Portuguesas de Genética. Porto, Portugal.

- **One oral presentation:**

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ABBREVIATIONS

7-AAD	7-amino-actinomycin D
APC	Aloficocianine
BSA	Bovine Serum Albumin
BVDV	Bovine Viral Diarrhea Virus
CMC	Carboximethylcellulose
CPD	Citrate Phosphate dextrose
DCs	Dendritic Cells
DMSO	Dimethyl sulfoxide
FAQs	Frequently Asked Questions
FBS	Fetal Bovine Serum
FITC	Fluorescein isothiocyanate
FSC	Forward Scatter
GM-CSF	Granulocyte Macrophage Colony-Stimulating Factor
HES	Hydroxyethyl starch
IL-4	Interleucin-4
IPS	“Instituto Português do Sangue” (Portuguese Blood Institute)
Tc	T cytotoxic (lymphocytes)
Th	T helper (lymphocytes)
Treg/s	T regulatory/suppressor (lymphocytes)
LPS	Lipopolysaccharide
MFI	Mean Fluorescence Intensity
MHC-II	Major Histocompatibility Complex Class II
Mo-DCs	Monocyte-derived dendritic cells
MRBC	Monkey Red Blood Cells
NCS	Newborn Calf Serum
NK	Natural Killer (cells)
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PEG	Polyethylene glycol
PMN	Polimorphonuclear (cells)
PE	Phycoerythrin
PVP	Polyvinylpyrrolidone
SRBS	Sheep Red Blood Cells
SSC	Side Scatter

DISSERTATION

I. INTRODUCTION

I.1. The immune system

The human body possesses an important and highly discriminatory immune system that is essential for survival. This system is composed by specific organs, lymphoid tissue, and various types of cells and soluble factors, all of which are specifically adapted for their respective role. The lymphatic organs are responsible to trigger the majority of immunological responses that will efficiently protect the host (Arosa *et al.*, 2007).

The role of the immune system concerns much more than regulating the homeostatic equilibrium of the organism (Arosa *et al.*, 2007). Not only keeps invaders from taking advantage of the rich source of nutrients provided by the host but also protects the organism from altered internal cells. Thus, the immunological system has to be able to differentiate cells that belong to the host and the commensal flora that populate the skin, gut and other tissues from the pathogenic invading organisms. It is also important that the host is sophisticated enough not to reject tissue that is demonstrably foreign, as the case of the fetus (Male *et al.*, 2006).

I.2. The cells of the immune system

As already mentioned, the immune system is composed by various types of cells with specific characteristics and purposes. The white blood cells or leucocytes are responsible for regulating the immune response and are composed by polimorphonuclear and mononuclear cells. Fig.I.1 schematically represents the different types of leucocytes, as well as the respective molecules expressed in their membranes that allow their identification.

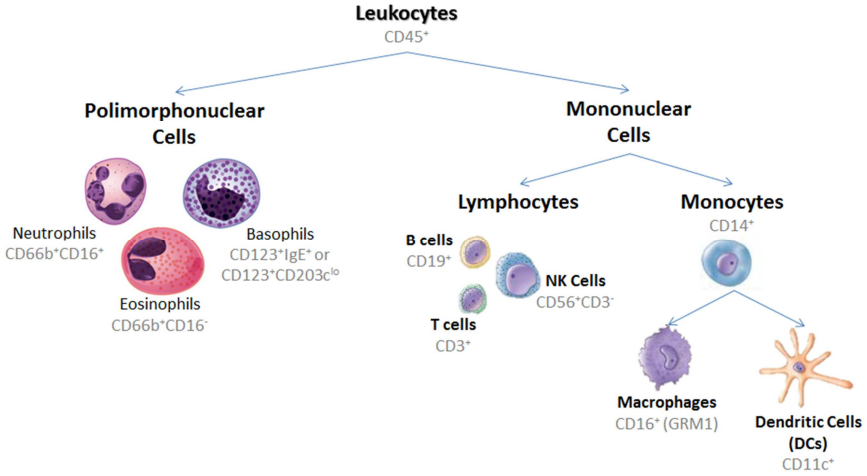

Fig.I.1 – Scheme portraying the different types of leucocytes (white blood cells). For each cell type is presented a representative image, as well as a suggestion of the molecules expressed on cell surface that can be used for their identification. Adapted from:

http://cnx.org/content/m46701/latest/; and

http://www.pc.maricopa.edu/Biology/rcotter/BIO%20205/LessonBuilders/Chapter%2014%20LB/cow95289_14_09b).

The polimorphonuclear (PMN) cells, also known as granulocytes, have cytoplasmic granules and a nucleus with variable shape, as indicated by the name. This type of cells can be distributed into 3 groups, differentiated by the type of the respective granules, namely: neutrophils, eosinophils and basophiles.

In the case of mononuclear cells, the name once more reflects one of the most general characteristics of this class: the existence of a single nucleus. The mononuclear cells are divided in lymphocytes and monocytes. Both these cell classes can present granules, although, they are more visible in some types of lymphocytes (Arosa *et al.*, 2007).

In Table I.1 it is possible to compare the percentages in which the previously mentioned cells can be found in the peripheral blood.

Table I.1 – Main cells of the peripheral blood (Arosa *et al.*, 2007).

Type of cells	**Cell concentration (cells/mm^3)**	**Approximate percentage (%)**
Erythrocytes	5 x 10^6	
Platelets	3 x 10^6	
Leucocytes	7 x 10^6	
Lymphocytes		30.0
T		75
B		15
NK		10
Neutrophils		62.0
Monocytes		5.3
Eosinophils		2.3
Basophiles		0.4

I.2.1. Lymphocytes

Lymphocytes result from differentiation of stem cells produced on the bone marrow and are divided in essentially two types that differ among themselves regarding the place where they are developed. Thus, B lymphocytes or B cells are maturated in the bone marrow of adult mammals, while T lymphocytes or T cells are maturated in the thymus (Male *et al.*, 2006).

These cells are programmed to recognize pathogens and to initiate the adequate immune response; however each type of cell has its specialized roles.

Pathogen recognition is possible due to antigen binding by a specific antibody. An antibody is a surface receptor expressed in B cells that is specific to a certain antigen. Each B cell produces a

particular antibody that upon the eventuality of finding its specific antigen, will bind to it, multiply and differentiate into a plasma cell. These cells will then produce great quantities of the antibody in a soluble form. Soluble antibodies are large glycoproteins that can be found in blood as well as tissue fluids. Being a soluble form of the original antibody, it will still bind the same antigen as the B cell that was initially activated (Male *et al.*, 2006).

The T lymphocytes can be divided in 3 different classes: T helpers (Th), T cytotoxic (Tc) and T regulatory/suppressors (Treg/Ts). Each type of cells is identified by the expression of a different molecule: Th cells express CD4, Tc cells express CD8 (Arosa *et al.*, 2007), and finally Treg/Ts cells can express one or the other (Arosa *et al.*, 2007; Tsai *et al.*, 2011).

Th cells, as indicated by their name, act by aiding other cells performing their respective jobs, namely the antibody production by B cells and the microbicide function of macrophages. Tc cells act for example on the destruction of cells that are neoplastic or infected by viruses, thus having a more direct effective role. Finally, Treg/s cells can suppress specific immune responses. On the contrary to what happens with B cells, T cells do not directly recognize antigens; instead, they have to be processed and presented on the surface of cells associated with the major histocompatibility complex (Arosa *et al.*, 2007).

Both types of lymphocytes are small, with little cytoplasm and a round nucleus where the chromatin is highly condensate. However, after cell activation, the amount of cytoplasm increases and the chromatin becomes less condensate (Arosa *et al.*, 2007).

Finally, there is a third type of lymphocytes, designated as Natural Killer (NK cells); this term results from the ability of this type of cells to recognize and destroy aberrant cells, not needing any previous activation. NK cells also produce chemokines and cytokines, which are soluble factors that can either promptly act as microbicides or activate other cells of the immune system. Similarly to B and T cells, the nucleus of NK cells is round, however in some occasions it presents a small indentation in one of the sides. Also, NK cells have many granules in their cytoplasm, resembling Tc cells. Regarding their size, NK cells are bigger than non-activated T cells (Arosa *et al.*, 2007).

I.2.2. Monocytes, macrophages and dendritic cells

Usually, monocytes are the biggest cells circulating in the peripheral blood; their nucleus presents a shape similar to a horseshoe due to its lobed morphology. Like the nucleus, the limits of these cells are also irregular and the cytoplasm can present vacuoles. Monocytes circulate temporarily in the peripheral blood; eventually they can enter in the tissues and differentiate into macrophages or dendritic cells (DCs) (Arosa *et al.*, 2007).

Macrophages exist in almost every tissue of the body and have an oval or angular shape, their nucleus is not centered and is also oval, but with an indentation that attributes it a kidney-like morphology. One of their main functions is presenting antigens to lymphocytes. Moreover, the evolution of monocytes into macrophages implicates an increase of the phagocytic capacity as well as in the number of lysosomes, which are responsible for carrying hydrolytic enzymes. These cells can

be activated when stimulated by specific soluble factors, resulting in further increase in their phagocytic capacity and secretion of many immunoregulatory soluble factors (Arosa *et al.*, 2007)

DCs are distinguished by their starry, dendritic shape, and when in an immature state, are responsible for collecting antigens. The posterior migration of these cells into the T areas of the lymphoid organs is very important for antigen presentation to T cells, and is concomitant with phenotypic and functional alterations in the dendritic cells. These alterations are caused by a variety of stimulus and result in the maturation of DCs, which at this point are responsible for T cell stimulation. There are two different lineages of DCs, lymphoid and myeloid, depending on their origin or phenotypic characteristics. According to different types of regulatory signals, DCs can direct T cells to different types of immune responses. It is also possible to identify different types of DCs according to their location in the human body: when in the epidermis of the skin, DCs are called Langerhans cells; immature DCs located in the interstitial spaces drained by lymphatic vessels from organs like the kidney, liver, heart, lung, pancreas and intestine are called interstitial DCs; DCs present in T zones of secondary lymphoid organs are called interdigitating; immature or maturating DCs, not yet presenting a ramified morphology and located in afferent lymph or blood are designated by veiled cells (or circulating DCs); DCs located in B zones of secondary lymphoid organs are called follicular DCs (Arosa *et al.*, 2007).

I.2.3. Granulocytes

There are 3 main types of granulocytes: neutrophils, eosinophils and basophiles.

Neutrophils have a nucleus that is divided into 2 to 5 lobules linked by thick heterochromatin covered by nuclear membrane, forming a circle that rounds up the centrosome. Neutrophils have a short life and are one of the first peripheral blood cells to reach an inflammation site. There, they phagocyte and eliminate the pathogens using many mechanisms. One of the main processes consists in using peptides or proteins, with specific microbicide properties that are accumulated in the granules; a selection of the type of granules that are used allows to destroy intracellular or extracellular microorganisms (Arosa *et al.*, 2007).

Eosinophils have a bilobed or eventually trilobed nucleus and act essentially against parasites. They are characterized by a low phagocytic capacity; instead they act by releasing the content of their granules into the extracellular region (Arosa *et al.*, 2007).

Finally, basophils have nucleus covered by granules and release substances as heparin and histamine. These pharmacological active substances are the reason why this type of cells is involved in allergic reactions. Also, basophils are non-phagocytic (Arosa *et al.*, 2007).

I.3. Cell separation

Whenever we need to perform functional studies on a particular cell type we have to isolate the cell population from others present in the sample. Therefore it may be necessary to use physical or immunological separation methods (Freshney, 2010a).

Regardless the technique used, the separation principles fall in one or in a combination of parameters related to the specific characteristics of each cell type. Cell adherence (Tomlinson *et al.*, 2012), cell density and the affinity of antibodies to specific molecules in the cell surface are 3 relatively simple and cheap methods where no high technology is required. Methods relying on cell size and light scatter or differences in the emission of fluorescence, detected by flow cytometry require more advanced and expensive material (Freshney, 2010a).

Thus, some methods involving these principles will be further developed in the following sections.

I.3.1. Cell density

Cell separation according to different density profiles can be performed on a density gradient solution by centrifugation (Al-Mufti *et al.*, 2004). Basically, in order to efficiently accomplish this purpose, a cell sample has to be centrifuged using enough centrifugal force, during a period of time that is long enough to allow cells to arrive the point in the gradient that equals their own density, i.e., their isopycnic density, reason why this process is referred as isopycnic centrifugation (Pretlow and Pretlow, 1989).

The density gradient medium needs to be precisely defined regarding its osmolarity, temperature, pH and composition in order to still allow reproducible results regardless eventual alterations that cell properties may suffer, caused by external conditions (Shortman, 1984). Thus, it is important that at high densities (1,10 g/ml), the media is not toxic nor viscous; also it should apply little osmotic pressure onto the cell solution (Pretlow and Pretlow, 1989; Freshney, 2010a). Probably, the most known density gradient media used to perform cell separation are Ficoll and Percoll. The first is a sucrose polymer, thought to cause less osmotic trauma to the cells than sucrose solutions themselves (Sykes *et al.*, 1970). Percoll consists in colloidal silica spheres coated with polyvinylpyrrolidone (PVP) that allow the formation of density gradients at high-speed centrifugation (Pertoft *et al.*, 1978). Compounds as bovine serum albumin (BSA) (Turner *et al.*, 1966), dextran (Schulman, 1967) and metrizamide (Munthe-Kaas and Seglen, 1974) have also been used. Obviously, each compound has determined particularities in what concerns the formation of the density gradient.

Another isolation process based on cell density properties is called rosetting. Basically, it refers to the ability of some cells, usually lymphocytes, to form clusters that are surrounded typically by erythrocytes (MeSH-NCBI: Rosette Formation). In this context, one of the oldest methods used to separate human T and B cells, is precisely rosetting of T cells with sheep red blood cells (SRBC) (Jondal *et al.*, 1972; Kaplan and Clark, 1974; Wong and Mittal, 1981). The type of erythrocytes most

used for this process is, as already mentioned, SRBC; dog and pig red blood cells also showed some adherence, however in a smaller extent. All these red blood cells have the ability to form rosette structures with T cells; monkey red blood cells (MRBC) also formed rosettes, but to lymphocytes identified as B cells (Pellegrino *et al.*, 1975). Eventually, this method can be combined with antibody binding technology as happens with the RosetteSep™ kit from StemCell. In this kind of separation, cells are incubated with an antibody cocktail solution against specific epitopes of the unwanted cells; consequently, these will crosslink the unwanted cells to erythrocytes, forming immunorosettes as represented in Fig.I.2.

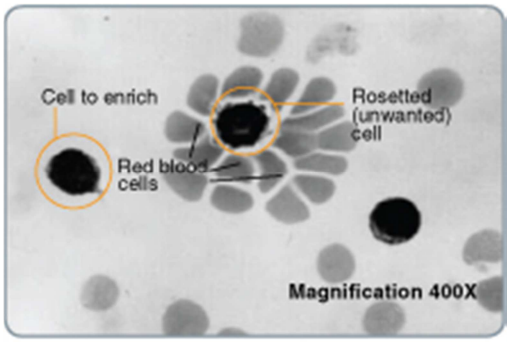

Fig.I.2 – Blood sample after incubation with the RosetteSep™ Cocktail from StemCell, and prior to perform a density gradient centrifugation. Erythrocytes (red blood cells) are linked to an unwanted cell, forming an immunorosette (StemCell).

Regardless the fact that antibody binding is or not involved, the isolation principle is the same: erythrocytes form complexes with the unwanted cells, thus creating immunorosettes. These complexes are considerably denser than the mononuclear cells of interest; therefore, after centrifugation it is possible to remove the wanted cell fraction in the mononuclear cell phase, while the immunorosettes will constitute the pellet (Strelkauskas *et al.*, 1975; Tomlinson *et al.*, 2012).

I.3.2. Cell size

Cell separation can also be performed based on cell size, which is a determinant factor for sedimentation velocity.

The majority of cell separations using this principle use a cell sorter (see section I.3.4.2) or centrifugal elutriation. Elutriation refers to when separation is performed by washing; therefore, in centrifugal elutriation the centrifugal force is opposed to a counter flow of medium. This process occurs in a separation chamber and it is the balance between these two forces that allows separation. With elutriation it is possible to process a large number of cells in a fast way; moreover, despite the shear forces applied to the cells, their viability is generally maintained (Sanders and Soll, 1989).

However, there is also a simpler process based on cell size that does not involve centrifugal methods and it is called unit gravity. This method only requires letting cells settle over a gradient medium (e.g. Ficoll); the different types of cells will settle through the medium accordingly to the

relation between their radius and their sedimentation velocity. This method is more useful when cells present major differences regarding cell size, or when separating aggregates; on the other side, it is generally not suitable to separate large number of cells, except if the mean size of the cells is different and each cell population is homogeneous in size (Sanders and Soll, 1989; Freshney, 2010a).

I.3.3. Cell adherence

The utilization of techniques based on cell adherence allows performing cell separation in a simple and not expensive way. However, these types of techniques depend on the affinity of the cells of interest to adhere to a certain material. Eventually, there may be more than one type of adherent cells, in these cases the target cells have also to be able to outcompete the other cell types for adherence. Moreover, the results are not specific, resulting in lower cell purity when compared to other separation methods (Tomlinson *et al.*, 2012).

One of the oldest methods used to separate T and B cells (besides the previously mentioned rosetting – see section I.3.1), rely precisely on specific adherence of B cells to determined materials. It has been proven that these cells have a preference to stick to glass beads (Rabinowitz, 1965), cotton wool (Hogg and Greaves, 1972) or nylon wool (Eisen *et al.*, 1972; Julius *et al.*, 1973; Wong and Mittal, 1981).

Regardless the fact that the theoretical basis for cell adherence to nylon wool separation is still unclear, this method has been widely used due to its simplicity and because it requires only standard laboratory equipment. Moreover, lymphocytes handled by this process are not exposed to antigens, complement, enzymes or electric fields. As a result, with this method it is expected to obtain a nylon adherent fraction, which is enriched with B cells and a non-adherent fraction, enriched with the T cells. Each fraction is expected to be depleted from the cells of the opposite type, resulting in high purity values (Trizio and Cudkowicz, 1974). On the downside, there is the fact that some antigen presenting cells also have adherence to nylon wool, thus being retained together with B cells. Moreover, it has also been described that T cells purified using a nylon wool column presented alterations regarding proliferation, activation response and production of cytokines (Wohler and Barnum, 2009). It has also been confirmed that is possible to use nylon wool as a solid support to which specific monoclonal antibodies may be associated, in order to separate T cells subpopulations (Kokkinopoulos *et al.*, 1992).

When comparing the nylon wool method to the SRBC rosette one, it is possible to state that despite the fact that SRBC rosette method provides B cells with higher purity, the nylon wool method has less non-lymphoid contamination, it has a simpler and faster protocol and requires less reagents (Wong and Mittal, 1981).

I.3.4. Antibody-based techniques

As portrayed by the name, antibody-based techniques relay on the specificity of a selected antibody to bind to the correct epitope on the surface of a specific cell type. This principle can be applied to many techniques in order to separate a large variety of cell types (Freshney, 2010a). Among the cell isolation techniques that have as basic principle antibody binding, the most commonly used are magnetic-activated cell sorting (MACS) and fluorescence-activated cell sorting (FACS).

In one hand, both techniques have in common the use of specific antibodies against cell surface antigens. On the other hand, MACS technology uses antibodies linked to iron oxide microbeads that will link to target cells, and consequently requires a magnetic field to separate those cells from the remaining ones; whereas FACS technology fluorescently labels its antibodies, and afterwards, the excitation of determined fluorophores above a specific threshold will signal the associated cell to be separated (Tomlinson *et al.*, 2012). Both methods will be approached in the following sections, with special highlight to MACS separation (section I.3.4.1).

I.3.4.1. Magnetic-activated cell sorting

MACS technology by Miltenyi (Klein *et al.*, 1994) is probably one of the most used for this type of separation. Nonetheless, there are other companies that also have their own magnetic sorting technologies as the EasySep™ platform from StemCell (Shin *et al.*, 2012). Both technologies have been used in our laboratory in order to separate different types of cells from human peripheral blood. Therefore, their main characteristics will be presented below.

MACS technology combines the use of high gradient magnetic fields with small superparamagnetic microparticles, as first suggested by Molday and Molday (Molday and Molday, 1984) and also with fluorescent labeling (Miltenyi *et al.*, 1990).

Separation by MACS (Miltenyi) is effective for small or large scale isolations and requires 3 different components: MACS MicroBeads, MACS Columns and MACS Separators (Miltenyi Biotec, 2013). MACS MicroBeads are biodegradable, non-toxic superparamagnetic particles conjugated to highly specific antibodies against a cell surface antigen. These particles are as small as 50 nm, reason why they do not saturate cell surface epitopes neither activate cells themselves; also they do not have to be removed from the cellular suspension prior to subsequent assays. Moreover, MACS MicroBeads are supplied in a colloidal suspension; this fact associated with the small dimensions of the beads enables short labeling steps, fast binding kinetics, and consistency among different lots and at the same time avoids cell clumping. Furthermore, after this process, cells are only minimally labeled in order to assure that concurrent antibody staining will still have enough free epitopes to bind to.

MACS columns comprise a set of cell-friendly coated ferromagnetic spheres, that in the presence of the magnetic field of the MACS separator will increase by 10000-fold their own magnetic field, resulting in the formation of a high gradient. Such increase is critical to compensate the previously mentioned minimal cell labeling. As the magnetically labeled cells are retained in the

column, they never get to bind to the column itself; instead, the cells stay in suspension, thus minimizing the stress caused to them. The remaining cells will simply be allowed to pass through the column as there is enough space between the spheres for that to happen.

Finally, MACS Separators can be found in different formats, which are specifically designed for the appropriated type of column, considering the isolation at hands and the volume that is being applied.

The basic overall principle of this method is described in 3 simple steps that are represented in Fig.I.3: magnetic labeling, magnetic separation and elution of the labeled cells (Miltenyi Biotec, 2013). First of all, a single cell suspension is prepared, magnetically labeled with the intended MACS MicroBeads and applied to an adequate MACS Column, previously set in a MACS separator for the magnetic separation. The unlabeled cells will be eluted and the labeled cells will be retained within the column. After washing the column, it can be removed from the separator in order to also elute the labeled cells. Consequently, this method allows both cell fractions to be isolated with high purity.

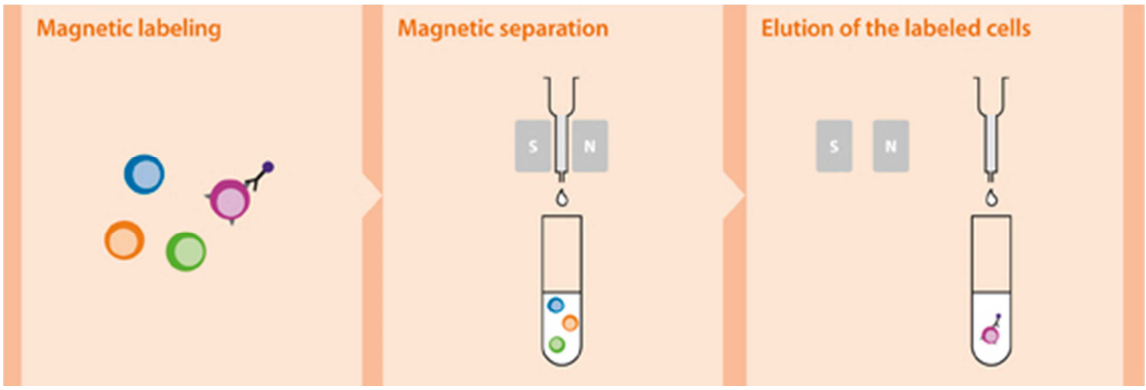

Fig.I.3 – Steps of MACS separation using technology from Miltenyi: magnetic labeling, magnetic separation and elution of the labeled cells. When applied to a magnetic field, the magnetically labeled cells will be retained in the column; those cells can be eluted after removing the column from the magnetic field (Miltenyi Biotec, 2013).

Concerning the labeling methods, they can be performed in a direct or indirect way. In the first case, the MicroBeads will specifically bind to the respective epitopes on the surface of the cells. This case represents the fastest way to label cells as it only requires one incubation step and a minimal number of washing steps, what also minimizes cell loss. On the case of indirect magnetic labeling, two steps are required. Firstly, the cells are labeled with an antibody against a specific cell marker. This primary antibody can be unconjugated, or conjugated with biotin or a fluorochrome. The second step corresponds to the magnetically labeling itself and consists on the binding of the MACS MicroBeads to the primary antibody (Anti-Immunoglobulin MicroBeads) or to the respective conjugated molecule (Anti-Biotin MicroBeads or Anti-Fluorochrome MicroBeads). Indirect labeling is useful to isolate untouched cells, as it allows the use of a cocktail of primary antibodies that will concurrently label the unwanted cells (Miltenyi Biotec, 2013).

MACS technology uses essentially 4 types of separation strategies: positive selection, depletion, untouched isolation (also referred as negative selection) and sequential sorting (Miltenyi Biotec, 2013).

In positive selection (see Fig.I.4), a specific cell type is magnetically labeled (direct or indirectly), thus being the one retained during separation, while the remaining cell types flow through. Then, after the column had been washed and removed from the magnetic field, the target cells can be eluted. To use this strategy is the fastest way to isolate a specific cell type.

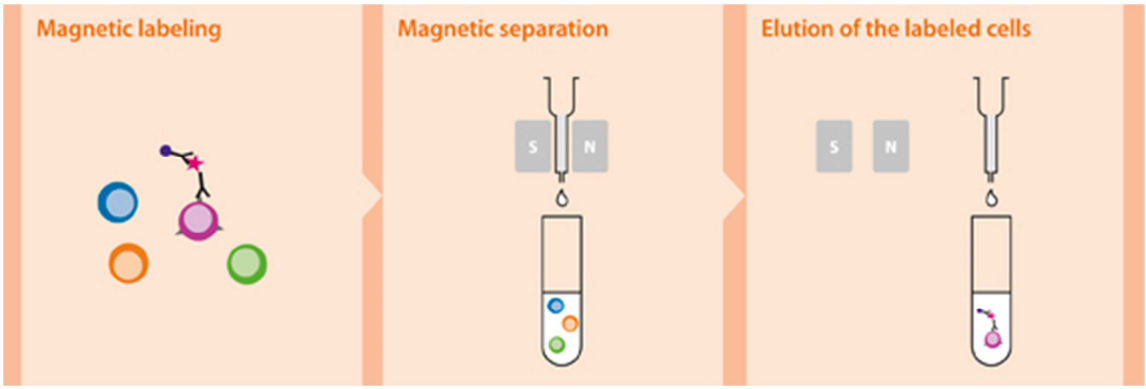

Fig.I.4 – Cell separation by positive selection using MACS technology. Target cells are magnetically labeled; then the cell suspension is applied in a magnetic field: unlabeled cells flow through the column while the target cells are retained, thus needing to be eluted after removing the column from the magnetic field (Miltenyi Biotec, 2013).

In a depletion strategy (see Fig.I.5), an unwanted cell type is magnetically labeled in order to be removed from a cell mixture. This cell type will be retained in the column, while all the remaining cells that represent the target population are collected in the flow-through. Nonetheless, the unwanted cell fraction can still be eluted after the column had been removed from the magnetic field.

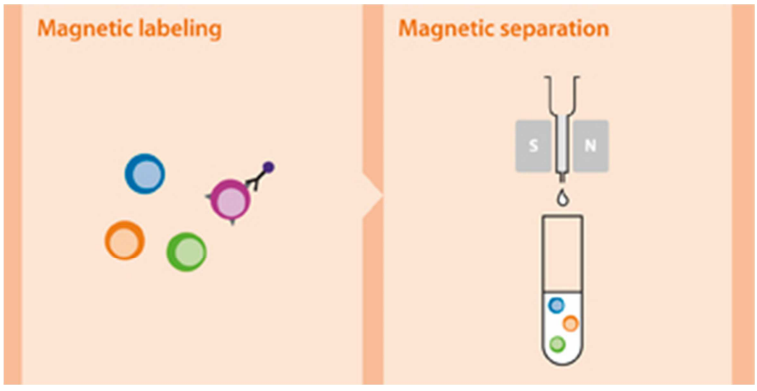

Fig.I.5 – Cell separation by depletion strategy using MACS technology. Unwanted cells are magnetically labeled. When the suspension is applied to the magnetic field, the target cells will be acquired in the flow through while the unwanted ones will be retained in the column and eventually discarded (Miltenyi Biotec, 2013).

Untouched isolation (Fig.I.6) is the most adequate strategy to obtain a target cell type without labeling it (untouched form). In this case, the unwanted cell types are the ones being magnetically labeled and consequently retained in the column and depleted. Being unlabeled, the target cells will be collected in the flow-through. If desired, it is also possible to elute the labeled fraction, after it had been removed from the magnetic field. In this type of isolation strategy it is used indirect labeling, therefore, the respective kits are supplied with a cocktail of titrated antibodies and MACS MicroBeads.

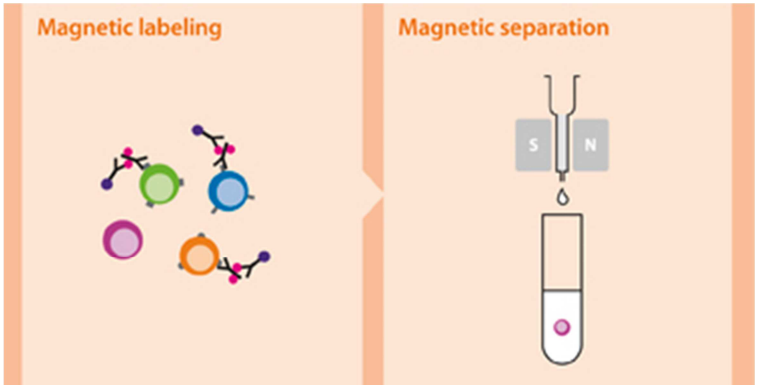

Fig.I.6 – Cell separation by untouched isolation using MACS technology. Unwanted cells are magnetically labeled and consequently retained in the magnetic field. Target cells are collected in the flow through (Miltenyi Biotec, 2013).

Sequential sorting combine two successive separations using more than one marker, in order to obtain a specific cell subset; even in situations where there is no marker defined for that type of cell. In these cases, the isolation strategies may be of different types (depletion followed by positive selection) or of the same type (two subsequent positive selections); both situations will be briefly described next.

When unwanted and target cells, both have the same marker, first of all it is necessary to deplete the undesired cells that also express that marker by magnetically labeling them via antigens different from the common ones. Then, the unlabeled cells obtained in the flow-through must be magnetically labeled with beads for the common marker in order to proceed for their positive selection. This strategy is represented in Fig.I.7.

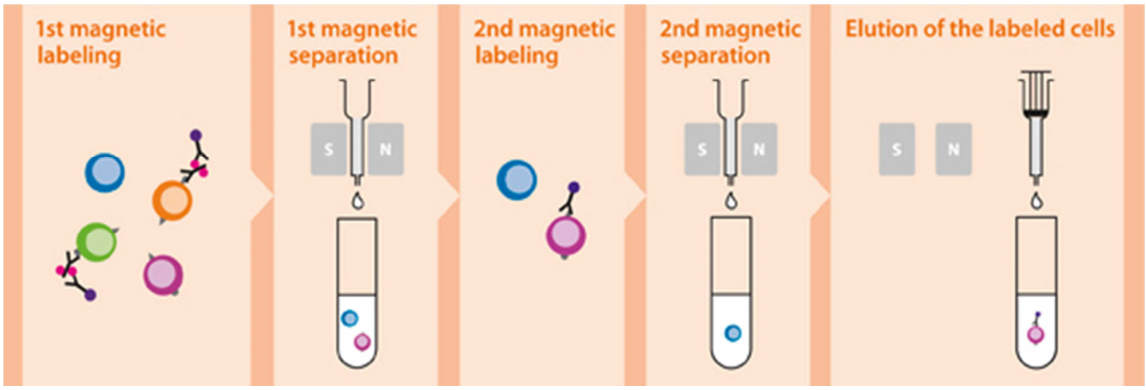

Fig.I.7 – Cell separation by depletion followed by positive selection using MACS technology. Unwanted cells that express a common marker with the target cells are labeled and depleted. Then the target cells obtained in the flow through are labeled for the common marker and obtained by positive selection (Miltenyi Biotec, 2013).

The other possible situation requires a multiparameter sorting using two different markers sequentially. In this case, the first step would be to use the appropriate MACS MultiSort Microbeads to the first marker and proceed to the first positive selection. Before labeling the cells against the second marker it is necessary to enzymatically remove the first beads by using the MultiSort Release Reagent. Only then it is possible to continue for the second positive selection. This process is represented in Fig.I.8.

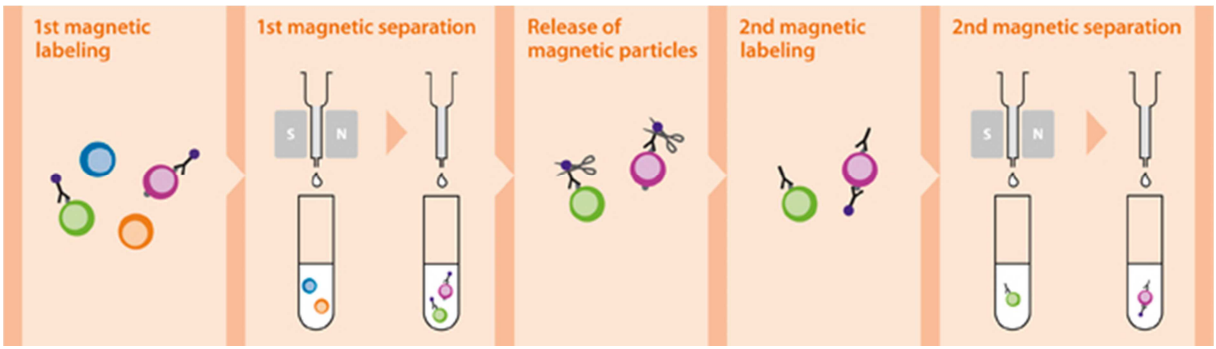

Fig.I.8 – Cell separation by two subsequent positive selections using MACS technology. First of all, cells are labeled for the first maker and collected by positive selection. Then the labeling is enzymatically removed in order to allow a second labeling with a second marker for the target cells, which will also be collected by positive selection (Miltenyi Biotec, 2013).

The EasySep™ platform (StemCell) uses the same magnetic separation principle, however has the advantage of not requiring columns or washes, resulting in a simpler and faster process that can be completed in about 25 minutes. In this case, instead of columns, standard polystyrene tubes can be used; those are directly placed into an EasySep™ Magnet, which is capable of generating a high-gradient field. There are different magnets available, suited for processing different sample volumes (which also happens with Miltenyi). This technology also allows obtaining highly pure

samples that do not require the removal of the magnetic particles prior to eventual downstream applications (StemCell, 2013).

In order to isolate cells using EasySep™, the target cells are linked to dextran-coated magnetic particles by antibody complexes (see Fig.I.9). The next step consists in placing the tube containing the cell suspension into the EasySep™ Magnet: the labeled cells (positive fraction) are attracted to the sides, thus remaining in the tube; however the unlabeled cells (negative fraction) can be poured off into another tube.

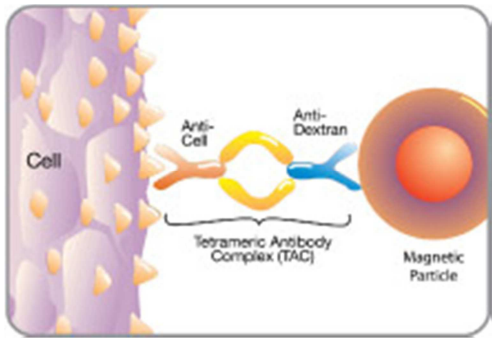

Fig.I.9 – Magnetic labeling using StemCell products. Antibody complexes link the target cells to the dextran-coated magnetic beads (StemCell, 2013).

This technology allows cell isolation through positive selection, negative selection and depletion (StemCell, 2013), much alike to what happens with the technology form Miltenyi, however in less time. The number of washes required by the protocols from Miltenyi, together with the variable time that the cell suspension and subsequent washes take to flow through the column, makes them more time consuming.

I.3.4.2. Fluorescence-activated cell sorting

As previously stated, fluorescence-activated cell sorting is an antibody-binding method that uses an instrument capable of separating cells according to different fluorescent markers (Bonner *et al.*, 1972).

The fluorescence-activated cell sorter acquires cells one-by-one by projecting them in a stream through a laser that detects the emission signals from each single cell. Then, the instrument processes the information, presenting it in a dot plot format, for example. Thus, it is possible to gate specific populations in the display, targeting them for separation. Consequently, the cell sorter will separate each cell that has properties matching the selected coordinates, regarding their light scatter and/or fluorescence (Freshney, 2010a).

This technology has been used to separate several types of cells; however hematopoietic cell separation seems to be the most common application (Yeung and So, 2009).

Comparing FACS technology to the MACS one, FACS has the advantage of allowing a cell to be marked with several antibodies at the same time, while MACS requires an enzymatic removal of

the MicroBeads in order to relabel the same cell. Moreover, the fact that FACS technology considers each cell at a time results in more specific results; on the downside this makes the separation much slower than with MACS technology (Tomlinson *et al.*, 2012).

I.4. Cell culture

I.4.1. Requirements of culture media

There are many types of culture media, adapted to better suit the needs of different cell types, however there are some properties that are overall indispensable, some of which will be briefly mentioned (Freshney, 2010b).

Thus, one of the first points is the pH level. The majority of cells require a pH of approximately 7.2–7.4 and its maintenance between these values is crucial for optimum cell culture conditions (Sigma Life Tecnhologies and ECACC, 2010). Regular assessment of the pH level of culture media is very important, reason why, commercial culture media, such as the RPMI-1640 from Sigma that we use in the laboratory, already include phenol red as a pH indicator. That facilitates a constant control of the pH status of the media: at pH 7.4 phenol red presents a red coloration, an increase in pH causes it to turn pink at pH 7.4 and purple at pH 7.6; on the other side, a decrease to pH 7.0 will turn the media orange, at pH 6.5 it will be yellow and bellow that value it will acquire a lemon yellow coloration (Freshney, 2010b). There are essentially two main forms by which the pH can be buffered, one natural and one chemical. In the natural option, the CO_3/HCO_3 content of the culture media is balanced with a CO_2 incubator, allowing that a 5-10% CO_2 to be maintained in this bicarbonate/CO_2 buffering system. The chemical option requires the use of HEPES, a zwitterion with a high buffering capacity in the range 7.2-7.4 that does not require a controlled gaseous atmosphere. Comparing this two methods, the first one has the advantage of being low-cost and non-toxic, what not always happens with the chemical system (Sigma Life Tecnhologies and ECACC, 2010).

Inorganic salts are also important because they provide sodium, potassium and calcium ions. They are necessary for cell matrix attachment as enzyme cofactors, to retain the osmotic balance of the cells and to regulate the membrane potential (Sigma Life Tecnhologies and ECACC, 2010). The osmolality of the cultured cells is also very important and should be kept between 260 mOsm/kg and 320 mOsm/kg (human plasma osmolality is 290 mOsm/kg so this is inferred as an adequate value for cells in culture). When incubation is performed using a Petri dish or open plates, eventual evaporations can be compensated by a faintly hypotonic medium. Changes in the osmolality may be necessary, for example to the addition of HEPES, and can be achieved by altering the sodium chloride concentration (Freshney, 2010b).

Carbohydrates are another important constituent of culture serum. Usually in the form of sugars, such as glucose and galactose, they represent the main source of energy. The concentration of carbohydrates vary accordingly to the cell types, usually in values of 1-4.5g/l (Sigma Life Tecnhologies and ECACC, 2010)

It is also critical to add amino acids (glutamine in particular) to the culture media as their concentration will determine cell proliferation: amino acids are the constituents of proteins, though they cannot be produced by cells themselves, therefore, from the time they are no longer available to be used by the cells, cell density will no longer increase. Non-essential amino acids can also be added in order to prolong cell viability in culture and stimulate growth (Sigma Life Tecnhologies and ECACC, 2010).

Regarding vitamins, those are usually obtained as part of serum (serum will be further developed in section I.4.2), but can also be added as supplements. Riboflavin, thiamine and biotin are examples of vitamins that are frequently used in culture media, although some media also require B group vitamins or increased levels of vitamins A and E. By supplementing culture media with vitamins, it becomes suitable for a larger variety of cell lines, as vitamins act as precursors for many co-factors (Sigma Life Tecnhologies and ECACC, 2010).

Proteins, peptides, fatty acids and lipids are usually present in serum, making them particularly relevant in culture media. Albumin, transferrin, fibronectin and fetuin are the proteins and peptides added to the medium as replacement. Cholesterol (fatty acid) and steroids (lipids) may also be added for specialized cells (Sigma Life Tecnhologies and ECACC, 2010).

Eventually, more specialized types of cells may also require to supplement the culture media with trace elements as copper, zinc, selenium and tricarboxylic acid intermediates (Sigma Life Tecnhologies and ECACC, 2010).

Factors as temperature and viscosity may also influence the cell culture. The optimal temperature may depended for example on the body temperature of the animal that “provided” the cells as well as on mechanical variations, however most warm-blooded animal cell lines can be kept at 37 ºC; usually, the temperature is set to a slightly inferior value as to prevent damage caused by eventual oscillations (overheating can be more dangerous than underheating). Viscosity takes particular importance when the cells are agitated or dissociated after tripsinization, mainly if there are low or inexistent concentrations of serum. Increasing the viscosity of the medium may be useful to decrease eventual damages upon these procedures and may be accomplished for example by adding carboxymethylcellulose (CMC) (Freshney, 2010b).

Regarding the use of antibiotics, it is only recommended in very specific occasions, such as primary cultures or highly specific experiments. Though antibiotics have been introduced to prevent contamination, they have several disadvantages in cell culture: favor the development of antibiotic-resistant microorganisms, hide infections or the presence of some contaminants, have an antimetabolic effect that can cross-react with the cells in culture, and their utilization may discourage aseptic technique. Mostly, the use of antibiotics is unnecessary if a laminar-flow hood and an efficient aseptic technique is used (Freshney, 2010b).

I.4.2. Serum

As already mentioned in the previous section, serum is composed by several constituents, such as albumins, growth factors, growth inhibitors and hormones. Besides providing nutrients, the main roles performed by serum in culture media are: increase the buffering capacity, protect against any mechanical damage caused by handling the cells, and bind and neutralize toxins. Serum has the advantage of suiting a large variety of cell types, regardless their distinct growth factors requirements. On the downside, standardization of serum production is difficult as it varies a lot from batch-to-batch; moreover, the presence of serum in the culture media may difficult downstream processes, as protein purification, for example (a 10% supplement of Fetal Bovine Serum (FBS) adds an extra 4.8 mg of protein per milliliter of the cell culture fluid). (Sigma Life Tecnhologies and ECACC, 2010).

It is also very important to access the quality of each serum batch, reason why several tests are performed in order to detect the presence or level of the following: virus contamination (e.g. bovine viral diarrhea virus – BVDV), *Mycoplasma* contamination, endotoxin, haemoglobin, total protein, immunoglobulin, osmolality, hormone testing, pH at room temperature and sterility (Sigma Life Tecnhologies and ECACC, 2010).

There are many types of serum commercially available, which may vary according to their range of applications. FBS, bovine calf serum, newborn calf serum (NCS) and human serum are probably the most commonly used.

FBS is one of the most popular types of serum due to its wide range of applications. Its contribution involves providing growth promoting factors as well as improving cell survival.

NCS is also widely used, not only for cell culture, but also for organ preservation. Its cell culture applications include oocyte maturation, tubular epithelial cell functional studies and primary cell culture (e.g. visceral adipocytes). Bovine calf serum is also available, although it has more restrict applications, such as propagation of viruses, *Helicobacter pylori* and *Plasmodium falciparum*.

Regarding human serum, type AB is probably the most used one, being associated with a great variety of applications: tissue engineering, transplantation and some kinds of cell therapy.

The most common way to isolate human sera is by allowing blood to clot. The resulting clear portion will be free of blood cells, fibrinogen and some coagulant factors, being designated as serum (Lewis and Tatsumi, 2006; MeSH-NCBI: Serum). However, if an anti-coagulant agent is added to the blood, it will not be allowed to coagulate; in such cases, the clear phase originated upon centrifugation will be plasma, not serum. Nonetheless, there are also protocols to isolated serum from plasma. As an example we have the method used by Sigma: calcium is added to the plasma in order to activate the clotting cascade; then the preparation is centrifuged and the resultant liquid phase will be serum (Sigma-Aldrich – FAQs).

Usually, the utilization of male AB human sera is preferred over other gender or blood type. The choice for male blood is essentially due to the fact that female donors may have more antibodies resultant from previous pregnancies. On the other hand the choice for AB blood type lays on the absence of anti-A and/or anti-B antibodies (Sigma-Aldrich – FAQs). However, in cases where immunoreactivity is not an issue, sera from other blood types can also be used, and are also available.

I.4.2.1. Heat inactivation

The heat inactivation procedure is performed in order to inactivate the complement cascade which if active, could lead to the activation of lymphocytes and macrophages (Sigma Life Tecnhologies and ECACC, 2010). The functions of the complement include opsonization (increase susceptibility to phagocytosis of antigens), chemotaxis (cell attraction based on concentration gradients) and cell lysis (Pacheco and Cardoso, 2007).

Heat inactivation also reduces the cytotoxic action of immunoglobulins without being harmful to polypeptide growth factors (Freshney, 2010b). Moreover, heat inactivation can also protect the cell culture from being contaminated by viruses, as some can be inactivated by this process (Sigma Life Tecnhologies and ECACC, 2010). On the down side, heat inactivation may cause the removal of labile constituents, and this can be prejudicial to the cell culture (Freshney, 2010b). Thus, heat inactivation of serum is not an obligatory requirement for cell culture (Sigma Life Tecnhologies and ECACC, 2010). By principle, in the case of cultures of PBMCs, the fact that the complement is or not inactivated should not be a problem as normal $CD4^+$ T cells do not express complement receptor in a large enough level to be of influence (Montefiori Laboratory, 2011).

The heat inactivation process is very simple, though it may present some variations. The most common method requires serum to be heated to 56 ºC for 30 minutes (Sigma-Aldrich, 2013c). However, there are protocols that suggest an incubation period of 1h if samples have not been previously heat-inactivated; when they have already been previously heat-inactivated for 30 minutes or less, a new incubation step at 56 ºC should be performed during 30-45 minutes (Montefiori Laboratory, 2011).

I.5. Cell cryopreservation

Cell cryopreservation, is an almost self-explaining term that refers to the process of conserving and protecting the structure, form and chemical composition of cells by means of freezing (MeSH-NCBI, 1990).

Frequently, in most laboratories, it is necessary to stabilize materials in order to preserve their sub-cellular components. Mainly, when working with cell lines it is vital to protect them from several mishaps such as genotypic drift, phenotypic instability, transformation and acquisition of non-desired characteristics, senescence and eventual extinction (Freshney, 2010c). Also, it is important to avoid contamination by other microorganisms or even by other cell lines (cross-contamination) (Capes-Davis *et al.*, 2010). So, before proceeding to the cryopreservation process itself it is important to check for contamination and register the cell line characteristics including eventual genetic modifications or infectiousness (Simione, 1992; Stacey and Dowall, 2007).

Moreover, for some research purposes, it is useful to store primary cells so that the same batch can be used at different times in the future. Also, in clinical applications, for cell-based long-term

treatments, such as DC-based vaccinations, it is useful to store some aliquots of PBMCs to differentiate into DCs later on (Westermann *et al.*, 2003; Heo *et al.*, 2009).

For all the referred reasons, cryopreservation is a practical solution; it is also a helpful solution when distributing samples to other investigators or even as a way to save time, by avoiding the maintenance of the cell lines that are not being used at that moment (Freshney, 2010c).

I.5.1. Principals of cryopreservation

The objective of any cryopreservation process is not only to preserve cells but also to allow the maximum recovery rate possible. In order to accomplish such goals it is important that certain principles are followed. First of all, it is essential that an adequate cryoprotectant is used, and then samples have to be frozen with an appropriate cooling rate and stored under restrict conditions. Finally, the thawing process also has to be controlled in order to maximize cell viability and recovery (Freshney, 2010c).

I.5.2. The cryoprotective agent

The utilization of a cryoprotective agent in a freezing process is fundamental; its main role in the process is to minimize damage caused by the formation of ice crystals and the subsequent osmotic imbalance.

Dimethylsulfoxide (DMSO - Me_2SO) and glycerol are clearly the ones most commonly used (Simione, 1992); however PVP (Suzuki *et al.*, 1995), polyethylene glycol (PEG) (Monroy *et al.*, 1997) and hydroxyethyl starch (HES) (Pasch *et al.*, 2000) are examples of compounds already suggested and tested as cryoprotectants.

Handling DMSO requires some caution as it is a penetrating agent that when in direct contact with skin is rapidly absorbed and may transport other potentially harmful substances along with it (Pope and Oliver, 1966). On the other side, this facility in penetrating cells comes as an advantage in its role as a cryoprotective agent, especially in larger and more complex types of cells, because it encourages the dehydration of the cell before ice crystals start to form in its interior. Glycerol is not as penetrating as DMSO, nonetheless is often preferred due to its inferior toxicity. Moreover, these 2 cryoprotective agents are not used simultaneously, in the same freezing media, unless we are handling plant cells (Simione and Thermo Fisher Scientific, 2009).

Regardless the preferred agent, it should be diluted in fresh media, and only then added to the cell suspension; this will allow a more uniform contact with the cells. In what DMSO and glycerol are concerned, they are usually applied in a concentration of 5-10% (v/v) in order to minimize the eventual aggressiveness of this contact (Simione and Thermo Fisher Scientific, 2009). Nonetheless other percentages of the cryoprotective agent may also be used, and the type of cell being frozen may play an important role on that decision (Baust *et al.*, 2002).

I.5.3. Sample preparation and equilibration

Usually, samples are frozen at a high cell concentration in order to compensate eventual low cell recovery after thawing (Freshney, 2010c); the values are highly variable, but 5 x 10^6-10^7 cells/ml are commonly used concentrations when freezing PBMCs (Stacey and Dowall, 2007). Also after thawing, the cryoprotective agent needs to be removed by centrifugation or diluted in the cell suspension. In the case of dilution, the initial cell concentration should allow a dilution factor of 10 or 20 fold. When centrifugation is not the preferred option, a higher cell concentration prior freezing will allow a higher sample dilution (Freshney, 2010c).

Another variable that is important to consider when freezing cells is the equilibration process. This refers to the period of time that starts when the cryoprotective agent is added to the cell suspension, and goes up until the beginning of the cooling process. This period of time should take place at room temperature and may be used to distribute the cell suspension through the intended cryovials. Equilibration should occur in a period of approximately 15-45 minutes, in order to allow the cryoprotective agent to enter the cells. However it may never exceed 60 minutes as the cryoprotective agent may be toxic to the cells after longer periods (Simione and Thermo Fisher Scientific, 2009).

I.5.4. Cooling rate

When cells are not able to use water for their usual chemical processes their metabolism stops. That is what occurs upon freezing, as all water is converted to ice (Simione and Thermo Fisher Scientific, 2009).

Cell freezing causes ice crystals to form inside and outside the cells. The rate at which cells are frozen has great influence on the overall success of the process. If the cooling rate is too slow, ice crystals will start forming outside the cell first, diminishing the amount of water in a liquid state and increasing solute concentration. To compensate this imbalance, intracellular water will start coming to the extracellular environment, thus increasing the concentration of solute inside the cell too. Therefore, the overall result of this osmotic imbalance will be less intracellular ice crystals being formed and higher solute concentrations. On the other side, if the cooling rate is too fast, ice crystals will form uniformly both inside and outside the cell, not causing a big alteration in solute concentration. However, as water did not have much time to migrate to the extracellular environment, there will be more ice forming inside the cell. The formation of too much ice crystals inside the cell may damage its components, and this, together with eventual damage caused by recrystallization during thawing can be very prejudicial to the cells (Simione, 1992; Simione and Thermo Fisher Scientific, 2009).

There are many factors that may influence the cooling rate, such as the ambient temperature, eventual leaking in the freezing containers, the specific heat (the quantity of heat required to raise the temperature of a substance by 1ºC), the volume occupied by the components being frozen and also the latent heat absorption (heat absorbed during a constant-temperature process) (Freshney, 2010c).

Different cells may require different rates of cooling but usually, a compromise is found at 1 ºC/min (Leibo and Mazur, 1971).

The easiest way of achieving a controlled freezing rate consists in using a programmable-rate cell freezing apparatus that according to its sophistication may allow the selection of one or more cooling rates throughout all the freezing period. These units have the disadvantage of being expensive, reason why cheaper and simpler options are available for similar purposes. For instance, Thermo Scientific Nalgene Mr. Frosty simulates a controlled-rate cooling process by allowing a decrease in temperature close to 1 ºC per minute. Nonetheless, this container still needs to be placed in a mechanical freezer at -60 ºC to -80 ºC (Simione and Thermo Fisher Scientific, 2009).

I.5.5. Storage

Cell recovery and survival rates are highly dependent of the temperature of storage. Usually, longer viable storage periods are associated with colder freezing temperatures, nonetheless it is important that the freezers are functioning properly and have no leaks (Simione and Thermo Fisher Scientific, 2009).

Short-term storage of cells is possible with temperatures of -80 ºC (Simione, 1992), however for long-term storage the best results are obtained with freezing temperatures below -130 ºC, because that temperature marks the limit of biological mechanisms (Mazur, 1984).

I.5.6. Cell thawing

Cell thawing is actually a very simple process, only requiring cells to be warmed as fast as possible, usually in a water bath at 37 ºC (Simione and Thermo Fisher Scientific, 2009). Delaying this step will increase the formation of ice crystals inside the cell, intensifying the risk of cell damage or even death. Afterwards, depending on the sensibility of the cells being handled, the cryoprotective agent might need to be removed by centrifugation; this is usually the case of cells capable of growing in suspension. On the other side, there are cells that only require to be gradually diluted or to have their media changed the following day (Freshney, 2010c).

I.6. Cell viability studies

Cell viability assessments are usually performed after a potentially traumatic event, such as cell separation or cryopreservation. The basic principle of this type of assays associates cell membrane integrity with either the uptake or release of a dye. In the first case, normal cells exclude the dye but when the membrane is damaged, the dye is able to penetrate the cell; as examples of this kind of dyes are trypan blue, propidium iodide, erythrosine and naphthalene black. On the second

case, viable cells have the ability of retaining a certain dye (e.g. diacetyl fluorescein, neutral red), but damaged cells loose that capacity (Freshney, 2010d).

One of the most used dyes is trypan blue: usually the cell suspension is diluted (1:2) in 0.4% trypan blue and mixed. Then, the suspension is loaded into a Neubauer chamber (hemacytometer) and allowed to set during 1-2 minutes so cells will focus in the same optical plan. Upon observation in the optical microscope, living cells will be easily distinguished from the dead ones, which will present a blue coloration. It is important that cells do not remain too long in the presence of the dye, or they will deteriorate and uptake it. Moreover, dye exclusion methods tend to overestimate cell viability, i.e., they only refer to the moment when they are performed, as not all living cells at that moment may be effectively able to perform their functions a few time later (Freshney, 2010d).

Furthermore, if these methods are performed after washing steps, the proportion of viable cells will only refer to the total of remaining cells at that point, i.e. the cells lost upon the washing process will not be considered (Freshney, 2010d).

I.7. Context and objectives of the work developed for this thesis

The work performed for this thesis lies within the Cell Biology Services @ CEDOC and have accompanied and contributed to its development. This platform offers other investigators the possibility of purchasing several products isolated from human peripheral blood, such as mononuclear cells, monocytes, B and T cells; differentiation of haematopoietic progenitors into antigen presenting cells (dendritic cells, macrophages) and human serum are also available.

The set-up of this platform entailed several aspects, ranging from its laboratory practical component to the estimation of costs associated with the services provided and to the creation of divulgation platforms and documents. This thesis covers those aspects, and we have delineated three main objectives:

1 - Optimization of laboratory protocols. It included the following secondary aims:

a. First of all, we intended to optimize a protocol to isolate serum from buffy coats of human peripheral blood. This serum is intended to be used for cell culture. In order to determine whether it is suitable for that purpose, cells were cultured with media supplemented with the serum obtained with our protocol and the results were compared to the ones obtained from parallel cultures with FBS and commercial human serum.
b. The second objective was to compare different protocols for the isolation of monocytes and lymphocytes from PBMCs. Several cell characteristics, such as isolation yield, purity, percentage of cell dead, cell activation and maturation levels were assessed and the results are presented. Knowing the influence each isolation process has in cells may help choosing the most appropriate method for a specific assay.
c. Finally, the preservation and evaluation of cells shelf life is also very important. Thus, the third objective concerned the determination of the cell recovery after different periods of

cryopreservation. Moreover, different protocols for removal of the cryoprotectant were also tested in order to determine the less harmful one.

2 - Cost estimations: we aimed to estimate the real cost associated with the provided services. In this thesis, we intended to estimate costs associated with each isolation protocol. For the estimation of these costs we had to gather recovery rate data for each type of cells isolated from buffy coats. Since the recovery rate is variable from individual to individual and was calculated based on the cell yields from cell isolations performed within the duration of this thesis. Since the quantities used for each reagent are dependent on the initial cell numbers, the costs with reagents are directly correlated with these recovery rates. We also compared different brands and methodologies, used to isolate the same type of cells.

3 - Cell Biology services divulgation: the divulgation of the services was crucial for its success. We have therefore created divulgation platforms, where investigators could get information on the services, the contacts, the prices and conditions and download order form templates. The divulgation was also done via email, flyers and a poster presentation at a scientific meeting.

II. MATERIALS AND METHODS

II.1. Human peripheral blood

All the work developed for this thesis required the use of human peripheral blood products/components. These were isolated from leuco-platelet concentrates (buffy coats) of healthy volunteers provided and ethically approved by the Portuguese Blood Institute (Instituto Português do Sangue – IPS). For more information concerning blood collection (Optimal Blood Use Project, 2010) and donor specifications consult Appendix I.

The posterior processing applied to the buffy coats is briefly summed up in Fig.II.1.

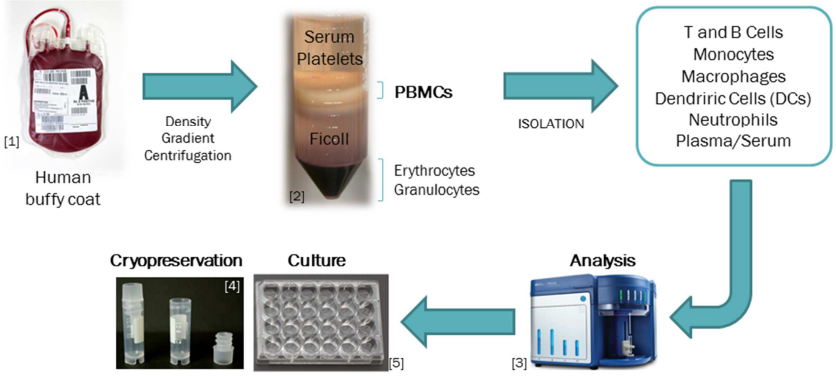

Fig.II.1 – Buffy coat processing workflow applied in Cell Biology Services @ CEDOC. Images adapted from:

[1] http://3.bp.blogspot.com/-93a19ZQeris/UFOmEnPInJI/AAAAAAAAAN8/rTibegsklPA/s1600/BloodBagImage.jpg;

[2] Glycoimmunology Group, FCM-UNL;

[3] http://cdn.medgadget.com/img/attune342.jpg;

[4] i00.i.aliimg.com/photo/v0/103930744/Cryo_Vials.jpg;

[5] http://globebio.com/media/catalog/product/cache/1/image/9df78eab33525d08d6e5fb8d27136e95/2/4/24-well-plate.jpg

II.2. Isolation of plasma and defibrinated plasma

As mentioned before in section I.4.2, plasma refers to the residual phase of non-coagulated blood that is obtained after removing the cells by centrifugation, while serum is the clear phase obtained after blood had clotted.

Plasma can be separated from other blood components by a two-step process. The first step consists in centrifuging the leuco-platelet concentrates at 1100 x g (2500 rpm) for 10 minutes, with no brake. At this point, three different layers became visible. The upper and clearer one corresponds mainly to plasma and must be transferred into a new tube. The intermediate thin white layer corresponds to leucocytes and platelets, and the inferior red layer is composed of erythrocytes; these remaining phases can still be used to proceed to blood cell isolation. The removed upper layer is then centrifuged once more, but now at 2000 x g (3400 rpm), for 15 minutes, with no brake. The resulting pellet must be discarded as it is composed essentially of platelets that failed to lower down to the

intermediate layer on the first centrifugation. The supernatant is the actual plasma, which can either be immediately analyzed or stored at -20 ºC in aliquots of desired volumes.

When blood is collected into a dry tube, the coagulation cascade is initiated. Part of this process consists in thrombin converting fibrinogen to fibrin, which will contribute to the formation of the blood clot (MeSH-NCBI: thrombin, fibrinogen, fibrin). Therefore, it is possible to separate serum from the remaining blood components simply allowing the blood to clot. However, this method cannot be applied to our situation as buffy coats are supplied in the presence of citrate phosphate dextrose (CPD), a preservative and anticoagulant solution that prevents the formation of the fibrin clot, and consequently, direct serum isolation.

In such situation, the alternative would be to isolate the plasma and afterwards apply a fibrinogen removal step. This can be accomplished by adding calcium to the plasma sample, thus inactivating the coagulation cascade, as it is performed by Sigma (Sigma-Aldrich – FAQs). However the exact process and calcium concentrations were not known to us. Therefore, we have adapted a method to precipitate fibrinogen from plasma using heat (Dintenfass and Kammer, 1976); this process had the advantage of being cheaper as it would only require the use of a water bath and a centrifuge.

First of all we have tried to simply remove fibrinogen from plasma and for that we have centrifuged the buffy coat at 1100 x g (2500 rpm) for 10 minutes, with no brake. Then, the upper clear phase (plasma) was removed and incubated in a water bath (General Electrics) at 58 ºC for 3 minutes. After that, the sample was centrifuged at 2000 x g (3400 rpm), for 15 minutes, with no brake. The resulting supernatant would correspond to the defibrinated plasma, and the pellet was discarded. This protocol will be referred as “method #1”.

On the other hand, we also intended to heat inactivate the serum after its isolation, so we have decided to test whether combining that step with the fibrinogen precipitation one would be successful. Therefore, in this case we have proceeded as in method #1 concerning both centrifugations, but the incubation was performed in a water bath at 56 ºC during 1 hour, being this protocol now on referred as “method #2”. The supernatant was immediately analyzed or stored at -20 ºC in aliquots of desired volumes.

II.3. Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were isolated by sequential density gradient centrifugations. The first step in this process consists in distributing the buffy coats into 50 ml tubes and diluting that volume in a phosphate buffered saline solution (PBS) (Appendix II) in a 3:2 ratio; then the solution was centrifuged at 1100 x g (2500 rpm), for 10 minutes, with no brake. At this point, the intermediate layer consisting mainly of leucocytes, were removed to a new tube Then, this leucocyte fraction was diluted with PBS until a final volume of 20 ml. After proper homogenization of the solution, it was carefully layered on top of a Ficoll (Biochrom AG) solution (12 ml), using a plastic Pasteur pipette. This step is followed by a new centrifugation at 1100 x g (2500 rpm), for 20 minutes, still with no brake. Ficoll is a hydrophilic polysaccharide with a particular density, superior to PBMCs, but

inferior to erythrocytes and granulocytes, allowing a specific separation of the different components, after centrifugation. Thus, at this point, blood components are separated by density gradient and four phases should be differentiable: erythrocytes and granulocytes, present as pellets, followed by Ficoll, and then the PBMCs right beneath the most abundant layer, which corresponds to plasma and the majority of the platelets. PBMCs were collected and transferred to a 15 ml tube. In order to remove the remaining cell debris and platelets, PBMCs were washed three more times, being for each time, diluted in PBS to a final volume of 10 ml. Firstly the cell suspension was centrifuged at 250 x g (1200 rpm), for 10 minutes, and then twice at 110 x g (800 rpm), for 5 minutes. These last two centrifugations may be performed at 4 ºC in order to improve platelet removal. Before the last centrifugation, a small aliquot should be removed and counted in the optic microscope (Nikon), using a Neubauer chamber, in order to determine the total number of PBMCs in the total solution.

The isolation of PBMCs is the starting procedure for the isolation of more specific cell types, such as CD3 cells (sections II.4 and II.5) and CD14 cells (section II.6).

PBMCs from the same donor were divided and simultaneously processed with different kits in order to compare different types of separation and different vendors.

II.3.1. Lyse solution

Occasionally, after performing the first centrifugation after the Ficoll density gradient separation step, the pellet still shows a reddish color. This might be due to erythrocyte contamination of the leukocyte layer, because some erythrocytes are trapped and fail to lower down during Ficoll separation. In order to clear the pellet from this undesired cells it is possible to include an extra step in which a lyse solution is used to eliminate them.

To evaluate the effect of this lyse step to the final quality of PBMCs, the amount of PBMCs was equally divided and with one half the protocol was followed as usual, while the other was incubated with the lyse solution as will be described next.

After centrifuging the PBMCs at 250 x g for 10 minutes, the supernatant was removed and cell pellet was resuspended in 1.5 ml of lyse solution (Appendix II) and incubated for 5 minutes, at room temperature. After this period, the cell suspension was diluted in RPMI-1640 (Sigma) to a final volume of 10 ml, before centrifugation at 110 x g for 5 minutes, resuming the usual protocol. Samples originated by both processes were counted in the Neubauer chamber and analyzed by flow cytometry.

II.4. Isolation of $CD3^+$ T cells using MACS technology from Miltenyi

T lymphocytes ($CD3^+$ cells) were isolated with two different kits from Myltenyi Biotec: CD3 MicroBeads (positive selection) and Pan T Cell Isolation kit (negative selection). Both kits were compared by equally dividing the amount of PBMCs (from the same donor) and simultaneously using each kit to separate T cells in one of the halves.

II.4.1. Positive selection

Once the approximate total number of PBMCs had been determined, and the supernatant originated from the last centrifugation has been discarded, the pellet was resuspended in beads buffer (Appendix II) in a proportion of 80 µl per 10^7 cells. Then cells were labeled by adding 20 µl of CD3 MicroBeads (Miltenyi Biotec) per 10^7 cells, in accordance with the supplier specifications. The cell suspension was well mixed and incubated at 4 ºC. After 15 minutes, cells were diluted by adding beads buffer to a final volume of 10 ml and centrifuged at 250 x g (1200 rpm) for 10 minutes, to wash the cells. Then, the pellet was diluted once more, now in a proportion of 10^8 cells for 500 µl. At this point, cells are ready for the magnetic separation.

Meanwhile, a LS column (Miltenyi Biotec) was placed in the magnetic field of a MidiMACS Separator (Miltenyi Biotec) and rinsed with 3 ml of Beads Buffer. Once the column reservoir had emptied, the cell suspension was applied onto it and the resulting effluent collected. The column was then washed 3 times, using 3 ml of beads buffer, never applying the next 3 ml before the column reservoir had emptied. At this point, the total effluent collected is composed only by unlabeled cells ($CD3^-$), representing the negative fraction of the isolation.

In order to obtain the labeled cells (positive fraction), the column was transferred to a 15 ml tube, where it was loaded with 5 ml of beads buffer. Then, the plunger supplied with the column, was immediately applied and the eluted fraction was collected. The eluted fraction corresponds to the wanted $CD3^+$ fraction. The isolated cells were then counted in a Neubauer chamber and further analyzed by flow cytometry for quality control assessment. The isolated cells were centrifuged at 250 x g (1200 rpm) during 10 minutes, and resuspended in freezing media in a concentration of 6.25 x 10^6 cells/ml and frozen at -80 ºC for posterior sell.

II.4.2. Negative selection

This separation method is very similar to the positive selection one; the existing differences focus mainly on the labeling steps, as in this case the target cells are the unlabeled cell fraction (untouched cells).

After the number of PBMCs had been determined, and the supernatant originated from the last centrifugation had been removed, the pellet was resuspended in 40 µl of beads buffer and 10 µl of Pan T Cell Biotin-Antibody Cocktail (Miltenyi Biotec), both per 10^7 total cells. Then, the cell suspension was well mixed and incubated at 4 ºC for 5 minutes. After, an extra 30 µl of beads buffer was added, followed by 20 µl of Pan T Cell MicroBeads Cocktail (Miltenyi Biotec), both per 10^7 total cells. Then, the cell suspension was once more well mixed and incubated at 4 ºC, for 10 minutes. Finally, the cells were resuspended in a minimum of 500 µl of beads buffer, (scale up for more than 10^8 cells), making them ready for magnetic separation.

The LS column preparation and sample application was carried as described in section II.4.1. In this case the T cells correspond to the unlabeled cells (negative fraction), on the contrary of what

happened with positive selection. Thus, the column flow-through after sample application and subsequent column beads buffer washing (3 mL) were collected in the same tube. Samples were taken for counting in a Neubauer chamber and analyzed by flow cytometry for quality control assessment. The isolated cells were centrifuged at 250 x g (1200 rpm) during 10 minutes, and resuspended in freezing media in a concentration of 6.25 x 10^6 cells/ml and frozen at -80 ºC for posterior sell.

II.5. Isolation of $CD3^+$ T cells by nylon wool fiber column

The first step in this process consists in preparing the column: an adequate stopcock was applied to the Luer tip of a Becton Dickinson syringe packed with 0.5 g of nylon wool fiber until the 5 cc mark (Polysciences). Then, the syringe was attached to a holder that allowed it to be maintained in the vertical position. The column was washed with RPMI-1640 medium supplemented with 10% FBS, at 37 ºC; during this process, the column was gently taped in order to ensure that all wool was wet. Leaving the column filled with media until the 10 cc mark, the stopcock was closed and the column was incubated at 37 ºC for 1 hour. After that period, the stopcock was opened just long enough to allow the media to drain until the top of the nylon wool. Then, 1-2 x 10^6 PBMCs, resuspended in 2 ml of media, were added to the column and allowed to enter the packed wool before the stopcock was closed. Then, the stopcock was re-opened and an additional 2 ml of media were added and allowed to enter the packed wool before closing the stopcock again. The syringe was once more filled with media up to the 10 cc mark and incubated at 37 ºC for 1 hour. Finally, the non-adherent T cells were collected by washing the column twice with media. The adherent fraction (non-T) was also collected by plunging the column after adding media until the 10 cc mark; this step was repeated twice. Finally, both factions were centrifuged at 250 x g (1200 rpm) for 10 minutes. After discarding the supernatant, the cell pellet was resuspended in 10 ml of media and an aliquot was taken for flow cytometry analysis.

II.6. Isolation of $CD14^+$ monocytes by positive selection

Monocytes ($CD14^+$ cells) were isolated with two different positive selection kits: CD14 MicroBeads (Miltenyi Biotec) and EasySep® Human CD14 Selection Kit (StemCell). The procedures for $CD14^+$ cell isolation were generally as recommended by the manufacturer, except for the amount of beads. The amount of beads to be used with the Miltenyi kit had already been optimized in our laboratory and determined to be 1/3 of the recommended concentration. However, the StemCell kit was not frequently used in our laboratory, and thus, we lacked data regarding an eventual optimization. Consequently, both kits were compared by dividing the amount of PBMCs in three parts which were simultaneously processed using the referred kits. The MACS kit was used in 1/3 of the

recommended beads concentration and the StemCell kit was used as recommended by the manufacturer, but also using 1/3 of the recommended beads concentrations.

II.6.1. Isolation of $CD14^+$ monocytes using MACS technology from Miltenyi

Once the number of PBMCs had been determined, and the resultant supernatant from the last centrifugation had been discarded, cell labeling was performed as described next. PBMCs were resuspended in 80 µl of beads buffer and 20 µl of CD14 Microbeads (Miltenyi Biotec) per 3 x 10^7 cells. The cell suspension was well mixed and incubated at 4 ºC for 30 minutes. After, cells were washed by adding beads buffer to a final volume of 10 ml and centrifugation at 250 x g (1200 rpm) for 10 minutes. Once the resulting supernatant had been discarded, the cell pellet was resuspended in 3 ml of beads buffer.

The LS column preparation and sample application was carried as described for T cell isolation in section II.4.1. The wanted $CD14^+$ cells (monocytes) correspond to the positively selected fraction, obtained after the plunger had been applied to the column. In order to analyze the resultant cell population, aliquots were taken for cell counting in the Neubauer chamber and for flow cytometry analysis. The isolated cells were centrifuged at 250 x g during 10 minutes and resuspended in culture media in a concentration of 1 x 10^6 cells/ml (see section II.7 for more detailed information on cell culture and generation of DCs).

II.6.2. Isolation of $CD14^+$ monocytes using EasySep platform from StemCell

Although based on the same principals as the kit from Miltenyi, in this case the protocol is somewhat different, mainly due to the fact that it does not require the use of a separation column and less washing steps have to be performed. Also, the EasySep kit protocol implies that 5 ml polystyrene round-bottom tubes are used.

The first step consists in diluting the PBMCs in recommended buffer (Appendix II), at a concentration of 1 x 10^8 cells/ml. Then, EasySep positive Selection Cocktail (StemCell) was added at a proportion of 100 µl per 1 x 10^8 cells (or 100 µl per 3 x 10^8 cells); the cell suspension was well mixed and incubated at room temperature for 15 minutes.

The following step consisted in adding the EasySep Magnetic Nanoparticles (StemCell): first of all, the nanoparticles had to be homogenized by being vigorously pipetted up and down, and only then, added to de cell suspension at a proportion of 50 µl per 1 x 10^8 cells (or 50 µl per 3 x 10^8 cells). Once again, the suspension was well mixed and incubated at room temperature, for 10 minutes. After, the recommended buffer was used to bring the cell suspension volume up to 2.5 ml. Cells were gently mixed and the tube was placed into the EasySep Magnet (StemCell). After 5 minutes, the magnet, and consequently the tube, was inverted in a continuous motion in order to pour off the supernatant; the magnet was left inverted for 3 seconds; it is very important that at any time the tube is shaken. At this

point, only the magnetically labeled cells ($CD14^+$) remain in the tube. Then, the tube was removed from the magnet and 2.5 ml of the recommended buffer were added to it. Once more the cell suspension was mixed and the tube was put back in the magnet for other 5 minutes and, once more, discarded. This process was repeated for a total of 3 incubations in the magnet, all 5 minutes each. After the last supernatant had been discarded, the remaining cells were resuspended in 5 ml of RPMI-1640. Aliquots were taken in order to perform cell count in the Neubauer chamber and for flow cytometry analysis. Finally, the cell suspension was centrifuged at 250 x g (1200 rpm) for 10 minutes and resuspended in an adequate volume of culture media (1 x 10^6 cell/ml); see section II.7 for more detailed information on cell culture and generation of DCs.

II.7. Generation of dendritic cells

After the isolation of monocytes ($CD14^+$ cells) was complete, they were resuspended at a concentration of 1 x 10^6 cells/ml in culture media (Appendix II) supplemented with 750 U/mL of Interleucine-4 (IL-4) (R&D Systems) and 1000 U/mL of granulocyte macrophage colony-stimulating factor (GM-CSF) (R&D Systems) to promote differentiation into DCs. The cell suspension was then distributed in a 24 well culture plate (1 ml/well) (Greiner Bio-one).

Cells were transferred into an incubator (Heraeus) and kept in culture at 37 ºC, in a humidified atmosphere with 5% CO_2. Every two days, half the culture media was gently removed and replaced by an equal amount of fresh one, supplemented with IL-4 and GM-CSF in the same concentration as previously referred. After 7 days in culture with cytokines, monocytes had already differentiated into DCs and were removed from culture in order to access their maturation and percentage of cell death.

At this point might be necessary to determine the efficiency of differentiation, which relates the number of DCs to the number of monocytes that were initially to be differentiated.

$$Efficiency\ of\ differentiation = \frac{number\ DCs\ \times DC's\ sample\ purity}{number\ of\ monocytes\ \times\ monocite's\ sample\ purity}$$

II.8. Testing of serum samples

In order to determine whether our adapted protocols for serum isolation from buffy coat plasma were or not effective we had to determine if fibrinogen had been successfully removed. Therefore, the first step was to perform a protein electrophoresis: this method separates the different components of serum, allowing identifying the presence of fibrinogen. At this point, if the isolation methods did in fact work, fibrinogen will be absent, meaning that we are dealing with a serum sample.

The second step was evaluating the quality of the serum isolated in our laboratory, i.e., how it influenced cells that had contacted with it.

II.8.1. Protein electrophoresis

Protein electrophoresis was used to determine whether plasma samples still contained fibrinogen. For this purpose, we have tested samples isolated from the same donor using method #1 and method #2. Simultaneously, we also tested a plasma sample from the same donor and a serum sample isolated from clotted blood of a different donor.

First we have prepared the cellulose acetate plate by soaking it in electrophoresis buffer for at least 20 minutes. At this point, the electrophoresis chamber should also be prepared, starting by washing it with electrophoresis buffer and then by filling it with new electrophoresis buffer.

The following step consists in loading the sample. First, approximately 10 µl of each sample are used to load the correspondent number of wells of the sample plate. In order to transfer the sample to the applicator, its tips have to be depressed into the sample wells 3 or 4 times. This first loading should be applied to a blotter paper and the applicator loaded again, as this will make the second load more uniform.

Then, the cellulose acetate plate has to be drained from excess of electrophoresis buffer and transferred, cellulose acetate side up, to the electrophoresis chamber, where it has to be secured and covered with the lid of the chamber. After loading the sample applicator for the second time, the samples should be applied to the plate, by fitting the applicator on the proper support of the chamber and gently pressing its tips down for about 5 seconds.

The chamber was confirmed to be fully closed by the lid and then the plate was electrophoresed for 25 minutes at 200 volts.

At the end of the electrophorese, and in order to visualize the protein bands, the plate was removed from the chamber and soaked in enough volume of Ponceau S stain to make sure it is covered. After approximately 10 minutes, the plate was removed and destained in 3 successive washes of 5% acetic acid by the end of which its background was white.

It is possible to identify which protocols were efficient for fibrinogen removal, by identifying the protein bands, namely the one that corresponds to fibrinogen, if existent.

II.8.2. Performance when used in culture media

At this point it had already been determine that method #2 was the only one that allowed efficient defibrination of plasma. However, it was still imperative to determine whether it was adequate for cell culture. Hence, two different sera isolated in our laboratory, one from a type A donor and other from a type AB donor, were tested in parallel with FBS (which is the standard serum used in culture media in our laboratory) and with two different commercial human AB sera (sera H4522 from Sigma). Both commercial human sera were also obtained from plasma defibrination, although with a different method requiring the addition of calcium to the plasma in order to inactivate the coagulation cascade. Moreover, one of these commercial human sera, here on refered as H4522_#1, was not heat inactivated, while the other, H4522_#2, was inactivated by incubation in our laboratory using a water

bath at 56 ºC during 30 minutes. Consequently, a total of 5 culture media had to be made, differing from the standard recipe referred in Appendix II on the type of serum used; also no antibiotics were used in order to facilitate the detection of eventual contaminations.

Each serum was tested with PBMCs from 6 different donors, except for H4522_#2, which was only tested with 3 donors.

PBMCs were resuspended in the different culture media at a concentration of 1 x 10^6 cells/ml. Then they were distributed in a 24-well plate (1 ml/well).

Additionally, PBMCs from 3 donors were stimulated with 10 µl lipopolysaccharide (LPS; Sigma). LPS stimulates the expression of MHCII molecule, therefore if it is expressed in high levels, means that cells are responding to stimulus. After 24h, cell stimulation was evaluated by flow cytometry regarding the expression levels of MHCII. The remaining cells (from all 6 donors) were kept in culture for a 7 day period, when they were analyzed by flow cytometry; cells from 3 donors were also counted in the Neubauer chamber, with trypan blue.

Three of the donors were left in culture for a total period of 11 days, when they were once more analyzed by flow cytometry.

During the time the PBMCs were kept in culture, they were periodically checked macro and microscopically for contamination. At the tenth day, representative photos were taken of cells in culture with all culture media except for H4522_#2.

II.9. Cell count using the Neubauer chamber

Cell count was always performed using the Neubauer chamber.

The total number of cells per milliliter (total, alive or dead), is determined as follows:

$$no.cells/ml = total\ cells\ counted\ \times DF\ \times 10^4$$

Where DF, is the dilution factor and 10^4 refers to the volume of the Neubauer chamber. In order to obtain the total number of cells existent in a cell suspension, the result of the previous formula has to be multiplied by the solution final volume (ml).

Dilutions were performed with PBS and the dilution factor was adapted according to each sample, and generally varied from 2 times to 10 times. The dilution factor should be optimized so that the number of cells counted in each large square fall between 50 and 200. Usually, a 2 times dilution factor was used to count thawed cells, monocytes and T cells, while higher dilution factors were required to access the number of PBMCs after isolation.

Upon the necessity of differentiate dead cells from the living ones, trypan blue was used in a ratio of 1:2. Knowing the number of dead cells (cells with blue coloration) it is possible to calculate the viability of the sample using the following formula:

$$Cell\ viability = \frac{number\ of\ living\ cells}{total\ number\ of\ cells}$$

II.10. Isolation yield

Cell yield was used as a parameter to define the efficacy of a cell separation process; by default, the formula used for this calculation was the following one:

$$Isolation\ Yield\ = \frac{number\ of\ cells\ after\ isolation}{number\ of\ cell\ before\ isolation\ (PBMCs)} \times 100$$

Basically, this formula makes it possible to establish a relationship between the number of separated cells and the amount of cells that existed originally (PBMCs). Knowing the average yield of a specific type of isolation, allows predicting the number of wanted cells that will be possible to obtain after isolation. However, it is still important to confirm by flow cyometry that all the separated cells are of the desired type.

II.11. Cell preservation

For the purposes of this thesis, it was important to optimize protocols for the removal of the cryoprotective agent (DMSO; Sigma), in order to preserve cell viability and function. Another important issue was to determine shelf life in a deep freezer (Thermo Electron Corporation), at -80 ºC, of blood primary cells isolated as described in this thesis.

II.11.1. Cell freezing and thawing

In order to prepare cells for the freezing process the first step was to carefully resuspend cells in RPMI-1640 medium supplemented with 20% FBS, at a concentration of 12.5 x 10^6 cells/ml. At this point, cells were ready for the addition of the freezing medium, which is composed of RPMI-1640 medium supplemented with 20% FBS and 20% DMSO, and was added to the cell suspension drop-by-drop. As a result we have a final concentration of 6.25 cells/ml, in RPMI-1640 medium with, 20% FBS and 10% DMSO. The cell suspension was well mixed and distributed by the adequate number of cryovials, in a way that each would contain a volume of 800 µl, corresponding to 5 x 10^6 PBMCs. Next, the cryovials were transferred into a “Mr. Frosty” container (Nalgene) and placed in a freezer at -80 ºC. After a minimum of 24 hours, the cryovials were removed from the cryocontainer and stored for future use in a freezer at -80 ºC.

Posteriorly, cells were thawed by removing the cryovials from the freezer and incubating them in a water bath at 37 ºC. At this point, an aliquot was taken in order to compare to the original number of PBMCs before freezing.

Cells from 4 different donors were thawed after periods of 24h, 48h, 1 month, 2 months, 3 months and 6 months, in order to study the effect of time on frozen cells. To remove DMSO, cells were resuspended in PBS until 10 ml and centrifuged at 110 x g (800 rpm) for 10 minutes. After the

supernatant had been removed and the pellet resuspended in 800 µl (the original volume), a new aliquot was taken to once more determine the number of PBMCs in the sample. Cells were counted in the Neubauer chamber with Trypan blue. Other protocols for DMSO removal were tested, being described in section II.11.2.

II.11.2. DMSO Removal

Two main aspects were tested regarding DMSO removal: the effect of the centrifugation speed and whether it should be done immediately or after 24 hours. In both assays were used PBMCs from 4 different donors that have been frozen for 3-4 months.

Regarding the speeds of sample centrifugation upon the step of DMSO removal we have proceeded as will be described next. After thawing the cryovial and removing an aliquot for cell count, the remaining volume was equally divided into two 15 ml tubes and in both cases diluted in PBS up until 5 ml. Then, one half of the sample was centrifuged at 110 x g (800 rpm) for 10 minutes, while the other half was centrifuged at 250 x g (1200 rpm), for 5 min. After DMSO had been removed, each pellet was resuspended in 400 µl in order to restore the initial volume, and an aliquot was taken in order to determine the number of PBMCs.

In order to determine which was the most effective moment for DMSO removal, whether immediately or only after 24 hours, a parallel assay was performed, comparing both methods. As soon as the sample was thawed, an aliquot was taken for cell count; the remaining sample was equally divided into two 15 ml tubes (400 µl, each).

In one case, PBMCs were washed in order to remove DMSO: the sample was resuspended with RPMI-1640 at 37 ºC, up to a final volume of 5 ml, and centrifuged at 110 x g (800 rpm) for 8 minutes. After that, the supernatant was discarded and cells were resuspended in 4 ml of culture media; then 3 ml were distributed in a 24-well plate (1 ml/well). In each of the following 3 days, the content of one of the wells was removed to be used for cell count.

Regarding the other half of the sample it was diluted 10 times by adding 4 ml of culture media at 37 ºC, then 3 ml were transferred to a well of a 6-well plate. The following day, the well content was removed and centrifuged at 110 x g (800 rpm) for 8 minutes; once the supernatant was removed, the pellet was resuspended in 3 ml of culture media. From that volume, 2 ml were distributed by 2 wells of a 24-well plate (1 ml/well), from the remaining volume was taken an aliquot for cell count.

In both cases (immediate removal and after 24 hours), the cells were kept in culture for a total of 3 days, not including the thawing day.

II.12. Flow cytometry

II.12.1. The flow cytometer: basic principals

A flow cytometer is composed essentially by three systems: fluidics, optics and electronics.

The fluidics system draws up the cell suspension and organizes the cells so that they will flow one by one, at the time they are presented to a light source provided by laser beams. This process is usually performed by hydrodynamic focusing as it is controlled by a stream of liquid, called the sheath fluid that has different velocity and pressure than the cell sample. The higher velocity of the sheath fluid keeps cells focused on its center, and the regulation of the sample pressure controls the flow rate.

The optical system is composed by a set of lasers and lenses responsible for excitation, and by a set of mirrors and filters that make up the collection optics. As each cell is hit by the laser beam, light bounces off in a particular way, making it possible to obtain information about the physical characteristics of that cell. The collected information may refer to the way the single cell scatters light, but also to its fluorescence, if any was present. Information on how light is scattered is provided by measuring the scattered light in two different angles: in the same direction of the laser trajectory and with an inclination of 90º. In the first case, the scattered light measures cell size and is called forward scatter (FSC), as in the second case the information obtained refers to the granularity of the cell and is called side scatter (SSC). If cells have been previously attached to a fluorochrome, by the time they pass through the laser beam, the fluorochrome is excited and will fluoresce, emitting a specific color. Then, the emitted light is filtered and directed to adequate photodetectors. One of the parameters that can be analyzed regarding fluorescence is the amount of fluorescently labeled antibody that binds to the cells, which is referred to as Mean fluorescence intensity (MFI).

Finally, the electronic system is responsible for converting all the collected analogue data to a digital form, which can be stored and analyzed in a computer in order to obtain information about the cell populations present in the sample (Applied Cytometry: http://www.appliedcytometry.com/flow_cytometry.php#work).

All flow cytometry data presented in this thesis was obtained using Attune® Acoustic Focusing Cytometer and analyzed with Attune® Cytometric Software v1.2.5. This flow cytometer has the particularity of using an acoustic pressure focusing system instead of the more common sheath-based hydrodynamic focusing system. On the contrary to what happens with usual flow cytometers, with Attune® Acoustic Focusing Cytometer the alignment of the cells does not depend of the total fluid flow, but on an acoustic resonant device. Although the diameter of the sample core may be altered by the total fluid flow or by adjustments on the amount of sample that is injected, the resolution of the cytometer remains unaltered as cells are being constantly focused in the more adequate position for laser analysis. This has the advantage that resolution is maintained even if the sample is much diluted (Life Technologies Corporation, 2012).

II.12.2. Data analysis

By default, all cell acquisitions were performed using a threshold of at least 500000 on FSC; eventually, when a high number of events that were clearly debris was identified, the threshold might have been increased until 1 x 10^6. However, when cells had been stained with 7AAD (7-amino-actinomycin D), their acquisition required the use of no threshold. Though, it was determined that there were no $7AAD^+$ events under a 5 x 10^5 threshold, only debris.

Acquiring the cells at an appropriate concentration helps decreasing the number of events that are doublets, however, even in these optimized conditions some might still occur.

In what this thesis concerns, and by suggestion of the vendor, doublets were identified in a stained sample, using a fluorescence-Heigh *vs.* fluorescence-Area dot plot. On this kind of dot plots, the majority of the events will appear aligned in a diagonal; consequently, events that fall outside this diagonal represent doublets.

In regular analysis, when representing a percentage inferior to 5%, the doublets were not discriminated in the analysis. When calculating purity, both singlets and doublets were taken into account; in phenotypic analysis, such as activation (CD69 staining) and maturation (MHCII staining) studies, only the singlet population was taken into account. The antibodies used for cell staining were the following: anti-CD3-APC (aloficocianine*),* anti-CD14-FITC (fluorescein isothiocyanate), anti-CD69-PE (phycoerythrin), anti-MHCII-APC, anti-MHCII-PE, all from Biolegend, except for the last one that was acquired from Bencton Dickinson.

II.13. Isolation purity

To evaluate the level of purity of the samples, the isolated cells were fluorescently stained with appropriate antibodies and analyzed by flow cytometry to identify their percentage. Both singlets and doublets were taken into account for this percentage; it is also important to consider if eventual "contamination" is caused only by debris or if other cell types, besides the target ones, are present. After each isolation procedure, cell isolation purity information was written in a quality control report.

II.14. Cell recovery rate

Cell recovery rate can be applied in 2 slightly different contexts. In one hand, it may refer to cell isolation; in this case, a sample of PBMCs is stained with a specific antibody for the target cells and after isolation, the resulting sample is also stained with the same antibody. Then, both samples are analyzed by flow cytometry in order to determine the percentage of target cells in each case. The recovery rate is calculated as follows:

$$Cell\ recovery = \frac{percentage\ of\ positively\ stained\ cells\ after\ isolation}{percentage\ of\ positively\ stained\ cells\ before\ isolation\ (among\ PBMCs)} \times 100$$

On the other case it may refer to the characteristics after cell thawing and DMSO removal. In this case, cell recovery establishes a relation between the amounts of cells viable for use (living cells) and the initial number of viable cells, and it was calculated as follows

$$Cell\ recovery = \frac{number\ of\ living\ cells\ after\ thawing\ and\ DMSO\ removal}{number\ of\ viable\ cells\ before\ freezing} \times 100$$

II.15. Economic viability evaluation of different types of cell isolations

One of the main objectives of this work was the optimization of protocols for cell isolation, in order to have the highest yields and highest quality possible with each procedure. Besides that, when choosing an isolation protocol we also have to consider the time and money that is spent on the procedures. For that, we present here what we call an economic evaluation of some procedures. The calculations were based on prices practiced by the vendors, obtained either online or provided by sales representatives of the respective products. In this comparison, we only took into account the prices of the kits and related components. Other reagents, such as media and tubes, despite being slightly different in each case, were considered equivalent in economic terms.

The costs of positive cell isolation were evaluated for kits from different companies. For that, $CD14^+$ monocytes isolation using MACS separation (Miltenyi) and EasySep Positive Selection (StemCell) were also compared regarding the costs of their utilization. We also compared different isolation methods: $CD3^+$ T cell immunomagnetic isolation with MACS separation kits (Miltenyi) and a Nylon Wool Fiber Column (Polysciences).

II.16. Statistical analysis

Experimental data were analyzed using GraphPad Prism 5 (GraphPad Software, Inc). Statistical differences were analyzed using Student's t-test, considering a p-value<0.05 as statistically significant.

II.17. Divulgation of the Cell Biology services

The most part of the divulgation work concerning the Cell Biology services was done on-line, through our facebook page (Glycoimmunology at Cedoc) and official website:

http://www.fcm.unl.pt/projectos/glicociencias/index.php?option=com_content&view=article&id=43&Itemid=52

Updating the official website required the use of Joomla! software as well as htlm programming skills.

Also, the divulgation of the poster “Cell Biology Services @ CEDOC/FCM” (see Appendix III) together with flyer distribution, both at the XXXVIII Portuguese Genetics Conference (XXXVIII Jornadas Portuguesas de Genética) in Oporto, have also contributed to divulge the Platform.

III. RESULTS

III.1. Optimization of a protocol to obtain human serum from buffy coat plasma

Human serum can be obtained in different manners. The most commonly used one is the collection of human whole blood to a dry tube and allowing the cells to clot and precipitate. Afterwards, a centrifugation step is used to allow proper separation of the solid (clotted cells and coagulation proteins) and the liquid phase (serum). However, our source to obtain human serum is the buffy coat, which is supplied diluted in a CPD anti-coagulant solution; therefore, we had set up a protocol to isolate serum from plasma. Essentially, the difference between plasma and serum is the inexistence of fibrinogen in the second one. Consequently, the strategy was to separate the plasma and then precipitate and remove the fibrinogen. The resulting blood component can be designated as defibrinated plasma or serum, but for simplification purposes, here on it will be referred by the second term.

Aiming to take the maximum advantage of each buffy coat, we intended to isolate cells and serum from the same buffy coat. Therefore, plasma separation from cells was the first step and consisted in centrifuging the buffy coat at 1100 x g for 10 minutes, removing its upper clear layer, centrifuging it at 2000 x g for 15 minutes and discarding the pellet (platelets); both centrifugations were performed without brake. The second step consisted in finding a protocol for efficient fibrinogen removal. For that purpose, two different methods were tested. With “method #1” the objective was simply to defibrinate the sample; while with “method #2” it was also intended to simultaneously heat inactivate the complement system present in the sample. Therefore, it was preceded as follows: equal volumes of a buffy coat were distributed into two tubes and centrifuged at 1100 x g for 10 minutes, without brake. The upper layer (plasma) was removed to new independent tubes. Then, one tube was incubated for 3 minutes in a water bath at 58 ºC (method #1), and the other tube was incubated for 1 hour in a water bath at 56 ºC (method #2). Finally, the tubes were centrifuged at 2000 x g, during 15 minutes, also without brake; the supernatant corresponds to plasma, defibrinated or not depending on the eventual success of the process; the pellet was discarded.

All samples were analyzed in the optic microscope in order to detect eventual remaining cells; none were identified in any of the cases. In order to confirm the efficiency of the process, the fibrinogen removal was assessed by protein electrophoresis.

III.1.1. Electrophoretic profile of serum samples

Protein electrophoresis allows the separation of proteins according to their size and electrical charge. When this method is applied to a serum or plasma sample, their protein components will be divided in 5 fractions: albumin, alpha-1 globulins, alpha-2 globulins, beta globulins and gamma globulins (see Fig.III.1 – A). On the contrary of what happens with serum, fibrinogen will be present in plasma samples, and will be seen in the gamma region.

Samples isolated with “method #1” and “method #2” were compared to a plasma sample isolated from the same donor (C_{plasma}) and to a serum sample from blood collected to a dry tube (clotted blood) from an independent donor (C_{serum}). The electrophoretic profiles were revealed by Ponceau S staining, which was removed afterwards with acetic acid and the results are represented in Fig.III.1 – B.

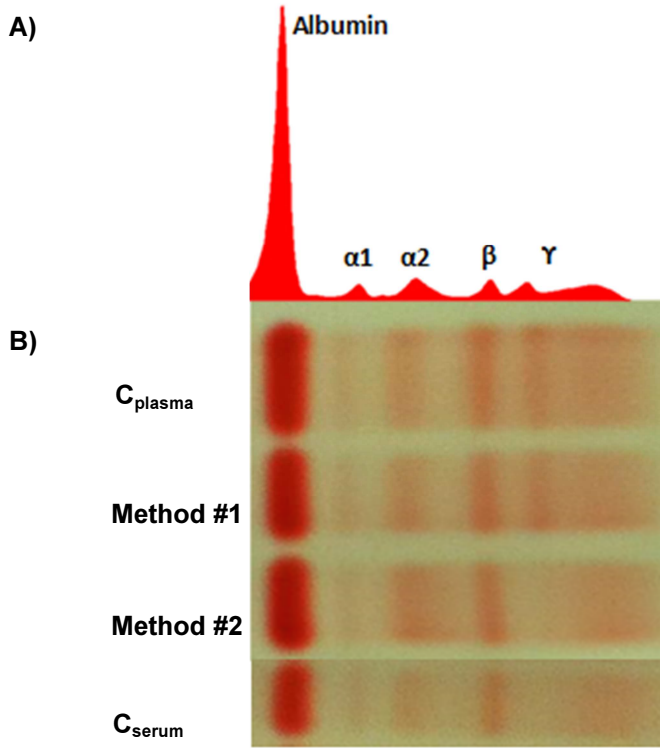

Fig.III.1 – Electrophoretic profile of plasma and serum samples. A – Profile of a normal serum protein electrophoresis, with identification of the composing zones. B – Electrophorectic profile of samples isolated in our laboratory using method #1 and #2; a plasma sample isolated from the same donor (C_{plasma}) and a serum sample from blood collected to a dry tube from an independent donor (C_{serum}) were used as controls.

In the electrophoretic profile of C_{plasma} it is possible to identify 5 electrophoretic bands, correspondent to the albumin and the 4 globulin fractions. The last band corresponds to fibrinogen, and proves that this is in fact a plasma sample.

The profile of the “method #1” sample also presents the 5th band, correspondent to fibrinogen. This result proves that incubating the plasma sample in a water bath at 58 ºC for 3 minutes it is not a viable protocol for fibrinogen precipitation and subsequent removal.

Finally, in the profile of the “method #2” sample, there are only 4 electrophoretic bands visible, as happens with C_{serum}. The absence of the fibrinogen band means that incubating the plasma sample in a water bath at 56 ºC for 1 hour allowed the efficient removal of fibrinogen, making this the only protocol viable to achieve the desired results.

III.1.2. Influence of using sera isolated in our laboratory in culture media

At this point we had chosen “method #2” for simultaneous fibrinogen removal and heat inactivation. So, this method was applied to subsequent sera isolations from buffy coats; after isolation, plasma samples were stored at -20 ºC. Our main objective was to evaluate if cells were suitable for functional studies after being kept in culture during long periods of time, in culture media supplemented with this type of serum. However, at some point we also studied the relevance of heat inactivating serum, by comparing two batches from the same sera, one heat inactivated and other that had not been heat inactivated. We have used PBMCs isolated in the same day, from different donors which were kept in culture for a maximum of 11 days. PBMCs from each donor were tested using culture media that had exactly the same composition except for the type of serum: FBS, not heat inactivated human serum (H4522_#1), heat inactivated human serum (H4522_#2), and 2 sera isolated in our laboratory. Sera isolated in our laboratory was originated from 2 donors with different blood types: AB (the most commonly used sera) and A.

By comparing macroscopically all culture media, even before they were in contact with the PBMCs, different colorations could be distinguished, as shown in Fig.III.2.

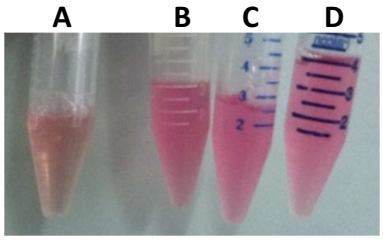

Fig.III.2 – Culture media supplemented with different types of sera. A – FBS; B –H4522_#1 (not heat inactivated); C – human serum, type A, isolated in our laboratory; D – human serum, type AB, isolated in our laboratory. The remaining supplements of the culture media were identical in all cases.

The coloration of the media varies accordingly to the pH levels. Usually, commercial culture media, such as the RPMI-1640 from Sigma that we use in the laboratory, already include phenol red as a pH indicator in order to facilitate a constant control of the pH status of the media. At pH 7.4, phenol red presents a red coloration; pH levels higher than 7.4 make the media pink and above 7.6 it turns purple; at pH 7.0 media turns orange, at pH 6.5 it turns yellow and below that, lemon yellow.

In this case, culture media supplemented with FBS (Fig.III.2: A) presents a more orange coloration, characteristic of pH 7.0; culture media supplemented with human sera (Fig.III.2: B-D) clearly have a pink coloration, meaning pH 7.4. Therefore, it is possible to state that FBS turned the culture media more acid, while human serum turned it more alkaline.

Throughout the time they were kept in culture, cells were periodically checked macro and microscopically for contamination, which if existing would be easily detected as no antibiotics were added to the culture media. No contamination was detected in any case. However, PBMCs in culture media supplemented with H4522_#1 (the only one that had not been heat inactivated), at some point caused the formation of white fibers, with variable sizes. These structures, although difficult to spot microscopically, apparently corresponded to cell aggregation in a string-like way.

III.1.2.1. Influence of different sera in the viability of the PBMCs and in the proportion of different cell types

After being kept in culture for 7 days, part of the PBMCs were removed, and immediately stained with 7AAD and analyzed by flow cytometry in order to determine the impact of different sera on cell populations (Fig.III.3 and Fig.III.4).

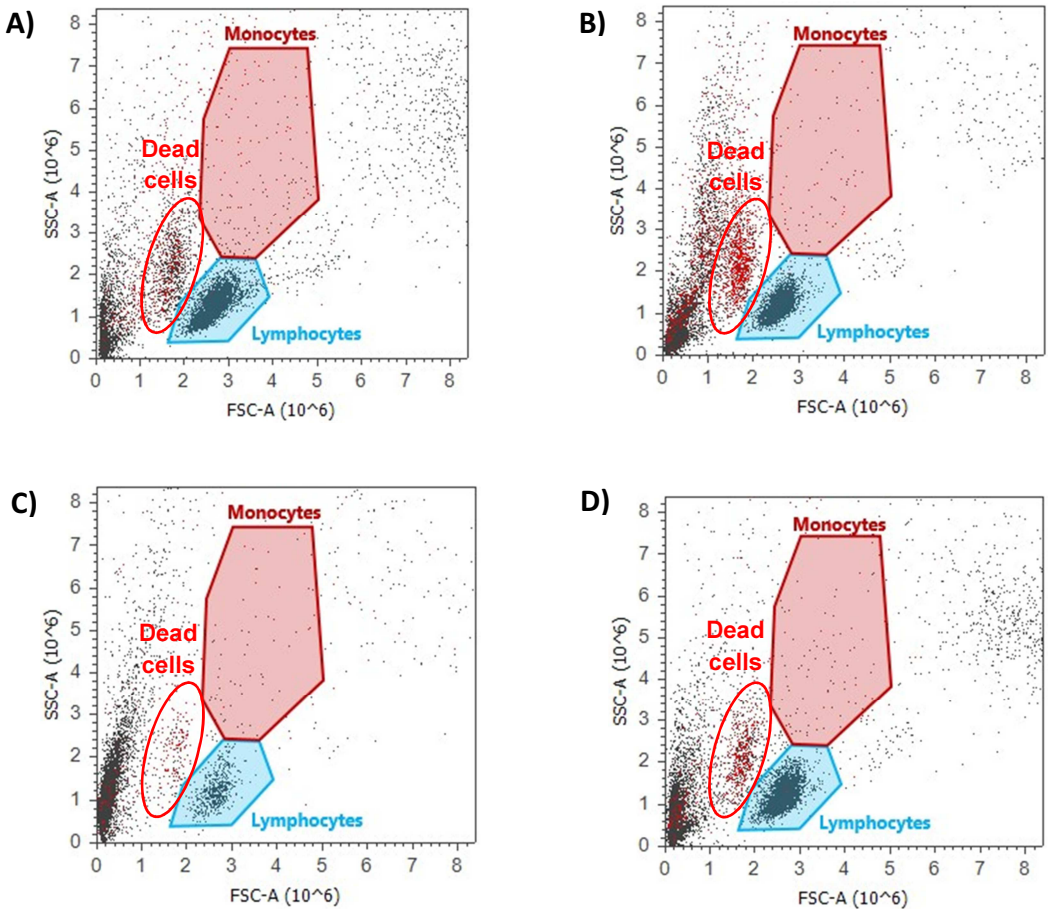

Fig.III.3 – Flow cytometry analysis of PBMCs kept in culture for a 7-day period. Each donor was kept in culture using media supplemented with 5 different sera, though only 4 are here represented: A – FBS; B – sera isolated in our laboratory from a type AB donor; C – H4522_#1 (non-heat inactivated); D – H4522_#2. Lymphocytes and monocytes were identified by their FCS *vs*. SSC. profile and are gated in blue and red, respectively. Dead cells were identified by 7AAD staining and are circled in red. The events appearing in the right of the dot plots may refer to monocytes already differentiated into macrophages. (These images are representative for one of the donors; n=6, except for H4522_#2 that has n=3.)

By observing Fig.III.3 it is obvious that the most preserved cells were the lymphocytes, on the other hand monocytes were almost inexistent. Tough, in some cases a new population seems to be visible in the right side of the dot plot, which might be a macrophage-like population. Also by analyzing these dot plots, it became obvious that 7AAD staining was not enough to evaluate cell death in the

different culture media. Thus, it was also necessary to consider the percentage of debris as well (see Fig.III.4).

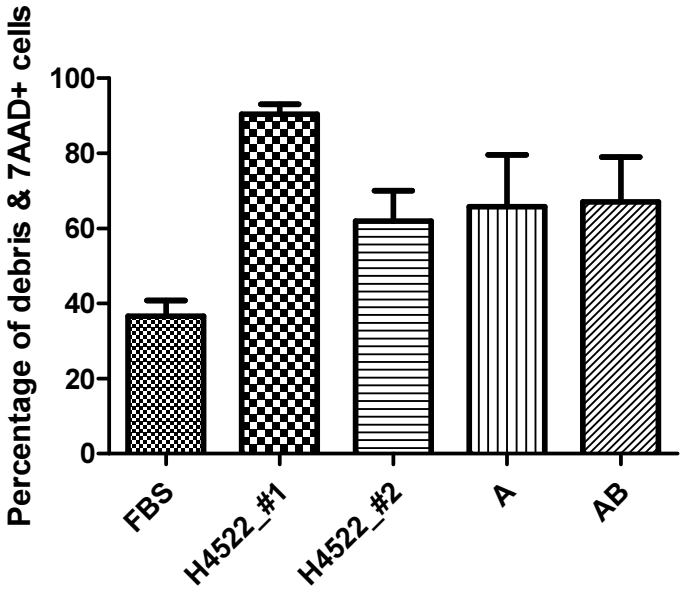

Fig.III.4 – Percentage of debris and dead cells. Each donor was kept in culture using media supplemented with 5 different sera: FBS, H4522_#1 (non-heat inactivated), H4522_#2 and sera isolated in our laboratory, one from type A and other from type AB. Dead cells were identified by 7AAD staining; debris correspond to the events in the left side of the dot plot (low FSC); all percentages resulted from flow cytometry analysis (n=6 except for H4522_#2 which had n=3.)

By far, FBS was the serum that originated the lowest percentage of cell death and debris. The results obtained for both sera isolated in our laboratory showed similar percentages (around 60%); this value is close to the one obtained with the heat inactivated commercial human sera (H4522_#2). Finally, H4522_#1, which was the only non-heat inactivated serum, had the worst results, presenting a percentage of dead cells and debris of approximately 90%.

Three of the donors were left in culture, until the 11th day (data not shown), and then analyzed; this was not performed using H4522_#2 sera. After this period, in all cases, lymphocytes still represented the highest percentage of living cells, followed by the undefined events and finally monocytes.

To sum up, after a 7-day period it was still possible to recover viable cells, and by this time, the relative percentage of the different cell populations seemed to be affected by not heat inactivating the serum. Moreover, among sera isolated in our laboratory, type AB originated a highest percentage of recovered cells.

At the 10th day, photographs were taken from cells that were in culture media supplemented with all sera but H4522_#2; representative images can be seen in Fig.III.5.

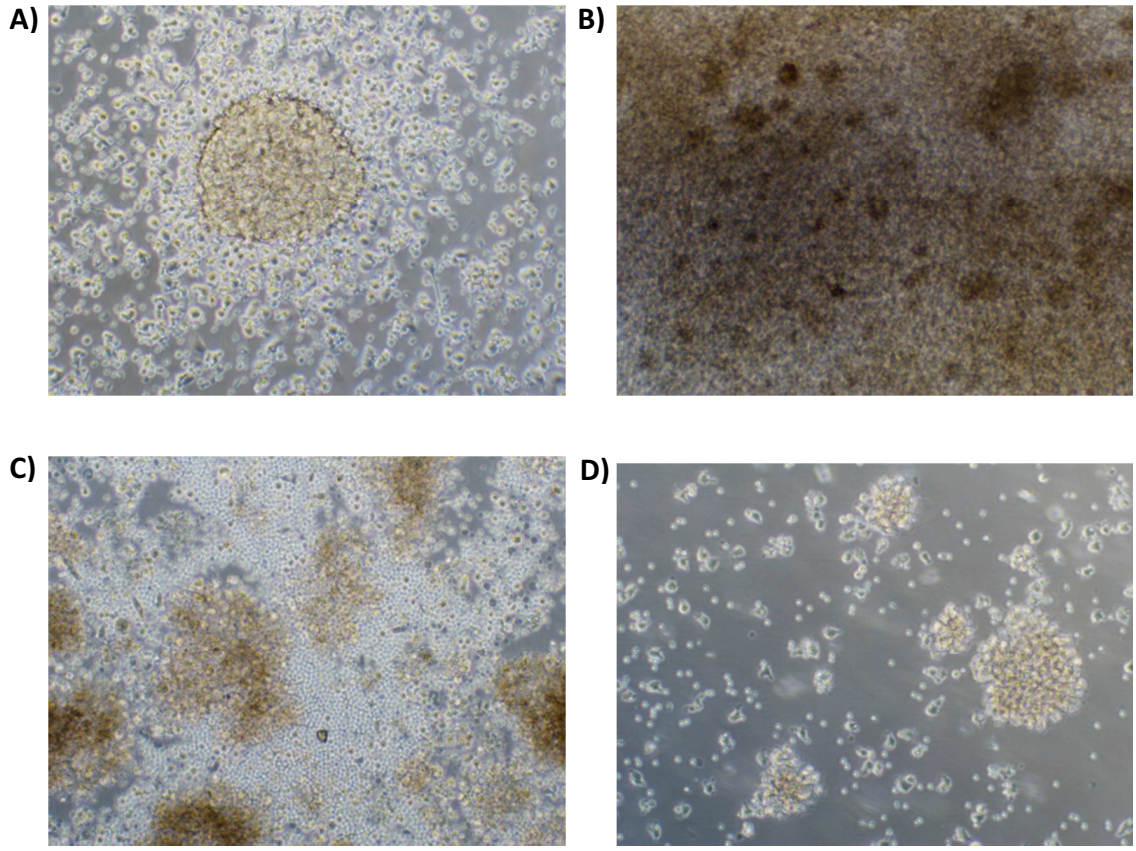

Fig.III.5 – PBMCs in culture using media supplemented with different types of sera. A – FBS; B – H4522_#1; C – human serum, type A; D – human serum, type AB. These photos are representative of one donor for each specific type of sera.

PBMCs in culture media supplemented with sera isolated in our laboratory (Fig.III.5: C and D), both present morphology similar to the one observed in PBMCs kept in culture with FBS (standard serum used in our laboratory). In these cases cell present a round shape, being more or less aggregated. When PBMCs were kept in culture using commercial human serum that had not been heat inactivated (H4522_#1), the results were less satisfactory, as cells had a more irregular shape; moreover a higher quantity of debris was visible. Taking into account these results, heat inactivating serum seems to be of great importance when serum is to be used in cell culture.

III.1.2.2. Response of PBMCs to a LPS stimulus

Major Histocompatibility Complex Class II (MHCII) molecule is expressed upon LPS stimulation and therefore a higher fluorescent staining is a quantitative measure of cell response to the stimulus.

Therefore, as to determine if the type of serum used had any influence on cell stimulation, by the time the PBMCs were first put in culture, some were stimulated with LPS, while others were left non-stimulated. After being incubated for 24 h, stimulated and non-stimulated cells were stained with

HLA-DR (human leukocyte antigen; a marker for cell stimulation) and analyzed by flow cytometry. Then the MFI of the non-stimulated cells were compared to the correspondent MFI of the stimulated cells, as represented in Fig.III.6; this comparison was performed for both lymphocyte (A) and monocyte (B) populations.

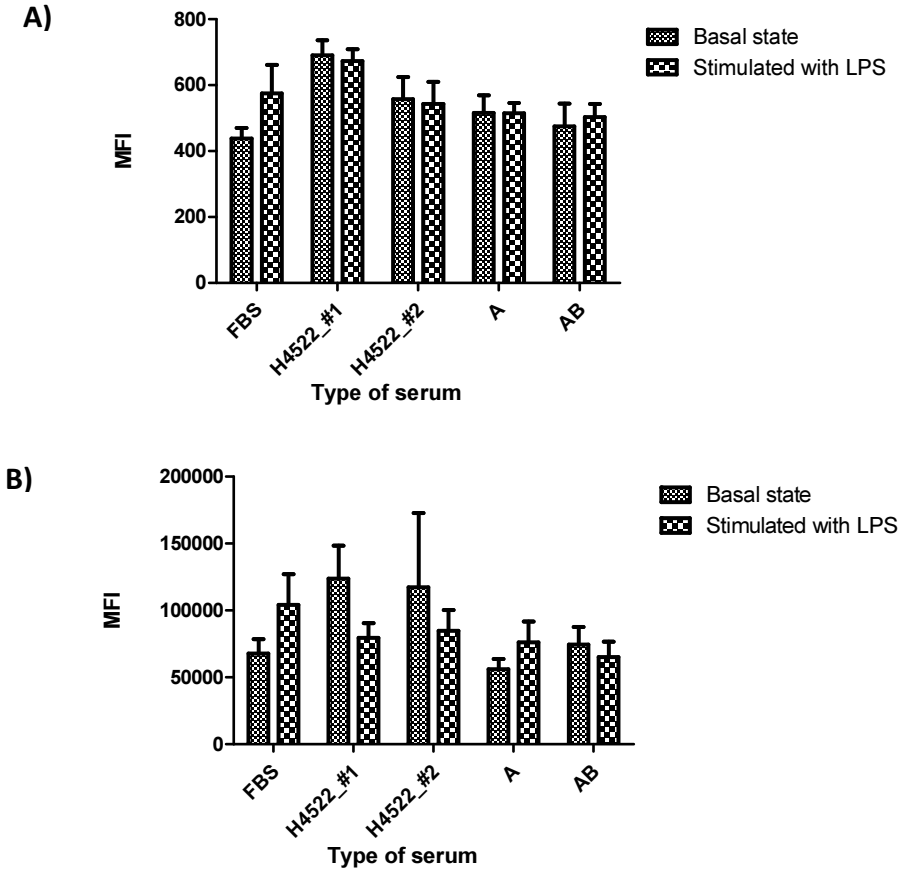

Fig.III.6 – Analysis of the MFI values obtained with HLA-DR staining in samples of PBMCs kept in culture using media supplemented with different types of sera. For each case, the bar on the left corresponds to the MFI value of the non-stimulated sample (basal state), and the bar to its immediate right correspond to the MFI value of PBMCs stimulated with LPS for 24 h. A – lymphocyte population; B – monocyte population. (n=3)

It was expected that when PBMCs were stimulated, their MFI values for HLA-DR staining would be superior to the ones obtained in the basal state. However, Fig.III.6: A (lymphocytes), shows that it only happened when FBS and serum type AB isolated in our laboratory were used. The increases in their MFI values were of 136.7 ± 58.81 and 28.00 ± 79.53, respectively. In the remaining samples the stimulated cells had lower MFI values than the basal ones, nevertheless, the difference was never superior to 16.67 (H4522_#2).

Regarding the monocyte population (Fig.III.6: B), it was only possible to verify an increase of MFI in stimulated populations when PBMCs were kept in culture media supplemented with FBS and serum, type A (an increase of 36591 ± 14414 and 20071 ± 8673, respectively). In all the remaining

cases, the MFI values of the stimulated cells were inferior to the ones of the basal state, with a difference of at least 9079 (sera isolated in our laboratory, type AB).

Overall, it seems that sera isolated in our laboratory is suitable to be used in cell culture, as cells kept in culture with any of them originated stimulation levels between the range of the ones obtained with the commercial sera (FBS and H4522). Sera H4522_#2 originated the highest MHCII expression, both with and without LPS stimulation, what is probably related to the fact that it was not heat inactivated.

III.2. Isolation Yield

In our laboratory, blood cell isolation from buffy coats is a routine procedure, sometimes with a frequency of two or three times a week and 2 to 3 buffy coats per day. For the most part, these protocols concern the isolation of PBMCs, and eventually, a subsequent isolation of lymphocytes (T or B cells) or monocytes. Due to the high frequency of this type of proceedings it was useful to determine what was the expected average number of PBMCs per isolation. This way, work planning would be facilitated in such things as defining the number of necessary donors.

Taking that into account, we determined that, in average, it is possible to obtain 303.9 x 10^6 ± 15.13 x 10^6 PBMCs from one buffy coat. Each buffy coat corresponds to a different donor and this average derives from data of 89 donors; which were processed in our laboratory between 8th May 2012 and 18th June 2013.

It was also important to determine whether, the number of PBMCs that were obtained with each isolation somehow varied or was dependent on the age or blood type of the donor; the results are represented in Fig.III.7 A and B, respectively. Data concerning the different age groups and blood type resulted from isolations of PBMCs performed to buffy coats of 82 and 81 donors, respectively. The number of donors was not equally divided through the different groups in each parameter.

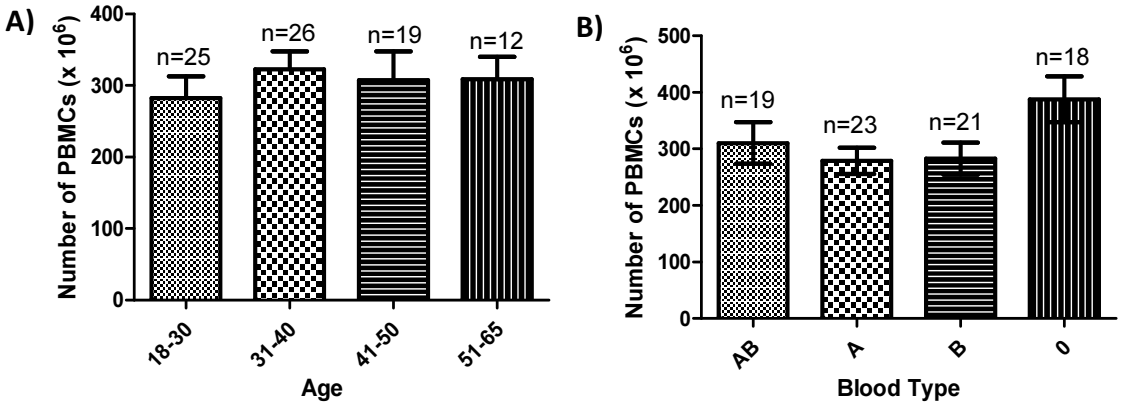

Fig.III.7 – Variation of the average number of PBMCs obtained per isolation, according to: A – the age group and B – the blood type. The number of donors was not equally divided through the different groups in each parameter. Age group: 18-30 (n=25); 31-40 (n=26); 41-50 (n=19); 51-65 (n=12). Blood Type: type AB (n=19); type A (n=23); type B (n=21); type 0 (n=18).

First of all, in a more general overlook, it is possible to say that donors from 31-40 are more common than the remaining ones, although donors from 18-30 are almost as many. Donors with ages between 51 and 65 are the less frequent. Regarding the blood type, type A is the most numerous and type 0 the least, although very close to type AB.

The age group of 18-30, seems to be the one from which the lowest number of PMBCs was isolated ($282.1 \times 10^6 \pm 30.82 \times 10^6$) while the highest was obtained in the age group of 31-40 ($322.4 \pm 25.38 \times 10^6$). The remaining age groups 41-50 and 51-65 have very similar results, situated between the previously referred ones ($307.6 \times 10^6 \pm 39.87 \times 10^6$ and $308.9 \times 10^6 \pm 31.21 \times 10^6$, respectively). Despite the fact that there are differences among groups, they are not significant.

Concerning blood type, type 0 donors clearly have the highest average number of isolated PBMCs ($387.6 \times 10^6 \pm 40.45 \times 10^6$), while type A has the lowest ($279.0 \times 10^6 \pm 23.42 \times 10^6$). Type B donors ($282.8 \times 10^6 \pm 28.29 \times 10^6$) have values slightly higher than the type A ones. And finally, type AB donors have the second highest value ($310.3 \times 10^6 \pm 36.93 \times 10^6$). These differences are not significant.

Once PBMCs have been isolated, they still may be used to select T cells, or monocytes, for example. In such cases it may be useful to have an estimation of the yield associated with each type of isolation. In other words, for instance, knowing the average relation between the number of T cells obtained after separation and the initial number of PBMCs, allows estimating approximately how many T cells it will be obtained from a given number of PBMCs. The results concerning cell yield for T cell separation are represented in Fig.III.8 and reflect data from 26 T cell separation processes, performed using 3 different protocols (described in sections II.4.1 and II.4.2).

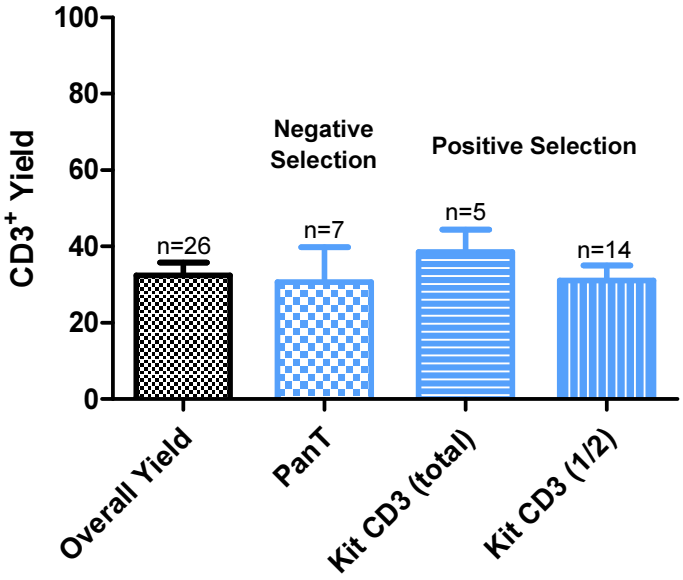

Fig.III.8 – Isolation yield for $CD3^+$ selection using protocols for positive and negative selection. Overall yield (n=26); Pant T (n=7); kit CD3 – total (n=5); kit CD3 – ½ (n=14).

The lowest cell yield was of $30.68 \pm 9.114\%$, and was obtained when T cells were separated using a negative selection kit. However, the yield obtained when only half of the recommended volume

of beads for the positive selection kit was used, was only slightly higher (31.13 ± 3.901%). Using the amount of beads recommended by the vendor for positive selection, originated the highest cell yield, corresponding to 38.54 ± 5.899%. In a more general approach, and without discriminating neither the type of isolation nor the amount of beads used, T cells can be selected from PBMCs with a yield of 32.44 ± 3.325%.

The results related to cell yield for $CD14^+$ monocytes separation are represented in Fig.III.9 and reflect data from 21 isolations, performed using 3 different protocols.

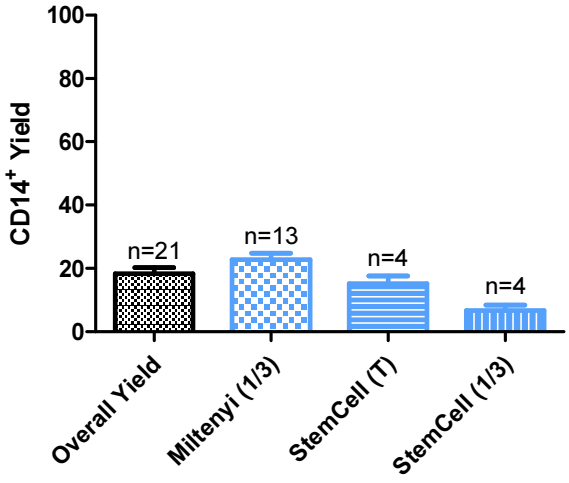

Fig.III.9 – Isolation yield for $CD14^+$ monocytes selection using protocols from different brands (positive selection). Overall yield (n=21); Miltenyi – 1/3 (n=13); StemCell – total (n=4); StemCell – 1/3 (n=4).

In this case, the lowest cell yield was of 6.728 ± 1.644%, and was obtained when $CD14^+$ monocytes were separated using 1/3 of the recommended volume of the StemCell kit. The yield obtained when the recommended volume of StemCell beads was used was more than the double (15.17 ± 2.447%). Separating monocytes with the kit from Miltenyi provided the highest yield (22.76 ± 2.040%), even though only 1/3 of the recommended volume of beads had been used. When taking into account all types of protocols that were used, the overall yield of the process is 18.20 ± 1.943%.

III.3. Influence of using a lyse solution when isolating PBMCs

Upon isolation of PBMCs, after the separation step using Ficoll, cell pellet occasionally presents a reddish coloration corresponding to remaining erythrocytes. To avoid this, a lyse solution was used, to selectively lyse the remaining erythrocytes and therefore clear the cell pellet.

The use of this solution does in fact remove the reddish coloration from the pellet; however it is still necessary to determine if its utilization is prejudicial to the remaining cells. Therefore, PBMCs from 3 different donors were equally divided after the step using Ficoll, and with one of the halves the isolation proceeded as usual, while to the other half the lyse solution was applied. Then, after isolation

was complete, cells were stained with trypan blue and counted in the Neubauer chamber to discriminate between live and dead cells. Cells were also analyzed by flow cytometry to identify and compare lymphocyte and monocyte populations.

First of all, it was determined that using a lyse solution when isolating PBMCs, resulted in an isolation yield 39.23% inferior than when that same solution was not applied. Despite this fact, the percentage of living cells was similar in both cases: 82.16 ± 4.170% with lyse solution and 83.84 ± 1.315% without lyse solution.

When PBMCs were also analyzed by flow cytometry, their SSC-A vs FSC-A profiles allowed the identification of monocytes and lymphocytes as can be observed in Fig.III.10.

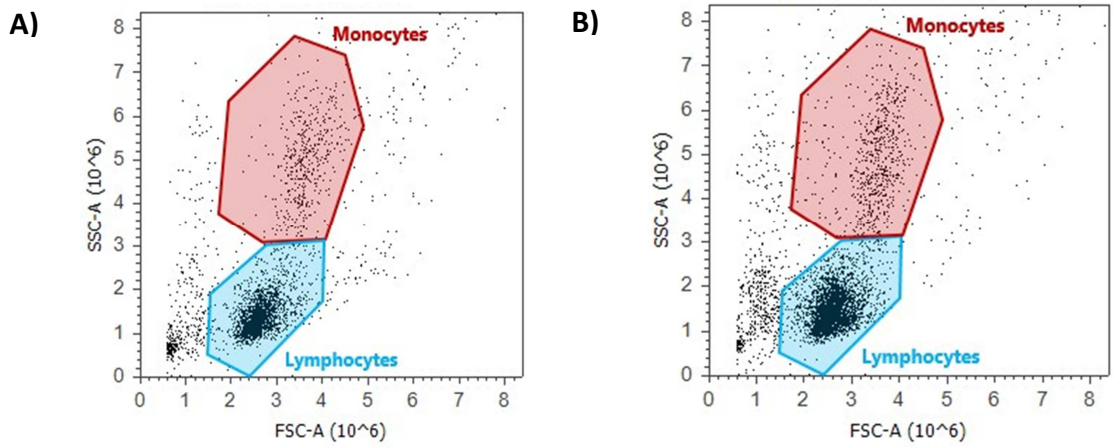

Fig.III.10 – Comparative analysis by flow cytometry of PBMCs isolated using lyse solution (A) and without using lyse solution (B). In both samples, the monocyte and lymphocyte populations were identified based on forward and side scatter profiles. Images are representative for the 3 donors.

Then, the percentage of lymphocytes and monocytes was related to the percentage of actual living cells, originating the results shown in Fig.III.11.

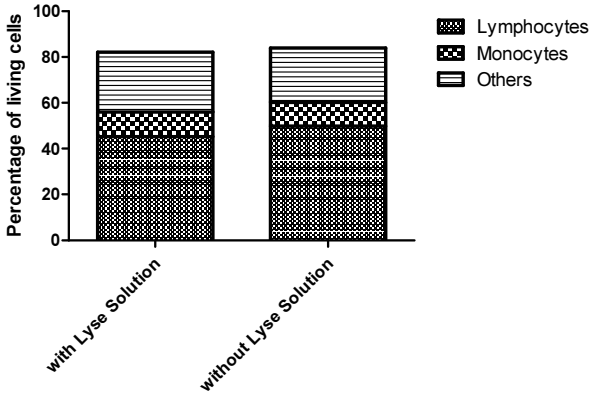

Fig.III.11 – Percentage of lymphocytes and monocytes upon isolation of PBMCs, depending on the utilization, or not, of a lyse solution. The percentage of living cells was determined by manual cell count using trypan blue; the lymphocyte and monocyte percentages were determined by flow cytometry (n=2).

As it has already been stated, the percentage of living cells obtained with each method was very similar. Moreover, in both cases lymphocytes were the predominant cell population, followed by unidentified events and finally by monocytes. Unidentified events, once more refer to events which SSC and FSC profiles did not match the lymphocytes or monocytes one; frequently it corresponded to debris.

To sum up, the utilization of lyse solution decreases the isolation yield, but does not seem to affect the relative proportions of the lymphocyte and monocyte populations.

III.4. MACS $CD3^+$ T cell separation: positive selection *versus* negative selection

In order to compare T cell isolation using positive and negative selection kits, PBMCs from 3 different donors were equally divided and each half isolated with one of the kits. The isolation yield was calculated for both situations and is indicated in section II.10.

Cells were also analyzed by flow cytometry: CD3 Ab was used to identify the $CD3^+$ population. The activation of T cells induces the expression of the CD69 molecule; therefore, cells were stained with CD69 Ab in order to compare the activation levels for both methods. The results are summarized up in Table III.1.

Table III.1 – Comparison between positive and negative selection

	Positive Selection (n=3)	**Negative Selection (n=3)**
Isolation Yield (%)	32.35 ± 7.943	23.29 ± 5.924
Purity (% of $CD3^+$ cells)	73.78 ± 9.077	92.81 ± 1.149
CD69 expression (MFI)	652.3 ± 63.01	522.7 ± 76.25

Regarding the isolation yield, the results show that positive selection is more successful than negative selection in almost 10%. Moreover, T cells obtained by positive selection expressed higher levels of the CD69 molecule, indicating a more activated state. Regarding the purity of the samples, the best result was obtained with negative selection, portraying a percentage 19.02% superior to the one resultant from positive selection.

Purity refers to the percentage of T cells (singlets and doublets) among all the events registered by the flow cytometer. However, not all cells that stained $CD3^+$ were registered as singlets. In Fig.III.12 it is possible to see dot plots representing the $CD3^+$ staining of the sample for positive (A) and negative (B) separation; singlets are gated in green and doublets are identified in red. The identification of the doublets was based on the profile of the sample in a CD3-APC-H *vs.* CD3-APC-A dot plot. Therefore, among cells stained $CD3^+$, singlets and doublets were separately considered. Their relation to the total amount of $CD3^+$ cells is also represented in Fig.III.12; as we can see in the CD3-APC-A *vs.* SSC-A dot plots, positive selection induces the formation of doublets representing 15.260 ± 4.8890% of the $CD3^+$ cells, while negative selection only originated 2.950 ± 0.8228%.

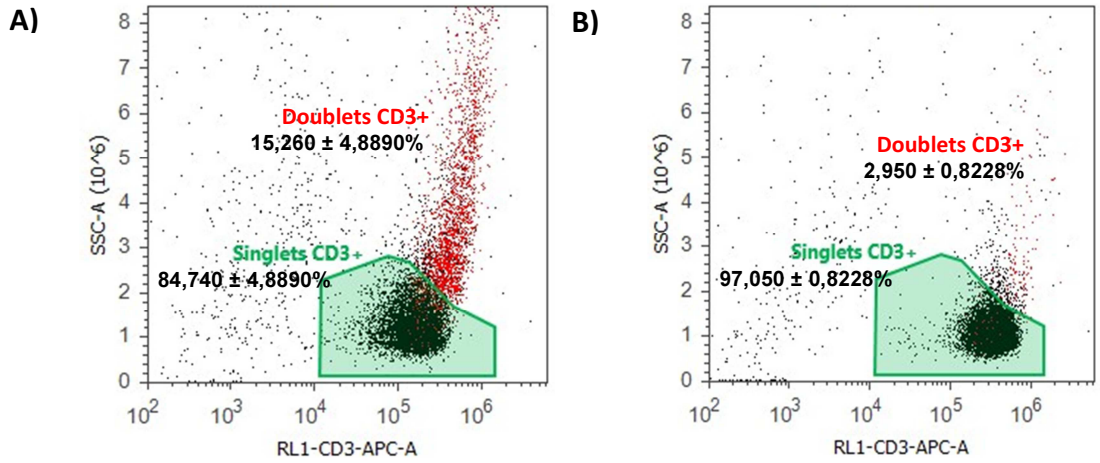

Fig.III.12 – Flow cytometry analysis of the $CD3^+$ population isolated using: A – positive selection and B – negative selection. Doublets are represented in red and were identified based on the profile of the sample in a CD3-APC-H *vs.* CD3-APC-A dot plot. Singlets are gated in green. Images are representative for the 3 donors.

III.5. T cell separation using nylon wool fiber columns

The isolation of T cells was also tested without resorting to immunomagnetic methods. The chosen method requires the use of a nylon wool fiber column, and is based on cell adherence properties. After PBMCs have been loaded into the column they are incubated in contact with the packed wool. Then, T lymphocytes, as non-adherent cells, can be collected by simply washing the column with media. On the other side, B lymphocytes and monocytes, as adherent cells would get stuck in the wool, having to be dislodged and plunged out of the column.

This method was tested using PBMCs from 3 different donors; both fractions, adherent and non-adherent, were collected and analyzed by flow cytometry. In this case, doublets represented a percentage inferior to 5%, and consequently they were not taken into account independently from singlets. The samples were stained with CD3 and CD14 Ab, in order to identify T lymphocytes and monocytes, respectively. Consequently, cells that were simultaneously $CD3^-$ and $CD14^-$ were considered to be B cells. This is exemplified in Fig.III.13.

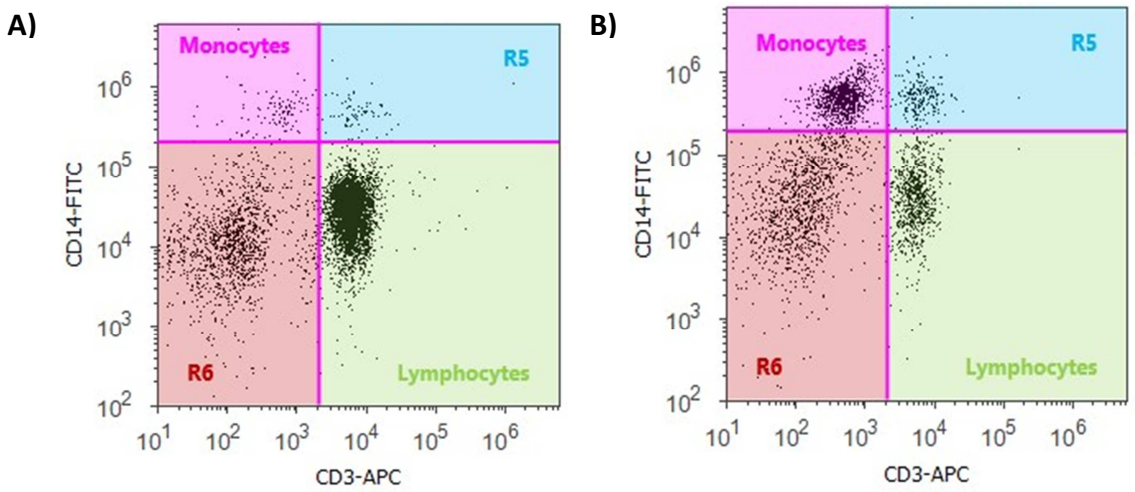

Fig.III.13 - Flow cytometry analysis of cells isolated using a nylon wool fiber column and stained with CD3-APC and CD14-FITC. A – Non-adherent fraction; B – Adherent fraction. The $CD3^+/CD14^-$ cells are T lymphocytes (green); the $CD3^-/CD14^+$ cells are the monocytes (pink), and $CD3^-/CD14^-$ cells, by exclusion, are B lymphocytes (red). $CD3^+/CD14^+$ cells, represent cells that stained with both antibodies not specifically (blue). Images are representative for the 3 donors.

Then, the relative percentage of the different cell types was compared for both fractions and is graphically represented in Fig.III.14.

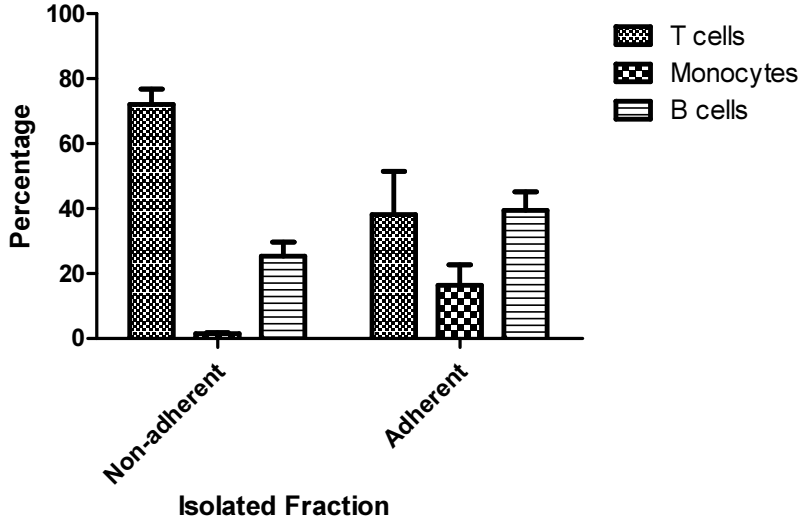

Fig.III.14 – Relative proportion of different cell populations among PBMCs separated using a nylon wool fiber column. Both fractions, adherent and non-adherent, are represented. (n=3)

Regarding the non-adherent cell fraction, the highest cell percentage matched the population of T cells (72.13 ± 4.645%). However, B cells were still present in the sample in a considerable amount (25.38 ± 4.348%). This fraction was in fact almost depleted of monocytes, which represented only a small percentage (1.441 ± 0.3146%).

In the adherent cell fraction, as expected the most abundant population was of B cells (39.44 ± 5.706%). On the contrary, T cells were almost as abundant (38.15 ± 13.28%), what should not happen. In this fraction, monocytes represent 16.38 ± 6.212% of the total events.

III.6. $CD14^+$ monocytes positive selection: Miltenyi *vs.* StemCell

In our laboratory, monocytes selection is usually performed using a positive selection kit from Miltenyi (CD14 MicroBeads). However, we had the opportunity to test a kit for the same type of selection but from a different vendor, StemCell (EasySep® Human CD14 Selection Kit). Moreover, the Miltenyi kit was already optimized to 1/3 of the recommended volume of beads for $CD14^+$ isolation; therefore it was also relevant to test if the same optimization could be applied to the kit from StemCell.

Hence, after the PBMCs of each donor had been isolated and counted in the Neubauer chamber, they were equally divided into 3 tubes, in order to proceed to monocyte isolation using the three protocols in parallel: Miltenyi (1/3), StemCell (total) and StemCell (1/3). For this part of the assay, 4 different donors were used.

After monocyte isolation was complete, cells were counted in the Neubauer chamber in order to determine the isolation yield. The results are represented in Table III.2.

Samples were also stained with CD14-FITC Ab and analyzed using flow cytometry. By comparing the percentage of isolated cells that stained $CD14^+$ to the number of PBMCs that were $CD14^+$ (also analyzed prior monocyte isolation) it is possible to determine the recovery rate which is indicated for each protocol in Table III.2.

The percentage of $CD14^+$ cells after isolation represents the purity of the sample (see Table III.2). Once more this value correspond to singlets and doublets altogether, however, the percentage of cells that were acquired as doublets is inferior to 5%, reason why it was not discriminated here.

Finally, the $CD14^+$ population was compared regarding its FSC and SSC profiles in order to detect eventual alterations caused by the different protocols. It is essential that only singlets are taken into account for these considerations as doublets would immediately affect the parameter values. The average median FSC and SSC values for each protocol are indicated in Table III.2.

Table III.2 – Comparison between $CD14^+$ monocytes isolated using positive selection kits from different vendors: Milteniy and StemCell

	Miltenyi	StemCell (total)	StemCell (1/3)
Isolation Yield (%) n=4	25.92 ± 3.205	15.32 ± 2.368	8.08 ± 9.170
Recovery rate (%) n=3	86.70 ± 14.29	34.54 ± 4.890	28.20 ± 2.871
Purity (%) n=4	95.01 ± 2.737	90.61 ± 1.447	87.34 ± 2.129
Median FSC (x 10^6) n=4	3.591 ± 0.0277	3.504 ± 0.0399	3.527 ± 0.0383
Median SSC (x 10^6) n=4	4.195 ± 0.1806	6.071 ± 0.4472	5.111 ± 0.2563

First of all, regarding the isolation yield, Miltenyi isolation clearly had the best results, 10.6% higher than the ones obtained when the StemCell kit was used with the recommended amount of beads. When 1/3 of the recommend volume of the StemCell beads was used, the isolation yield was even 7.24% inferior, resulting in only 8.080 ± 9.170%.

The same hierarchy occurred regarding cell recovery, where over 85% of all $CD14^+$ cells existing prior monocyte isolation were successfully selected. On the other hand, results obtained with the StemCell selection were much inferior, respectively 65.46% and 73.11% less, for total and1/3 of the recommended volume of beads.

Concerning the purity of the sample, all three protocols allowed high values, but once more the highest one corresponded to Miltenyi, followed by StemCell (total) and finally StemCell (1/3). The exact values are expressed in Table III.2.

Taking into account the last 2 parameters (recovery rate and purity), it would have been expected that with such high purities, almost all $CD14^+$ cells existing prior to cell separation would have been present in the final cell suspension. However, StemCell originated recovery rates of less than 35%, which means that a lot of cells were lost throughout the isolation process.

Just by comparing the general position of the $CD14^+$ population in a FSC-A *vs*. SSC-A dot plot (see Fig.III.15) it is possible to state that in all three protocols, FSC is approximately the same, reflecting identical cell size regardless of the protocol used. On the other hand, cells isolated with the kit from StemCell had a higher SSC profile, which decreased when the volume of used beads also decreased. Nonetheless, in both cases SSC was superior to the one of $CD14^+$ monocytes isolated with Miltenyi, what represents a greater cell complexity. The correspondent values are indicated in Table III.2.

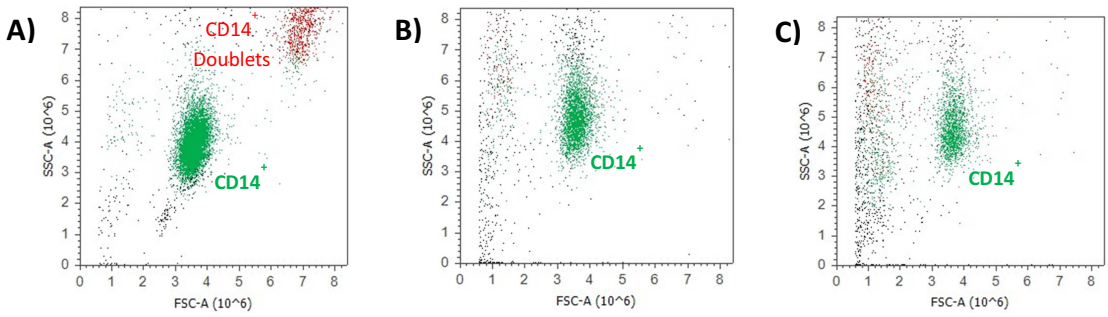

Fig.III.15 – Flow cytometry analysis of FSC and SSC profiles of the $CD14^+$ singlet population isolated by positive selection using: A – CD14 MicroBeads kit from Miltenyi (1/3 of the recommended amount of beads); B – EasySep® Human CD14 Selection Kit from StemCell (recommended amount of beads); and C – EasySep® Human CD14 Selection Kit from StemCell (1/3 of the recommended amount of beads). CD14-FITC Ab was used to stain monocytes, and then $CD14^+$ cells were identified in CD14-FITC *vs*. SSC-A dot plot. Finally, a back gate of these cells was selected in this dot plots. $CD14^+$ singlets are identified in green and $CD14^+$ doublets are colored in red. In order to compare FSC and SSC profiles using the different methods, only the singlet population was taken into account. Images are representative of the 4 donors.

Moreover, the resulting monocytes of 3 of the donors were kept in culture in a 24-well plate (1 million cells/ml). The cytokines GM-CSF and IL-4 were added to the culture media in order to allow monocyte differentiation into mo-DCs. At the 7th day the mo-DCs were removed from culture and counted in the Neubauer chamber in order to determine the percentage of monocytes that were successfully differentiated into DCs. Cells were also stained with 7AAD and HLA-DR and analyzed by flow cytometry. By using 7AAD it was possible to determine the average percentage of cell death for each method; MFI values of HLA-DR staining measure the expression of MHC-II which is a marker for cell maturation. All data previously mentioned is summed up in Table III.3 and correspond to 3 donors, except for HLA-DR staining, which was only performed in 2 of them.

Table III.3 – Comparison between mo-DCs differentiated from CD14$^+$ monocytes isolated using positive selection kits from different vendors: Miltenyi and StemCell.

DCs	Miltenyi	StemCell total	StemCell 1/3
Efficiency of differentiation (%) n=3	43.84± 1.723	45.91 ± 7.018	50.86 ± 14.11
Cell Dead (7AAD staining) (%) n=3	13.71 ± 3.285	21.224±1.098	20.89 ±2.840
MFI (MHCII staining) n=2	4171 ± 2001	13341 ± 10484	11212 ± 9728

Regarding the efficiency of differentiation of the selected CD14$^+$ cells into mo-DCs (see formula in section II.7), the best results were obtained with monocytes isolated with the StemCell kit using 1/3 of the recommended amount of beads; the utilization of the recommended amount of beads resulted in a decrease of 4.95% in cell differentiation efficiency. In this case the mo-DCs differentiated from monocytes isolated with the kit from Miltenyi had the worst results with a decrease of 7.02% in comparison to the most successful one.

The percentage of cell dead was the same for both bead volumes tested with the StemCell kit. Monocytes isolated with the Miltenyi kit differentiated into mo-DCs with a dead cell percentage 7.514% inferior to that. In Fig.II.16 it is possible to see for each protocol a representative histogram comparing a sample stained with 7AAD (black), the respective unstained sample (purple), and the resulting 7AAD$^+$ selection.

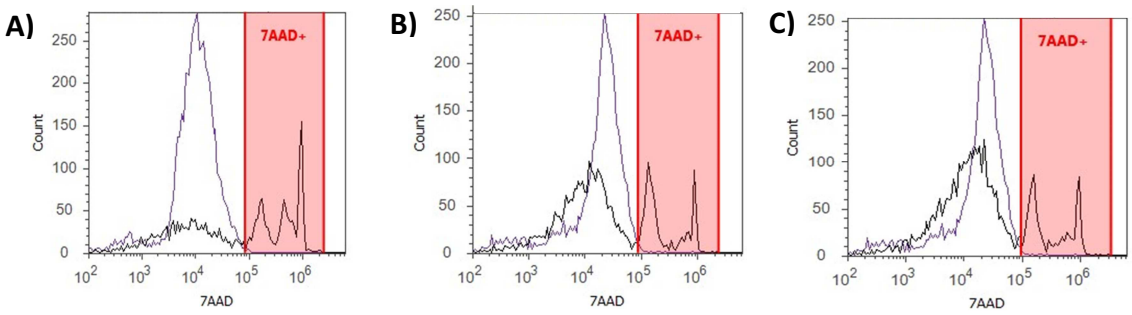

Fig.III.16. – Flow cytometry analysis for 7AAD staining of mo-DCs differentiated from $CD14^+$ monocytes isolated by positive selection using: A – CD14 MicroBeads kit from Miltenyi (1/3 of the recommended amount of beads); B – EasySep® Human CD14 Selection Kit from StemCell (total amount of the recommended beads); and C – EasySep® Human CD14 Selection Kit from StemCell (1/3 of the recommended amount of beads). Each histogram portraits the profile of the sample stained with 7AAD (black line) and the respective unstained sample (purple line). The resulting 7AAD population is selected in red. Images are representative for the 3 donors.

Finally, concerning the MFIs obtained from the HLA-DR staining, it is possible to determine that both bead volumes tested for StemCell resulted in similar maturation levels, with a difference of only 2129. However, even though when 1/3 of the recommended volume of beads was used, the value was inferior, it was still 7041 higher than the one obtained for Miltenyi, where the separation process resulted in the lowest maturation and activation levels amongst all 3 conditions tested.

III.7. Cell preservation

In this section, we intended to evaluate the shelf life of frozen cells, as well as to optimize the protocols used to remove the cryoprotective agent (DMSO). For that, aliquots of PBMCs from 4 different donors were frozen, immediately after separation, in the same conditions: cell concentration, total volume, freezing media, storage temperature, etc. (as described in section II.11.1).

III.7.1. Shelf life of PBMCS at -80ºC

To determine the shelf life of cells we have evaluated cell viability after thawing over a six month period post freezing. After periods of 24h, 48h, 1, 2, 3 and 6 months, aliquots of PBMCs from all donors were thawed in a water bath at 37 ºC for approximately 1 minute. Then, the samples were washed for DMSO removal (110 x g, 10 minutes) and resuspended in their initial volume. Finally, each sample was counted in a Neubauer chamber using trypan blue, to distinguish live from dead cells. Two parameters were evaluated: recovery rate and cell viability. Recovery rate refers to the number of living cells after cell thawing and DMSO removal in relation to the total number of living cells before

freezing; and cell viability was defined here as the number of living cells divided by the total number of cells (after thawing and DMSO removal).

Keeping track of cell recovery rate and viability allowed a better knowledge on how PBMCs were influenced by freezing and thawing over a 6 month period, under the tested conditions. The results can be observed in Fig.III.17.

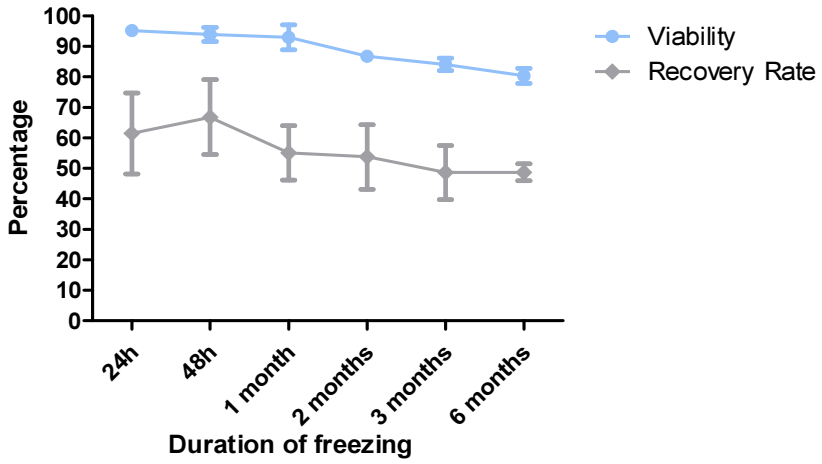

Fig.III.17 – Evolution of cell viability (blue) and cell recovery rate (grey) throughout a 6 month period. Cell viability refers to the percentage of living cells among the total number of cells. The recovery rate reflects the percentage of the initial living cells (before freezing) that still remain alive in the sample after thawing and DMSO removal. (n=4)

Once an aliquot had been frozen, if it is thawed on the following two days, it is still possible to recover at least 61.45 ± 13.26% living cells, after DMSO removal. After that initial period, and for the following 6 months, the recovery rate only further decreases 6.39%, seeming to stabilize just beneath 50% (at 48.69 ± 8.861 %).

After a 6 month freezing period and subsequent thawing and DMSO removal, the viability of the samples was higher than 80% (84.15 ± 2.098%). During this period, the most abrupt decrease occurred between the 1st and 2nd months and corresponded to 6.12%. These observations allowed us to conclude that, under the tested conditions, PBMCs shelf life was at least 6 months.

III.7.2. DMSO immediate removal *vs.* after 24 hours

Since we have observed that most cell losses occurred during thawing procedures we aimed to optimize this procedure. After cells had been thawed, it is still necessary to remove DMSO; generally, this step can be performed in one of two different moments. One option consists in washing the cells right after being thawed, thus immediately removing DMSO. The other option requires that DMSO removal only occurs after cells, and consequently the DMSO, had been diluted and kept in culture for 24 hours. In order to compare how these two methods influence cell recovery rate and viability they have both been tested in PBMCs from 4 different donors that had been frozen for 3-4

months. After being thawed, each sample was equally divided, allowing both DMSO removal protocols to be used in the same sample. In Fig.III.18 it is possible to observe the results regarding cell viability and recovery rate for both methods: immediate DMSO removal and after 24 hours. The presented data only refers to the period after DMSO had been washed from both cell samples; after that, cells were kept in culture for 2 more days, in order to determine if there was a less immediate effect caused by any of the methods.

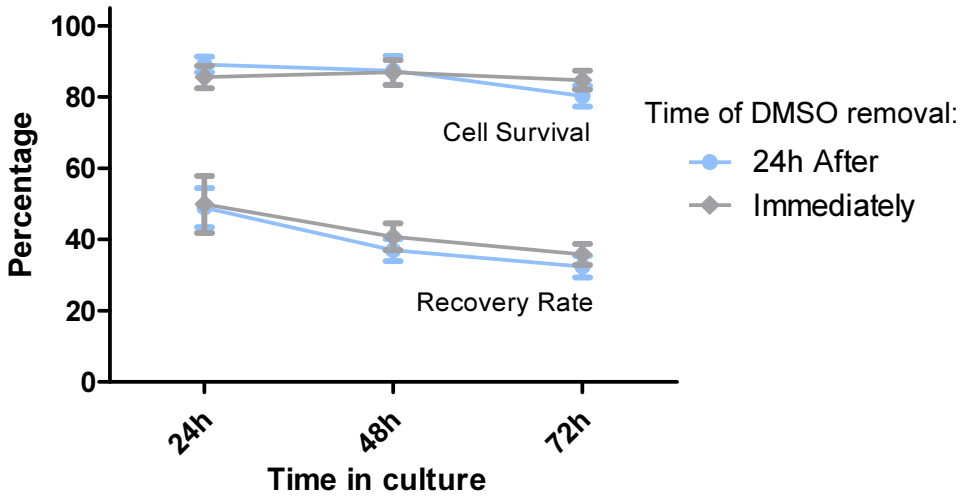

Fig.III.18 – Comparison between immediate DMSO removal (grey) and after 24 hours (blue). The results shown refer to the 3 days that PBMCs were kept in culture starting from the point both cell sets were already DMSO-free. Cell survival and cell recovery rates are presented for both methods. (n=4)

When DMSO is immediately removed, the recovery rate of PBMCs varies from 49.89 ± 8.00% to 35.86 ± 2.98% and its viability starts in 85.65 ± 3.16% and decreases to 84.78 ± 2.64%. When PBMCs are diluted, and DMSO only removed 24h later, the recovery rate goes from 48.97 ± 5.54% to 32.41 ± 3.06%, while cell viability varies from 89.15 ± 2.21% to 80.32 ± 2.89%. Overall, both methods present a very similar behavior; however, when DMSO is immediately removed, there seems to be less variation throughout time, and after 72h in culture it is possible to recover 0.92% more cells; also, in this case, cells are 3.5% more viable than the ones obtained after 72h in culture if DMSO had initially been diluted.

III.7.3. Effect of centrifugation speed: 110 x g *vs*. 250 x g

Here, we intended to test the effect of centrifugation on cell viability during thawing protocols. In order to determine the influence of centrifugation speed, during DMSO removal, samples were equally divided and washed in different conditions. One half was centrifuged at 110 x g for 10 minutes and the other was centrifuged at 250 x g for 5 minutes. The data collected from PBMCs of the 4 tested

donors allowed to determine the average recovery rate and cell viability for both conditions, and can be consulted in Fig.III.19.

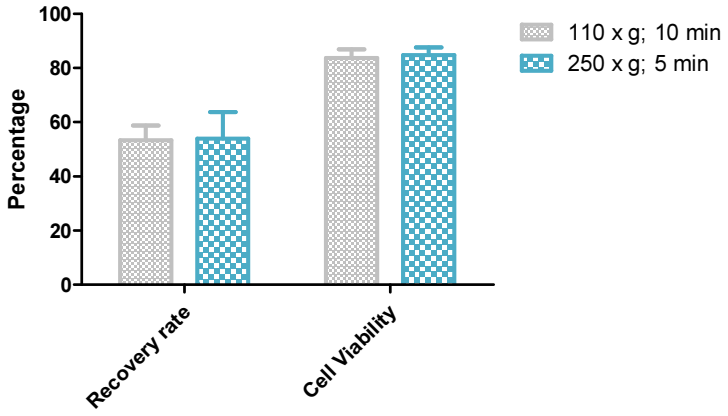

Fig.III.19 – Cell recovery rate and cell viability using different centrifugations parameters. Centrifugation at 110 x g for 10 minutes (grey) was compared to centrifugation at 250 x g for 5 minutes (blue). (n=4)

In average, when cells were centrifuged at 250 x g, the recovery rate was 53.98 ± 9.816% and viable cells corresponded to 84.80 ± 2.814% of the total. Those values were only 0.69% and 1.13% higher than when centrifuged at 110 x g, respectively. Thus, in both cases, differences are near 1%, portraying an almost identical behavior between both methods. Therefore, as a standard procedure, after thawing, cell samples were washed by performing a centrifugation at 110 x g, for 10 minutes.

III.8. Economic viability evaluation

When comparing different methodologies, different kits or even different vendors, it is important to take into consideration parameters such as isolation yield, cell purity and others. However, it is also crucial to relate these characteristics with the costs associated with each process. It is important to make sure that a good relation quality-price is reached.

In our laboratory, the most frequently isolated cells are monocytes and T cells, thus, economic viability evaluations were performed in order to compare different kits available for each case.

Only the kits and other specific material directly related to them were taken into account in this comparison; the remaining reagents and materials were considered as equivalent.

III.8.1. Isolation of $CD3^+$ T cells

In our laboratory, $CD3^+$ T cells are usually isolated using a positive selection kit from Miltenyi (CD3 MicroBeads). However, more recently, T cell separation has also been tested using a nylon wool

fiber column. Consequently, it would be relevant to know whether this was a good alternative. Prices for all the referred methods are represented in Table III.4. Only purity and costs will be compared because information regarding isolation yield was only calculated for the Miltenyi kit and not form cell isolation with the nylon wool column.

Table III.4 – Comparison between prices and limitations of $CD3^+$ T cells using different types of selection.

Product	**Miltenyi** **CD3 MicroBeads,** human	**LS Columns**	**Polysciences** **Nylon Wool Fiber,** Sterile
Catalog #	130-050-201	130-042-401	21759-1
Price w/ IVA (€)	814.26	514.14 (25 units)	211.00 (10 syringes)
Total volume supplied (ml)	2.0	-	-

The prices do not include other reagents.

Regarding cell purity, using the positive selection kit or nylon wool fiber result in very similar average percentages: 73.78% (see section III.4) and 72.13% (see section III.5), respectively. Although, it is important to consider that with the Miltenyi kit the contamination is essentially due to debris, while when the nylon wool fiber column is used contamination is due not only to debris but also to the presence of other cell types.

Even without further calculations it is possible to state that using a nylon wool fiber column is less expensive. In this case, the only expenses would be the syringe packed with nylon wool fiber, costing 21.10€. Regardless the amount of cells being separated, positive selection will always be more expensive because it requires not only the use of a specific column (20.57€ each), but also of an immunomagnetic kit.

III.8.2. Isolation of $CD14^+$ monocytes

In our laboratory, most of monocyte isolation is performed using the Miltenyi kit. In order to determine if it was economically more viable to use the StemCell kit, the inherent costs of both of them were compared. The prices for the main components are listed in Table III.5. In each case there are shipping and handling associated costs: in the case of StemCell they represent 50€ of each shipment (added to the price of the kit, for an estimate total value), while Miltenyi does not specify the value but it is already applied to the price.

Table III.5 – Comparison between prices and limitations of $CD14^+$ monocytes positive selection kits from Miltenyi and StemCell.

Product	MACS **CD14 MicroBeads**, human	**LS Columns**	StemCell **EasySep™** Human CD3 Positive Selection Kit
Catalog #	130-050-201	130-042-401	18058
Price w/ IVA (€)	814.26	514.14 (25 units)	607.00+50**
Total volume supplied (ml)	2.0	-	1.0 + 1.0*

The prices do not include other reagents.

*This kit contains 2 components: EasySep® Human Positive Selection Cocktail (1.0 ml) and EasySep® Magnetic Nanoparticles (1.0 ml), however, since both are required for each isolation, the limiting volume was considered to be 1.0 ml.

**Additional 50€ referring to shipping and handling.

First of all, each isolation using the MACS kit implies the use of a LS column that costs 20.57€.

In order to determine which one was the most economic kit, all of them were compared regarding the costs to isolate 24 million monocytes, the equivalent amount to fill a 24-well plate, with the usual 1 million cells/ml rate. The calculations for Miltenyi selection refer to the optimized utilization of 1/3 of the recommended volume of beads. The same calculations were made for StemCell, for both, total and 1/3 of the recommended volume of beads. The results are indicated in Table III.6.

Table III.6 – Comparison between costs associated with $CD14^+$ monocytes positive selection using kits from Miltenyi and StemCell.

	MACS (1/3)	StemCell (total)	StemCell (1/3)
# PBMCs used for isolation (x 10^6)	92.59	156.66	297.03*
Isolation Yield (%)	25.92	15.32	8.08
# $CD14^+$ selected cells (x 10^6)	24	24	24
Amount of kit used	12.35% (246.91µl)	15.66% (156.66µl)	9.90% (99.01µl)
Cost (€)	121.13	102.89	65.04

*The separation of such amount of cells would require 2 subsequent selections, as the maximum number of total cells that can be used each time is 250 x 10^6 (With Miltenyi the limit is 2000 x 10^6 cells per column).

The less expensive option would be using 1/3 of the recommended volume of StemCell beads; however the inferior isolation yield of this process would require a higher number of PBMCs to be used. In this case, that also implicates that the total amount of cells could not be isolated at once, as only a maximum of 250 million PBMCs can be processed each time. When the recommended amount of beads is used the process would be 37.85€ more expensive, but it would only require 1 selection. Finally, Miltenyi protocol was the most expensive: 56.09€ more than when 1/3 of the recommended volume of StemCell beads was used. However, this protocol allows performing monocyte isolation starting with a maximum number of PBMCs that is 1750 million cells higher.

It is also important to take into account that with the increase in costs also increased the purity of the selected cells.

III.9. Divulgation of the Cell Biology services

Most of the divulgation work concerning the Cell Biology services is performed through our official webpage. At the moment it is hosted in the FCM (Faculdade de Ciências Médicas) server and can be accessed directly in the website of the Faculty (1); moreover, the official site of CEDOC also allows direct access to our webpage (2). In alternative, it is possible to use our link (3). In Fig.III.20 it is possible to see the homepage of the service.

(1)http://www.fcm.unl.pt/main/index.php?option=com_content&view=article&id=623&catid=30&Itemid=208&lang=pt

(2)http://cedoc.unl.pt/services2/

(3)http://www.fcm.unl.pt/projectos/glicociencias/index.php?option=com_content&view=article&id=43&Itemid=52

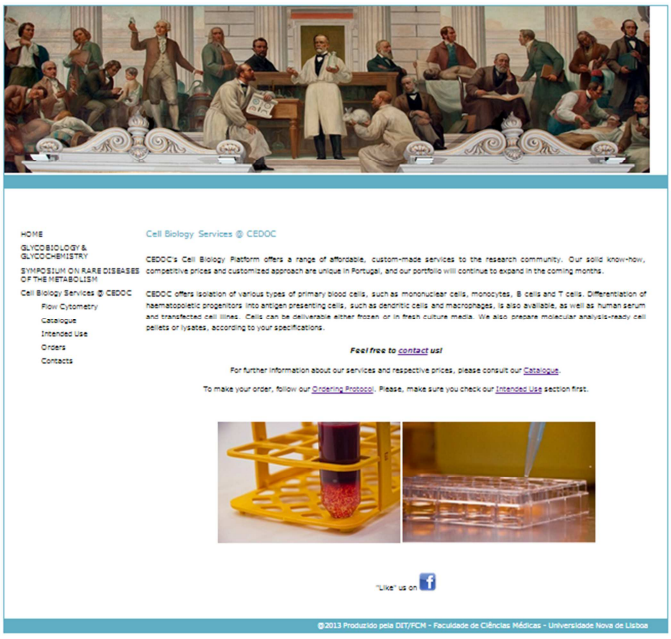

Fig.III.20 – Homepage of the "Cell Biology Services @ CEDOC".

Currently, the webpage includes the flowing subjects: brief description of the offered services, catalogue and ordering protocols, our intended use policy, main contacts and a FAQs (Frequently Asked Questions) section. It is also available a section describing the terms and prices for the Attune® Acoustic Focusing Cytometer utilization, which is accessible for fellow investigators. Updates are constantly being made in order to make sure that the website keeps up to the breakthroughs taking place at the laboratory. Any alterations require the use of the Joomla! software and eventually, html programming.

Our facebook page, Glycoimmunology at Cedoc (see Fig.III.21), has a less commercial focus, and generally approaches themes more related to the group instead of the service.

Fig.III.21 – Snapshot of the facebook page of “Glycoimmunology at Cedoc”.

IV. DISCUSSION

IV.1. Protocol optimizations

IV.1.1. Serum isolation from buffy coats of human peripheral blood and its performance in cell culture

Serum isolation is usually an easy process that only requires collecting peripheral blood directly into a dry tube and leaving it to coagulate. After removing the clot, we would have the serum. However, in our laboratory we use buffy coats from human peripheral blood, which have the anti-coagulant CPD to avoid cell clotting. Under these circumstances the solution would be to isolate the plasma and remove the fibrinogen in order to obtain just the serum. Most common protocols usually require the addition of calcium to the blood to remove the anti-coagulant solution. The calcium will activate clotting, thus allowing serum isolation (Sigma-Aldrich – FAQs). Despite the fact that this kind of protocols were known to us, the adequate calcium concentrations that were necessary were not. Consequently we have chosen an alternative method requiring fibrinogen precipitation by heat (Dintenfass and Kammer, 1976) that not only seemed simpler but also cheaper. However, in our hands when the referred protocol was used (3 minutes, at 58 ºC – method #1), fibrinogen precipitation was not complete. Since we intended to perform a subsequent heat inactivation (1 hour, at 56 ºC – method #2) to destroy complement we decided to test the efficiency of this protocol, for fibrinogen removal, which would ultimately allow serum isolation from a plasma sample obtained from a buffy coat. Accordingly to bibliography (Dintenfass and Kammer, 1976), method #1 should have been enough to precipitate fibrinogen from plasma. Eventually, it might have not worked because it required a steady temperature during 3 minutes and probably there were some oscillations avoiding fibrinogen precipitation. Moreover incubating the samples at 56 ºC (method #2) gives some kind of safety margin for some eventual temperatures oscillations as temperatures above 58 ºC will destroy the sample permanently (Montefiori Laboratory, 2011). To assess the ability of method #2 to remove fibrinogen we have performed a protein electrophoresis. Plasma processed by method #2 showed a protein electrophoresis' profile similar to the one obtained from a serum sample collected into a dry tube, proving that the plasma treated with this method did not contain fibrinogen.

The next step was to evaluate the effect of the serum isolated by method #2 on cells. For that we have used human PBMCs from donors that were different from the serum donors. For comparison purposes, we have cultured in parallel the same batches of PBMCs in medium with equal composition but using other sera, namely FBS and commercial human sera obtained from Sigma.

The first difference we could observe was in media coloration, even before the cells were resuspended in the culture media. It was possible to observe that supplementing the culture media with human serum, whether commercial or isolated in our laboratory, resulted in a stronger pink coloration than when FBS was used. The presence of phenol red in the media allows attributing the different colors to different pH. Thus, it was determined that FBS turned the culture more acid, while all human sera, either commercial or obtained in our laboratory, turned it slightly more alkaline.

Nonetheless, these variations were not a big concern as it is only essential to change or replenish the culture media in case it turns purple (alkali) or yellow (acid) (Sigma Life Tecnhologies and ECACC, 2010). In order to assess the importance of heat inactivation for serum performance we have evaluated two different batches of commercial sera both from Sigma: one was not heat inactivated (H4522_#1) while the other was inactivated for 30' at 56 ºC (H4522_#2). Interestingly, after 5 days of culture the cells started to aggregate, in the media containing H4522_#1 and we could macroscopically observe the formation of long white strings and cell clumps were visible by optical microscopy observation. Despite these differences, which were observed for PBMCs of all three donors cultured in the presence of the human inactivated serum H4522_#1, all the cultures were free of contaminations as assessed by microscopy observation. This observation is important since we wanted to be sure that our protocols for serum isolation preserved serum sterility.

As most of the protocols using PBMCs required relatively long culture of these cells (one week in average), we have established 7 days as reasonable time to test cell viability.

After 7 days in culture, both sera isolated in our laboratory resulted in percentages of death cells and debris similar to H4522_#2 (heat inactivated), and higher than with FBS, which is the standard serum used in our laboratory. The non-heat inactivated serum (H4522_#1) had the highest percentages among all. Moreover, as can be seen in Fig.III.3, although the lymphocyte population is still well defined, the monocyte one has almost disappeared. However, a new population localized in the right side of the dot plot has shown up. Therefore we suggest that due to the fact that PBMCs have been in culture for 7 days, monocytes had started differentiating into macrophages, originating this population.

We also aimed to check for cell function when in culture with different sera, for that we have chosen to test the responsiveness to LPS stimulation. This assay was performed by incubating freshly isolated PBMCs with or without LPS and evaluating their response after 24h. The increased expression of MHCII on the cell surface is a measurable parameter of cell response to LPS. Regarding the stimulation level of PBMCs, it was expected that both lymphocytes and monocytes presented a higher expression of MHCII after stimulation with LPS than in the basal state. That only occurred for both populations with FBS. In all the tested human sera, lymphocytes were not responsive to LPS, and monocytes were only slightly responsive to LPS in the media containing type A serum isolated in our laboratory. Surprisingly, when comparing both commercial human sera to each other, neither presented a relevant activation of the lymphocyte population, and strangely monocytes had a lower MHCII expression than the basal one in both cases. Moreover the basal levels of MHCII expression were already higher than the levels of expression of LPS stimulated cells in FBS serum. One explanation may be that the endotoxin levels of these commercial sera are already too high and that cells are "naturally" stimulated by virtue of its presence. However, in the case of human sera obtained in our laboratory the basal levels were not much different from the ones with FBS but the response to LPS was not effective. From this observation we cannot exclude that other factors present in human sera are precluding the responsiveness of PBMCs to LPS, at least under the tested conditions.

The importance of serum inactivation is stressed by our observations. In fact FBS serum was purchased already heat-inactivated and commercial human serum was inactivated or not in our laboratory. The non-inactivated serum promoted cell clumping and a higher percentage of dead cells and debris. This goes against the expected as bibliography states that inactivation of serum is not an obligatory requirement for cell culture (Sigma Life Tecnhologies and ECACC, 2010). By principle, in the case of cultures of PBMCs, the fact that the complement is or not inactivated should not be a problem as normal $CD4^+$ T cells do not express complement receptor in a level large enough to be of influence (Montefiori Laboratory, 2011).

It seems that complement inactivation does in fact improve cell recovery, cell morphology, and cell response to stimulation, nonetheless, the blood type of the serum sample is only relevant when performing immunoractivity assays (Sigma-Aldrich – FAQs).

To sum up, in an overall observation, serum isolated in our laboratory seems to be suitable to keep cells in culture during at least 7 days, and eventually prolonged to 11 days. Of course, these assays only refer to PBMCs, as other cell types may require culture media with different characteristics and human sera may no longer be adequate.

IV.1.2. Comparison between different protocols for the isolation of lymphocytes and monocytes from PBMCs

In this section we will refer first to the general consideration drawn from this study and that apply to decisions that are previous to cell isolation, such as buffy coat selection. Knowing that there are some differences between man and woman we have set the criteria of restricting to blood collected from man between 18 and 65 years old. In this study we compared specific age groups or blood type in terms of the cell yield obtained for PMBCs. Our results show that there are no significant changes in cell yields, related to the characteristics mentioned above. Thus, upon the request of buffy coats it is irrelevant to further restrict to a specific age group or blood type. The only situation when it is compulsory to select the blood type refers to the isolation of human serum from type AB donors; although, it is important to keep in mind, that this is not the only type of serum that can be used in cell culture. Secondly, regarding the use of the lyse solution, data presented in section III.3 has shown that besides removing the reddish coloration of the pellet, it brings no advantage to the isolation process itself, at least in terms of cell yield (in fact the use of lyse solution diminishes it). Thus, the utilization of lyse solution as a standard step in each isolation brings no advantage. Nonetheless, this solution did not seem to cause any relevant damage to the remaining cells (including viability), therefore, the best solution might be to selectively use the lyse solution in situations where the cell pellet presents a very evident red color.

In immunomagnetic isolation procedures, the beads represent the most expensive reagent and, sometimes, lower amounts give similar results in terms of cell yield and purity. For this reason optimization of the number of beads may result in a significant overall cost reduction of isolation

procedures. As stated before, the studies comparing different procedures were performed with PBMCs from the same donor, which were divided and processed in parallel.

For T cell isolation we aimed to compare the use of positive and negative selection kits from Miltenyi. We also wanted to optimize the number of beads to be used in each case. First of all, the average isolation yield obtained with different kits for the same donor, had a difference of almost 10% between them. However, by observing these results in a wider perspective, i.e. taking into account a larger number of isolations (though not from the same donor), the respective cell yield increased approximately 10% in each case. Stands the fact that, in order to isolate the same number of final T cells, the negative selection kit (PanT) requires a higher number of initial PBMCs than the two remaining protocols tested. Because the utilization of the recommended volume of MicroBeads of the positive selection kit provides the highest isolation yield, this is the process that would require the lesser number of initial PBMCs. The yield obtained with negative selection is comparable to the one obtained when half of the recommend volume of the positive selection MicroBeads was used. After this analysis it is possible to conclude that the optimized utilization of half the amount of positive selection MicroBeads is acceptable as it allows an isolation yield high enough to meet the necessities of our assays. However, in a case where a higher cell yield is required, for example, if we have a smaller number of initial PBMCs available, it might be justifiable to use the recommended volume of MicroBeads despite the correspondent cost increase. On the other side, negative selection provided much purer samples. Mainly due to the existence of less disperse and undefined events that probably represent debris or other unwanted events. Also, we have observed that when positive selection was used, a larger percentage of T cells were present in the form of doublets (at least upon acquisition on the flow cytometer). Despite the fact that Miltenyi states that using these MicroBeads will avoid ever dealing with any clumping artifacts (Miltenyi Biotec, 2013) this could be explained by cell aggregation caused by interaction with the MicroBeads. In order to assess if the basal activation state of T cells isolated by positive selection and negative selection was different, we checked for the expression of CD69 molecule, which is an indicator of the T cell activation status. In fact, the levels of CD69 expression were higher for positively selected cells. The fact that in negative selection, the target cells (T cells, in this case) do not directly interact with the MicroBeads, may be the reason for the differences in activation. However according to Miltenyi, even in positive selection, no activation should occur (Miltenyi Biotec, 2013). Indeed, the differences were not significant and further implications in terms of T cell function could only be assessed by performing other studies on T cell function.

Another non immunomagnetic method was tested for T cell separation: using a nylon wool fiber column. For this study, we have used different donors. We aimed to use a less expensive method for T cell isolation and test it in terms of purity in comparison with immunomagnetic separations. T cell purity percentages are similar to the ones obtained with positive selection. However, this can be deceitful as “contamination” present in T samples isolated using positive selection, only refer to eventual debris, while in the nylon wool case it clearly refer to B cells (25%) and monocyte contamination (1%). Moreover this method attained a low cell recovery as a considerable percentage of T cells fail to be isolated, and stays collected in the adherent fraction together with monocytes and

some B cells. Thus, this type of selection would only be adequate for assays where the presence of B cell populations besides T cells were acceptable. In order to improve cell recovery and purity, different media could be tested, besides RPMI-1640 supplemented with 10% FBS, as there are a wide variety suggested by the vendors of the nylon wool fiber columns (Polysciences).

To sum up, each process has its advantages and disadvantages: positive selection allows a higher isolation yield, but an inferior purity and higher activation levels. Negative selection has the opposite results: higher purity and an inferior cell activation level, but a smaller isolation yield. Finally, nylon wool column is faster than both MACS methods but the isolated sample is contaminated with other cell types. Consequently, the choosing of the isolation protocol will highly depend on the assay for which the T cells are going to be used and in considerations such as if untouched cells are required or the damage that "contamination" with other cell types may cause.

Other objective of this study was to test isolation kits from different brands and compare them in terms of the yield and purity of cells but also, in terms of economic costs and time consumption. We had chosen, to compare the positive selection kits for $CD14^+$, i.e, monocytes, from two suppliers: Miltenyi and StemCell. In terms of cell yield, higher values were obtained with Miltenyi kit. Despite the high purity of the samples, the recovery rates provided by StemCell are lower, which means that a lot of cells were lost during the isolation process, eventually by being trapped or lysed. In order to improve cell recovery, StemCell suggests to decrease the number of separations in the magnet from 3 times to only 2 times, and the addition of magnetic nanoparticles in a concentration twice higher (100µl/ml cells) (STEMCELL Technologies, 2008). However, this suggestion was not tested by us. Besides the aspects referred above, which deal with the efficiency of cell separations, we could observe also differences in the quality of monocytes isolated by both kits. Monocytes isolated by the StemCell kit had a higher SSC profile, reflecting a higher complexity of the cells. However, when only 1/3 of the recommended volume of beads was used, the SSC decreased. Therefore, this higher complexity of monocytes isolated with the StemCell kit is possibly a direct consequence of cell contact with the beads or beads still attached to the cells, what according to StemCell should not occur (StemCell, 2013).

To further study how the function of monocytes could be affected by the StemCell beads, we cultured both monocytes (Miltenyi and StemCell) and induced their differentiation into DCs. After 7 days in culture with the appropriate conditions, the differentiation into DCs and their maturation status were assessed by the expression of MHCII. Concerning differentiation into DCs, it was effective in all the cases and cell death also showed similar values. However, differences of SSC profile were still visible (results not shown). Assuming that beads are lost during culture, the effects produced in the cells do not disappear. The main differences were for the maturation status of DCs derived from Miltenyi-monocytes and StemCell-monocytes. The maturation levels were clearly superior with StemCell, indicating a higher maturation status for DCs. This was a surprising result, and led us to contact StemCell staff; according to them, this increased activation should not occur either. However, another group of investigators also compared both kits and their results agree with ours, also revealing that DCs resultant from monocytes isolated with the StemCell kit were more mature. Moreover, they showed that by 6^{th} day of differentiation in culture StemCell-DCs already present a semi-mature state,

while Miltenyi-DCs are still imatture, as they should be. Furthermore, the advanced justification for this early maturation: EasySep beads are dextran-coated thus facilitating their capture by DCs via a mannose receptor and their internalization through an endocytic pathway (Mucci *et al.*, 2009).

IV.1.3. Cell preservation: shelf time and cryoprotectant removal

The first parameter to be taken into account was the shelf life of cells isolated and preserved in our laboratory conditions. Although we could gather some precious indications on this matter from StemCell, which is a referenced provider of primary cells, we had to do our own testing because not all the conditions are the same. They provide aliquots of 15-100 million cells in a 1.8 ml solution (8.3 x 10^6 PBMCs/ml in the case of the smaller aliquot), which is a higher concentration than the one tested by us (6.25 x 10^6 PBMCs/ml). Moreover, StemCell uses the same DMSO percentage as us (10%), but on the other hand uses more FBS (40%). Regarding storage procedure, they advise temperatures of at least -135 ºC or storing in liquid nitrogen, but in neither case for more than 6 months; they also refer that storing cells at -80 ºC should be avoided, but is acceptable for periods inferior to 1 month. The importance of using cells immediately after thawing is also approached (StemCell, 2010). In our laboratory we were able to keep PBMCs stored at -80 ºC for 6 months with maximum cell losses of approximately 50%. Moreover, the recovery rates were stable throughout the storing period, as the major cell loss is associated with the cryoprotective agent removal process. Overall, this is a good indicator to the efficiency of our protocols.

Regarding the protocols for DMSO removal, it was determined that immediate removal or after 24h did not make much difference. Also, there is no relevant advantage on using 110 x g, over 250 x g. By comparing our thawing protocol, for example to the one from StemCell, they have some differences, mainly the fact that the latter has 2 centrifugation steps, and those are performed at 200 x g, during 15 minutes. However, they opt to remove DMSO immediately after thawing, as is our standard procedure. Moreover, StemCell refers that cell loss may be up to 30% (StemCell, 2010), which is a lower value than the one obtained by us in the 6 month period. This higher cell loss obtained by us may be related to the fact that our cells were preserved at higher temperatures.

IV.2. Cost estimations

The cost estimations presented in the results section set the basis for a more profound study on the economic viability of the Cell Biology Service @ CEDOC. Off course, the economic viability study will entail many more variables such as number of service demands by the potential customers, the number of persons needed to fulfill service workload and their salaries, the general costs with reagents, equipment and their maintenance, overheads, financial resources, etc.

The results presented in this thesis concerning cost estimation are important when, having different strategies and brands, the investigator needs to make an informed decision on what to

choose. That choice is always dependent on the specific objective of the study to be performed, i.e., the intended use for the cells to be isolated.

IV.3. Cell Biology services divulgation

The steps taken in order to divulge the Cell Biology services were indeed crucial for its success. At this time, the Cell Biology services are fully functional, presenting a set of products mentioned in our on-line catalogue.

The Cell Biology services supply cells (PBMCs, T and B cells, monocytes) and sera isolated from human peripheral blood, using a highly flexible routine that is adapted to each client specific necessities. Cells can be provided either fresh or in culture, depending on the preference of the client; also, the size of each aliquot can be negotiated in order to better suit requirements from the client.

Mainly due to the extensive online divulgation of our services, fellow investigators can have access to the type of products available and the respective costs. Even the order can be easily performed on-line. Moreover, upon delivery, each product is accompanied by a certificate of quality that certifies it for research use. The products are certified by our laboratory after quality analysis performed to them; the certificate of quality presents data regarding donor characteristics (gender, age, blood type), date of blood collection and processing, amount of product being supplied, isolation method used and cell purity (determined by flow cytometry).

Furthermore, the Cell Biology services are available to offer immunological expertise in scientific collaborations.

The Cell Biology services are a platform under constant evolution. Overtime we expect to improve the products already supplied by us but also to develop new and original products. It is also our policy to practice a good quality-cost relation in order to have prices that are competitive in the market. Thus we hope to support other investigation groups in the pursuit of scientific breakthroughs.

IV.4. Concluding remarks and future work

The cells used in this work were the surplus of cells used for blood transfusions that are otherwise treated as waste.

The Cell Biology Services @ CEDOC is unique in Portugal, because it makes use of these human surplus providing reliable high quality blood products to be used for research purposes, either by the academic community or companies. In this context, this work has greatly contributed to the implementation of methodologies and standardized practices, essential in this type of services. The experience on cell isolation, culture and preservation is planned to be shared with the research community by different means, which include our online divulgation platforms. Moreover, this work gave some new and unexpected results concerning differences in monocyte isolations using two different widely known commercial brands. The differences in the maturation status of DCs obtained

from monocytes isolated by the two kits stresses the idea that each kit may have a distinct functional impact on the cells, and caution should be taken when choosing the most adequate.

In the future, we plan to further investigate if the phenotypic differences observed in cells isolated with kits from different brands will have an impact in cells functions, such as the ability for T cell activation and consequent induction of citotoxicity.

Besides, in order to further investigate the questions that arose during this work, we plan to continue improving our methodologies and laboratory practices to match and expand the demands of the researchers requiring our services.

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APPENDIXES

APPENDIX I

Blood Collection and Handling Process by the Portuguese Blood Institute (Instituto Portugês do Sangue - IPS)

All steps of the work developed for this thesis required the use of blood products. These were acquired from blood of healthy volunteers provided and ethically approved by the Portuguese Blood Institute (Instituto Português do Sangue - IPS).

Approximately 450-500 ml of blood from one donor are collected to an adequate plastic bag already containing 63 ml of citrate phosphate dextrose (CPD), which is a preserving and anti-coagulant solution (Fig.1; Optimal Blood Use Project, 2010).

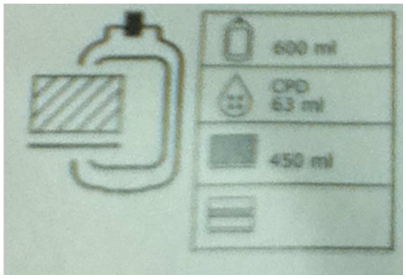

Fig.1 – Quantitative information of the blood bag contents. The total capacity of the blood bag is 600 ml; it contains 63 ml of CPD (anti-coagulant), and 450 ml of blood. This information is part of the label of each blood bag.

Then a serological control is performed for the following: Ac *Treponema pallidum*, Ag HBs, Anti-HBc, Anti-HCV, Anti-HIV 1/2, THIV/HCV/HBV (Fig.2); all of which should be negative for blood to be suitable for use. Usually, due to the duration of this process, we are only able to take the blood to our laboratory in the following day.

Each blood bag supplied for investigation purposes contains approximately 60 ml of blood and is generally designated as buffy coat. Each buffy coat is supplied with information such as the blood collection date, expiration date, results of the serological control and blood type of the donor; the gender and age of the donor may also be included if required (see Fig.2).

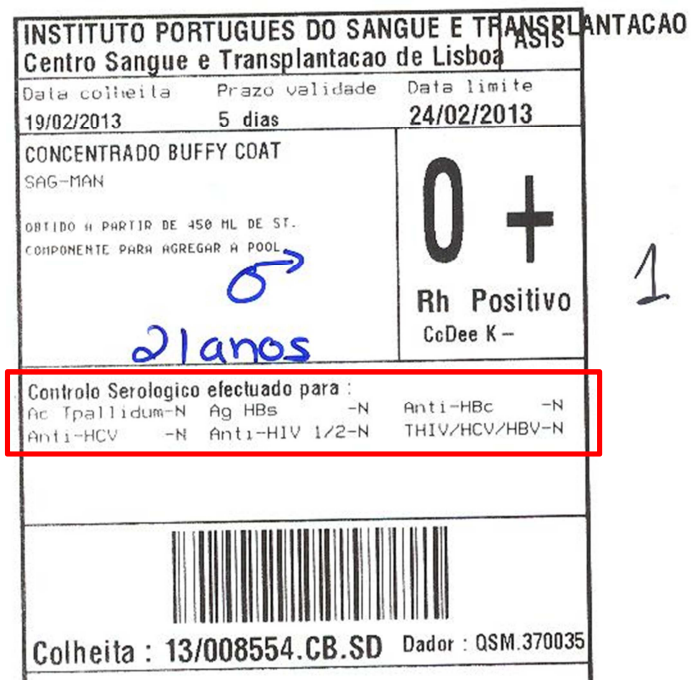

Fig.2 – Label from a blood bag. Each label contains information about the blood content of the bag, such as: blood collection date, expiration date, results of the serological control (identified in red); blood type of the donor and eventually, its gender and age.

Aiming to reduce variability as much as possible, the blood used in our laboratory was always collected in the previous day and corresponded only to male donors with ages between 18 and 65 years old. Age and blood type of each donor were always registered for statistical purposes.

APPENDIX II

Constitution of the buffer solutions and media used in this work

- **Phosphate buffer solution (PBS 1x):**
  Solution with 1.47 mM KH_2PO_4, 4.29 mM $NaHPO_4.7H_2O$, 137 mM NaCl e 2.68 mM KCL (pH=7.3) in distilled water.

- **Lyse solution:**
  Tris-HCl 0.17 M solution with 10% ammonium chloride 0.16 M (pH=7.65).

- **Recommended buffer for cell separation using the Miltenyi kits (beads buffer):**
  PBS 1x solution supplemented with 0.5% BSA and 2mM EDTA.

- **Recommended buffer for cell separation using the StemCell kits:**
  PBS 1x solution supplemented with 2% FBS and 1mM EDTA.

- **Culture media:**
  RPMI-1640 media supplemented with 10% FBS, 2 mM L-glutamine (R&D Systems), 100 µg/mL penicillin/streptomycin (Sigma), 1% non-essential amino acids (Gibco/Invitrogen), 1% sodium pyruvate (Gibco/Invitrogen).

- **Freezing media:**
  RPMI-1640 media supplemented with 20% FBS and 20% DMSO.

APPENDIX III

Abstract presented to the "XXXVIII Jornadas Portuguesas de Genética":

Cell Biology Services at CEDOC/FCM

Graça S. Marques, Inês Iria, Zélia Silva and Paula A. Videira

The Cell Biology Services at CEDOC is a non-profitable Cell Biology Platform that offers a range of affordable, custom-made services to the research community. The Platform offers isolation of various types of human blood products (cells and sera). Primary blood cells such as mononuclear cells, monocytes, B cells and T cells are delivered on time either fresh or frozen. Haematopoietic progenitor's differentiation into dendritic cells and macrophages is also available. The Platform is now expanding its services so as to offer genetic manipulation of primary cells.

The products are processed under rigorous sterile conditions and observing strict standard operation procedures resulting from our improved, time-tested team methodologies. During and after isolation procedures, the products are analyzed and quality-tested. This service makes use of assay techniques such as Flow Cytometry, Microscopy, among others. Here, we will give an overview of our standard techniques and present our recent data comparing different methods of cell isolation, and methods for transducing non-dividing primary cells. The impact of those methodologies will be evaluated regarding cell yield, purity, and cell functional characteristics.

A highly qualified and experienced team works, with gold standard criteria, to help researchers of several fields such as Immunology, Oncobiology or Genetics. With this Service we expect to establish collaborations with several Biotech companies and research groups, also to offer unique Cell Biology training to students and to develop in-house technology that may in the future lead to the setting of spin-off companies.