P-Glycoprotein functional activity in peripheral blood lymphocytes. Role of immunosuppressants, pharmacogenomics and alloimmune response

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CERTIFY

That Inés Llaudó Vallmajor, graduate in Biology by the University of Girona, has carried out under our direction, in the Laboratory of Experimental Nephrology from the University Hospital of Bellvitge, the research work to elaborate her Doctoral Thesis untitled **" P-Glycoprotein functional activity in peripheral blood lymphocytes. Role of immunosuppressants, pharmacogenomics and alloimmune response"** and through this writing they authorize its presentation to achieve the Degree of Doctor by University of Barcelona.

Núria Lloberas Blanch

Director of the Thesis

Josep Mª Grinyó Boira

Codirector of the Thesis

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INDEX

ABBREVIATIONS

ABC	ATP-Binding Cassette
ADP	Adenosin diphosphate
Ag	Antigen
APCs	Antigen presenting cells
ATP	Adenosin triphosphate
AUC	Area under the curve
BBB	Blood-brain barrier
BCRP	Breast cancer resistant protein
BSEP	Bile salt export pump
CNIs	Calcineurin inhibitors
CNS	Central nervous system
CNT	Concentrative nucleoside transporter
Cmin	Minimum concentration
CsA	Cyclosporine A
CYP	Cytochrome P450
DCs	Dendritic cells
ENT	Equilibrative nucleoside transporter
ER	Endoplasmic reticulum
FACS	Flow analysis cell sorter

FKBP	FK506 binding protein
HIV	Human Immunodeficiency Virus
iDC	Immature dendritic cell
IL-2	Interleukin-2
IL-6	Interleukin-6
IL-10	Interleukin-10
IFN-Y	Interferon gamma
LPS	Lipopolysaccharide
MDR	Multidrug resistance
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MLR	Mixed lymphocyte reaction
MRP	Multidrug resistance-associated protein
mTOR	Mammalian target of rapamycin
mTORi	mTOR inhibitors
NBD	Nucleotide-binding domain
NCT	Chronic allograft nephropathy
NFAT	Nuclear factor of activated T lymphocytes
NK	Natural Killers

OAT	Organic anion transporter
OATP	Organic anion transporting polypeptide
OCT	Organic cation transporter
PBMCs	Peripheral blood mononuclear cells
Pgp	P-Glycoprotein
Pi	Inorganic phosphate
Rapa	Rapamycin
Rho123	Rhodamine 123
SLC	Solute carriers
SNP	Single Nucleotide Polymorphism
SRL	Sirolimus (Rapamycin)
Tac	Tacrolimus
TAP	Transporter associated with antigen processing
TDM	Therapeutic drug monitoring
TMD	Trans-membrane domain
TNF-α	Tumoral necrosis factor
VP	Verapamil

INTRODUCTION

1. Introduction to drug transporters

The efficacy of many drugs depends critically on their ability to cross cellular barriers to reach their target. Lipophilic drugs may cross these barriers in the absence of specialized transport systems. On the other hand, hydrophilic and charged compounds often require specific transport mechanisms to facilitate cellular uptake and/or trans-cellular transport. The efflux mechanisms play a critical role in limiting the absorption and accumulation of potentially toxic substances and can effectively confer resistance to a diverse range of compounds in tumor cells. ATP-dependent drug efflux pump, P-glycoprotein (Pgp), has been the most widely studied regarding multidrug resistance in tumor cells [1-4]. Pgp was first described in hamster's ovary tumor cells, preventing access of anticancer agents to cells as a result of Pgp over expression [1, 5]. Pgp was just the first member of what is now a large and diverse super family comprising around fifty human ATP-binding cassette (ABC) proteins that perform many and different functions [6]. Of particular interest, family members that mediate drug transport, since these proteins can have a major impact on drug disposition and drug resistance to chemotherapy, as well as physiological homeostasis. These drug efflux proteins principally comprise the MDR (multidrug resistance) and MRP (multidrug resistance-associated protein) type transporters. Although these transporters tend to be over-expressed in tumors, their expression is widespread throughout many normal tissues, perhaps most notably in excretory sites such as the liver, kidney, and intestine, where they provide a formidable barrier against drug penetration, while providing a mechanism for drug elimination. The arsenal of ABC transporters that mediate drug efflux is supported by drug metabolizing enzymes, which modify drugs to yield metabolites that are themselves substrates for these transporters.

2. Transport of drugs across cellular barriers

The small intestine is the principal site of absorption. Oral administration is the most popular route for drug administration since dosing is convenient and non-invasive and many drugs are well absorbed by the gastrointestinal tract. As well as degrading and absorbing nutrients and solutes from the intestinal lumen, intestinal enterocytes form a selective barrier to drugs and xenobiotics. This barrier function depends largely upon specific membrane transport systems and intracellular metabolizing enzymes. The extent to which a compound is absorbed by the intestinal epithelium is therefore a critical factor in determining its overall bioavailability.

Transport into and across the cells of the human body is a prerequisite for the pharmacological action of drugs. Different pathways affect the transport of drugs across tissue barriers. The pathways can be divided in: 1) Passive trans-cellular transport; 2) Para-cellular transport; 3) Active efflux and 4) Active uptake (**Fig. 1**).

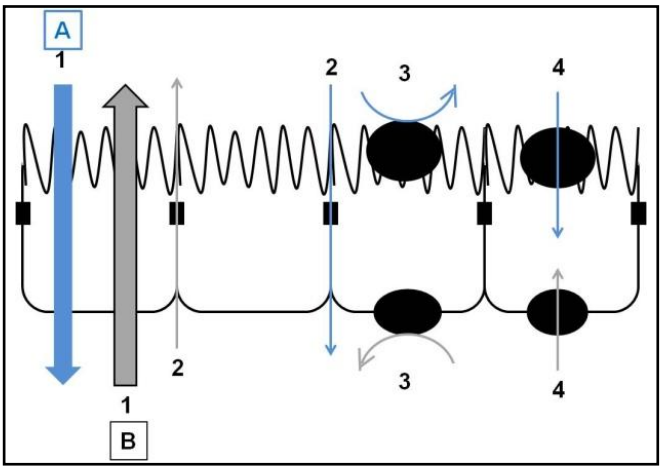

Figure 1. Drug transport pathways: 1) Passive trans-cellular transport. 2) Para-cellular transport. 3) Active efflux and 4) Active uptake. Passive

permeability can occur in absorptive (A) and secretory (B) directions, depending on local drug concentrations.

All of the transport processes may occur in both directions, depending on local drug concentrations (in the case of passive transport) and the directionality of the relevant transporter (in the case of active transport). Thus, drug transport proteins are expressed in both apical and basolateral membranes, and mediate active transport in absorptive and secretory directions.

To understand how drug accumulation can be reduced for instance in cancer cells and what an efflux transporter is, it is first necessary to examine how drugs get into cells. There are mainly two mechanisms of drug uptake. For water-soluble, hydrophilic drugs such as cisplatin, nucleoside analogue and antifolates, drugs cannot cross the plasma membrane unless they use the transporters or carriers, or enter through hydrophilic channels in the membrane. Resistance to such drugs resulting from decreased accumulation occurs because of individual mutations in the carriers, which produce single-agent resistance. For hydrophobic drugs, such as the natural products vinblastine, vincristine, doxorubicin, daunorubicin, actionmycin D, etoposide and paclitaxel, entry occurs by diffusion across the plasma membrane, without any specific drug carriers. The only way to keep such drugs out of the cells is by activation of energy-dependent transport system [7].

Using human genome sequencing, it has been estimated that approximately 500–1200 genes code for transport proteins [8]. The ABC transporters and the solute carriers (SLC) are the two dominating gene families among the plasma membrane transporters. ABC transporters utilize the energy from ATP hydrolysis to translocate their substrates across cellular membranes. Some members of the SLC family mediate

facilitated diffusion of their substrates along a concentration gradient, whereas others are secondary active transporters that use ion gradients to mediate concentrative substrate transport [9].

ABC and SLC transporters have the capacity to transport drug molecules. Based on their known and assumed tissue distribution, the activity of pumps has important pharmacological and toxicological consequences. They significantly affect pharmacokinetic processes such as intestinal absorption [10] distribution to the central nervous system (CNS) [11] and uptake into, and subsequent excretion from the hepatocyte [12] (**Fig. 2**). They belong to the following subfamilies: 1) the multidrug resistance protein (MDR); 2) the multidrug resistance-associated protein (MRP); 3) the organic anion transporter (OAT); 4) the organic anion transporting polypeptide (OATP); 5) the organic cation transporter (OCT); 6) the concentrative nucleoside transporter (CNT) and 7) the equilibrative nucleoside transporter (ENT) [13].

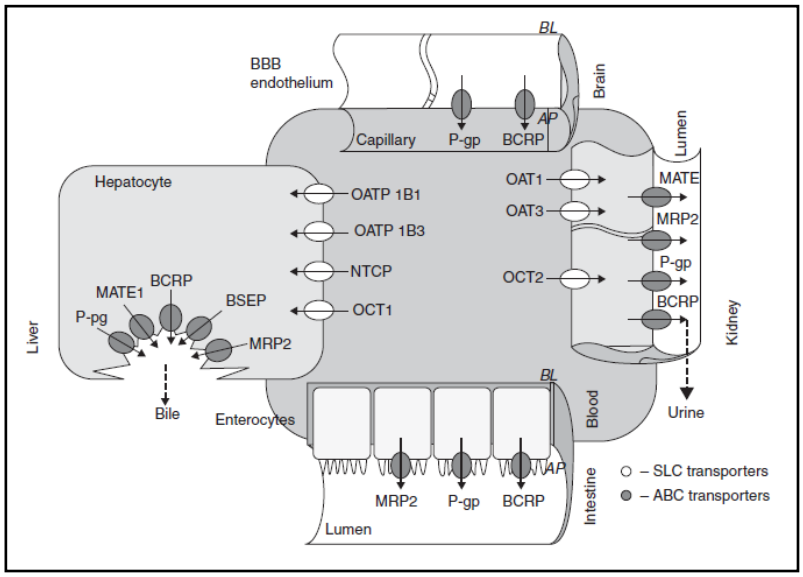

Figure 2. Localization of drugs transporters. Adapted from Y. Lai et al [14].

The first ABC transporter identified and characterized was Pgp or the product of the human MDR1 gene. Drug interactions involving transport pumps are particularly relevant for drugs with narrow therapeutic indices, while induction or inhibition of transport function can have a tremendous impact on drug efficacy and safety [15, 16]**.**

3. The ABC efflux proteins

3.1 Family of ABC transporters

The name of the ABC transporters was introduced in 1992 by Chris Higgins in an impressive review, a large group of proteins comprised of membrane transporters, ion channels, and receptors [17]. The ATP-binding cassette is found in a variety of prokaryotic and eukaryotic cells representing one of the largest and most diverse families of transport proteins. Based on sequence similarities, the human ABC protein family is categorized into seven subfamilies, labeled ABC A through ABC G, and consists of 49 members [18, 19]**.**

ABC transporters play a role in the transport of drugs and drugs conjugates. This role is exemplified by MDR1 (or Pgp), MRP1 (the multidrug resistance protein 1, ABCC1) and BCRP (breast cancer resistant protein, ABCG2) which can cause multidrug resistance in cancer cells. Some members of the MRP family mediate the export of drug conjugates. Others, MRP4 (ABCC4) and MRP5 (ABCC5) can transport cyclic nucleotides and nucleotide analogs. Mammalian secretory epithelia use ABC transporters to excrete endogenous metabolites. In the liver this compounds include bile salts, transported by BSEP (the bile salt export pump or ABCB11), phosphatidylcholine (ABCB4) and bilirubin glucuronides (MRP2 or ABCC2). Another ABC transporter is the transporter associated with antigen processing (TAP) which transports peptides for antigen presentation [20]**.**

Drug affinity of human ABC transporters is mainly found in the ABCB, ABCC and ABCG subfamilies (**Table 1**).

Table 1. Pharmacological important human ABC drug transporters.

Gene	Protein	Distribution	Location in plasma membrane	Preferred substrate type	Drug substrates	Patophysiology
ABCB1	MDR1/Pgp	Co,In,Li,Ki,Pa, Prox.tub, BBB	Apical	Hydrophobic and cationic	Digoxin, fexofenadine, indinavir, nelfinavir, ritonavir, saquinovir	Mutidrug resistamce
ABCC1	MRP1	Ubiquotious	Basolateral	GS-X, Gluc-X	Glucoronide, sulfate and glutathione conjugates.	Mutidrug resistance
ABCC2	MRP2	Ki,Li,In,BBB	Apical	GS-X, Gluc-X	Indomethacin, methotrexate, rifampin, doxorubicin.	Multidrug resistance and DJS
ABCC3	MRP3	Ki,Li,In,Co,Lu, Pa	Basolateral	Gluc-X, Sulf-X	Anionic glucoronide and glutathione conjugates, methotrexate	Mutidrug resistance
ABCC4	MRP4	Ubiquotious	Basolateral(Li) and Apical(Ki)	Gluc-X, Sulf-X	Adefovir,azathiopurine, methotrexate	Mutidrug resistance
ABCC5	MRP5	Ubiquotious	Basolateral	GS-X	Mercaptopurine, adefovir	Mutidrug resistance
ABCG2	BCRP	Li,In,BBB	Apical	Gluc-X, Sulf-X	Irinotecan,tamoxifen,imatinab	Mutidrug resistance

Co=Colon, In=Intestine, Li=Liver, Ki=Kidney, Pa=Pancreas, Prox.tub=Proximal tubules, BBB=Blood-brain barrier, Lu=Lungs, GS-X=Glutathione conjugate, Gluc-X=Glucuronide conjugate, Sulf-X=Sulfate conjugate, DJS=Dubin-Johnson Syndrome.

3.2 Role of ABC transporters

There is much interest as to how ABC transporters maintain such broad yet distinct substrate specificities. A model for Pgp function was described by [21]. The favored "hydrophobic vacuum cleaner" model predicted that Pgp pumps substrates either from the outer membrane of the lipid bilayer (e.g. before they cross the inner membrane leaflet to enter the cell cytosol) or from the inner membrane of the bilayer into the extracellular medium [22, 23].

The ABC transporters bind ATP and use the energy to drive the translocation of various substrates (ions, lipids, peptides, metabolites, chemotherapeutic drugs and antibiotics) across the plasma membrane as well as intracellular membranes of the endoplasmic reticulum (ER), peroxisome and mitochondria.

The discovery of the physiological role of ABC transporter proteins has offered a strong basis to rationalize some previously unexplained pharmacokinetic events. Moreover, Pgp and MRP proteins are becoming increasingly important to explain the pharmacokinetics of drugs.

Generally the drug transport is unidirectional. In eukaryotic cells the transport is exclusively in the exporting (efflux) direction, from the cytoplasm to the outside of the cell or intracellular compartments (ER and mitochondria). In prokaryotes, both ABC importers and ABC exporters have been identified.

The ABC transporters are distributed all over the organism, mainly in tissues with important metabolic activity and barrier function like the liver, kidney, lung, gastrointestinal tract, placenta, testis and bone marrow.

ABC transporters play several physiological roles and they are also related to human genetic disorders such as Tangier and Stargardt diseases, Dublin-Johnson syndrome and cystic fibrosis. Although some of the physiological roles are still unknown for many of the human ABC transporters, several have been described and exposed below.

The expression pattern suggests that Pgp plays a role in tissue protection and detoxification, with high expression in the apical membrane of intestinal enterocytes, in the bile canalicular membrane of the liver and in protective tissue barriers such as the blood-brain barrier (BBB), the blood-testis barrier and in the placental syncytiotrophoblasts that connect maternal and fetal blood circulation [24, 25].

In man and mammals, Pgp is found in the mucosal epithelium of the intestine, where it makes an important contribution to the direct excretion of transported compounds into the intestinal lumen [26] and is also a major determinant for the reduced uptake of orally administered compounds [27]. Most of the ABC transporter proteins, all homologues of either Pgp or MRP subfamilies, have been characterized in human hepatocytes where, by analogy with their intestinal function. Hepatocyte may be protected by returning hydrophobic toxic bile components to the bile. Pgp and MRP1 are also found in the epithelial cells of the proximal tubules of the kidneys, where they have both a direct excretory role for drugs. Both proteins have been found in epithelial cells of pancreas, along endothelial cells in small blood capillaries of the brain and of the testis, and in several other cells and tissues [28, 29].

One of the most important physiological role of Pgp, and possibly MRP1, is to prevent drug penetration in the brain [30]. The BBB maintains a nearly continuous physical barrier separating the brain compartment from the blood stream.

Pgp and MRPs proteins are becoming increasingly important to explain the pharmacokinetics of drugs. As a matter of fact, it has been shown that they have important consequences on the absorption, distribution, and clearance of many families of drugs, including the chemotherapic itself. The number of molecules (drugs, compounds of natural origin, food components, etc…) that have been shown to induce or modulate ABC transporter proteins is so huge that it is nearly impossible to keep track of all them.

More recently, data have also been accumulated on the expression and physiological relevance of ABC transporters in immune cells [31-37]. Apart from the protection function related to their capacity to extrude toxic compounds, various small inflammatory mediators such as prostaglandins, leukotrienes and cyclic nucleotides are among the natural substrates of ABC transporters. And this, strongly suggests additional regulatory functions of these pumps in immune cells [38-40].

4. MDR1 / P-glycoprotein (ABCB1)

4.1 General characteristics

Pgp is the most well characterized ABC transporter. Identified in the 1970s, Pgp was involved in cancer resistant cells against chemotherapeutic agents *in vitro* [41], and was subsequently found to be localized in blood capillaries in the brain [42]. Since then, plenty of literature has been published on the structure, mechanism, physiological and pharmacological roles of Pgp. Is encoded by the human multidrug resistance (MDR1, ABCB1) gene [43]**.** Human MDR1 spans over 100 kb on human chromosome 7q21. The MDR1 genome contains 29 exons that produce a 3843 bp sequence of transcripts encoding the 1280 amino acids Pgp protein with a molecular weight of 170 kDa [44]**.**

Pgp shows extremely broad substrate specificity, with a tendency towards lipophilic, cationic compounds. The list of substrates/inhibitors is continually growing and includes anticancer agents, antibiotics, antivirals, calcium channel blockers, and immunosuppressive agents. Naturally occurring substrates for Pgp include biologically active compounds found in normal diet, such as plant chemicals [45, 46], consistent with Pgp acting as part of a detoxification and excretion pathway.

4.2 Structure and transport mechanisms

There is a high sequence similarity between the two halves of the Pgp protein. Each halve consists of one hydrophobic trans-membrane domain (TMD) and one hydrophilic nucleotide-binding domain (NBD) which is located at the cytoplasmic face of the membrane. The two halves of Pgp interact to form a single transporter, and the major drug-binding domains reside in TMD domains 4,5,6,10,11 and 12 (**Fig. 3**). Pgp is glycosylated

at three sites in the first extracellular loop. The glycosylation appears to be required for the correct trafficking of the transporter to the cell surface, but is not required for the transport function of Pgp.

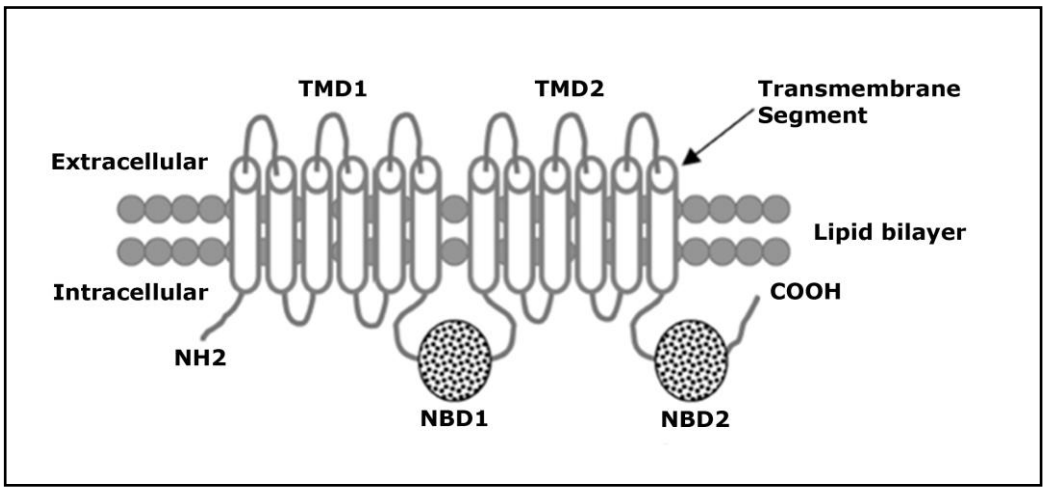

Figure 3. Diagram of a typical ABC protein in the lipid bilayer. The protein contains two TMD and the two nucleotide (ATP) binding domains (NBD). Adapted from S.Choudhuri and C. D.Klaassen [47].

ATP hydrolysis supplies the energy for active drug transport. In most ATP-driven transporters, ATP hydrolysis is tightly coupled to substrate transport, so that it is hydrolyzed only when substrate is concurrently transported.

Structural data in combination with biochemical and genetic studies of several ABC transporters have led to think about the mechanism of transport for Pgp as an ATP-switch model of function [48] **.** This model suggests a key in the two principal conformations of the NBDs: a) Formation of a closed dimer formed by binding two ATP molecules at the dimer interface and b) the dissociation of the closed dimer to an open dimer facilitated by ATP hydrolysis with release of Pi and ADP. Switching

between open and closed conformations of the dimer induces conformational changes in the TMDs necessary for transport of substrate across the membrane (**Fig.4**) [49].

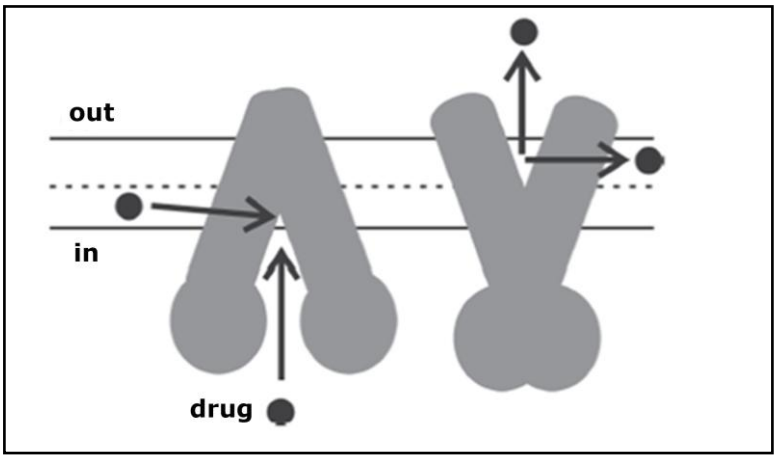

Figure 4. Internal-face (left) structure able to accommodate ligands from the cytoplasm and inner membrane. External-face (right) structure able to liberate ligands. Adapted from O.Wesolowska [50].

4.3 Tissue distribution and physiological role

Pgp is known to play a central role in the absorption and distribution of drugs in many organisms [51]. There is a different localization of Pgp in various tumors (where it confers the MDR phenotype) and at the apical surface of epithelial cells in several normal human tissues with excretory (liver, kidney, adrenal gland) and barrier (intestine, BBB, placenta, blood-testis and blood-ovarian barriers) functions [5, 25, 52]. Thus, suggests an important physiological role in cellular detoxification and protection of the body against toxic xenobiotics and metabolites by secreting these compounds into bile, urine and the intestinal lumen. In the end, it prevents their accumulation into the brain, testis and fetus (**Fig. 5**) [53].

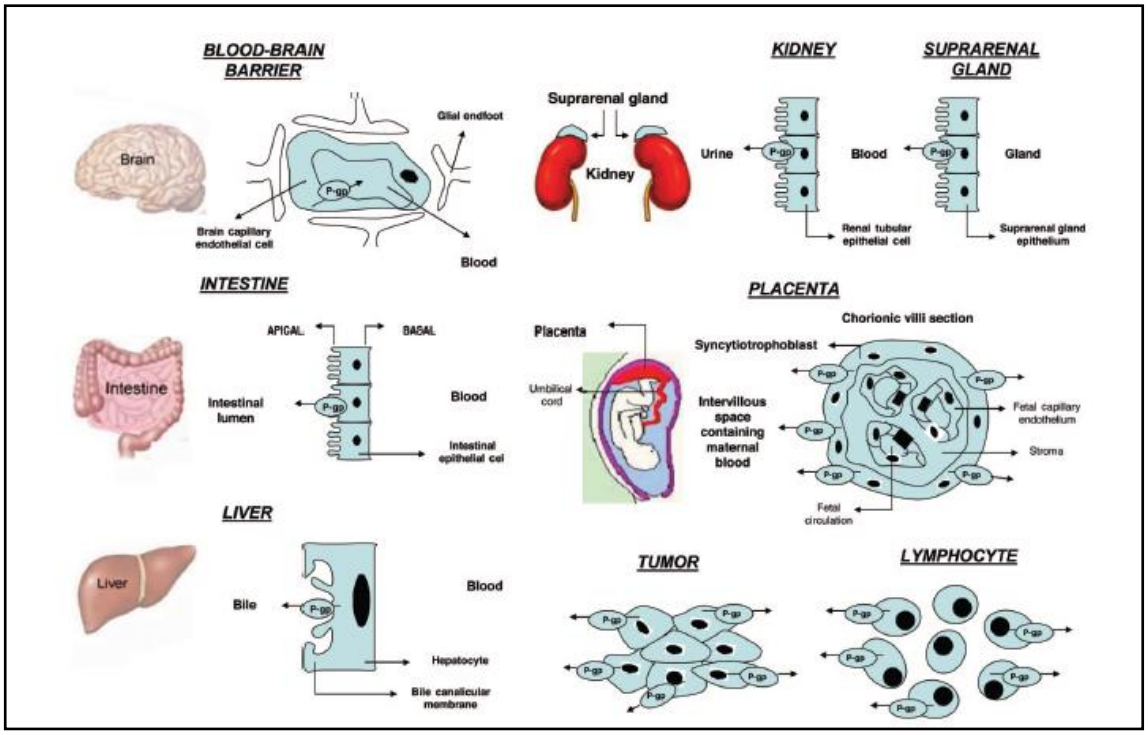

Figure 5. Schematic representation of the main sites of localization of Pgp in the body. Adapted from S. Marchetti et al [54].

4.4 Pgp substrates and Pgp inhibitors

One of the hallmarks of Pgp is its substrate promiscuity. Hundreds of structurally diverse compounds can interact with Pgp which are generally non-polar, weakly amphipathic compounds, and include natural products, anticancer drugs, steroids, fluorescent dyes, linear and cyclic peptides and ionospheres. Due to the nature of Pgp as an efflux pump for cell protection against a variety of substances, Pgp substrates vary in size, structure and function. Those substrates ranging from small molecules such as organic cations, carbohydrates, amino acids or some antibiotics to macromolecules such as polysaccharides and proteins (**Table 2**).

Although Pgp substrates are generally hydrophobic to amphiphilic compounds, there are no chemical characteristics that clearly distinguishe between Pgp substrates and non-Pgp substrates [55]**.** Even though, most Pgp substrates are lipophilic, uncharged or weakly basic, however, acid and hydrophilic substrates such as methotrexate have also been reported.

Potential physiological substrates for Pgp may include peptides, steroid hormones, lipids, and small cytokines, such as interleukin-2, intereukin-4, and interferon-α. However, there is little information on the extent to which endogenous compounds are transported by Pgp *in vivo*. Many drugs used in human disease treatments are Pgp substrates including anti-cancer drugs such as immunosuppressive agents, HIV protease inhibitors or antibiotics. Pgp can thus reduce the oral bioavailability of therapeutic drugs and the targeting of such drugs to the brain tissue, limiting the efficacy of treatment.

Because Pgp has an important role in drug resistance and drug pharmacokinetics, a number of studies have been undertaken to explain the molecular attributes required for interaction between Pgp protein and its small molecule substrates [56]. There are some physicochemical characteristics features such as lipophilicity, hydrogen-bonding ability, and molecular weight that contribute to a drug's binding ability to Pgp [57]**.**

Moreover, many Pgp drug substrates are also substrates of drug-metabolizing enzymes, particularly those from cytochrome P450 (CYP) 3A4. Both Pgp and CYP3A4 proteins act synergistically as a protective barrier in the bioavailability of orally administered drugs [58]. The overlap between CYP3A4 and Pgp substrates may have resulted in part from the coordinated regulation and tissue expression of CYP3A4 and

MDR1 in organs such as the liver and the intestine. This overlap can also explain why competitive inhibitors of Pgp, which are transported by Pgp, also inhibit CYP3A4. Pgp is also involved in the transport of drugs back to the lumen after passive absorption into the enterocytes. Therefore, the metabolism mediated by CYP3A4 in the intestine reduces the oral bioavailability of a drug by controlling the concentration of molecules entering the systemic circulation.

Co-administration of Pgp inhibitors with Pgp substrate drugs represents a potential strategy to overcome Pgp mediated drug resistance. However, clinical trials to date, which have focused exclusively on the use of Pgp inhibitors in combination with cytotoxic drugs (especially in resistant cancer treatments) have not proven to be successful due to pharmacokinetic and pharmacodynamic limitations [59].

Table 2. List of substrates and inhibitors of Pgp. Table modified from C. Marzolini et al [60], S. Choudhuri and C. D.Klaassen [47] and SF. Zhou [3].

SUBSTRATES
Anticancer agents: Actinomycin D, Daunorubicin, Docetaxel, Doxorubicin, Etoposide, Imatinib, Irinotecan, Mitomycin C, Mitoxantrone, Paclitaxel, Teniposide, Topotecan, Vinicristine **Antihypertensive agents:** Celiprolol, Losartan, Diltiazem, Talinolol **Antiarrhythmics:** Digoxin, Quinidine, Verapamil **Glucocorticoids:** Aldosterone, Cortisol, Dexamethasone, Methylprednisolone **Antibiotics**: Erythromycin, Levofloxacin, Rifampin, Tetracycline **Antiviral agents:** Amprenavir, Indinavir, Nelfinavir, Ritonavir, Saquinavir **Antidepressants:** Amitriptyline **Antiepileptics:** Phenobarbital, Phenytoin **Immunosuppressants:** Cyclosporine, Tacrolimus, Sirolimus **Antiacids:** Cimetidine, Ranitidine **Others:** Atorvastatin, Colchicine, Fexofenadine, Ivermectin, Loperamide, Mefloquine, Rho123
INHIBITORS
Antihypertensive agents: Carvedilol, Nicardipine, Reserpine **Antiarrhythmics:** Amiodarone, Propafenone, Quinidine, Verapamil **Antibiotics:** Clarithromycin, Erythromycin **Antiviral agents:** Indinavir, Nelfinavir, Ritonavir, Saquinavir **Antidepressants:** Fluoxetine, Paroxetine, Sertraline **Immunosuppressants:** Cyclosporine, Tacrolimus, Sirolimus **Opioids:** Methadone, Pentazocine **Others:** Atorvastatin, Bromocriptine, Dipyridamole, Emetine, Mefloquine, Progesterone

The Pgp inhibitors, also called modulators are able to reverse MDR in cells *in vitro*, by interfering with the ability of Pgp to efflux drug and thus generate a drug concentration gradient. The clinical importance to selectively block Pgp action aim to achieve more efficacious cancer chemotherapy, improve drug bioavailability and uptake in the intestine, or deliver drugs to the brain.

Numerous pharmacologic agents have been identified as Pgp modulators (**Table 2**). Both inhibitors and substrates present structural diversity. They appear to interact with the same binding site(s) as drugs, and compete with them for transport. Many inhibitors (*e.g.* verapamil, cyclosporine A (CsA), *trans*-flupenthixol) are themselves transported by the protein. Cells are generally not resistant to killing by modulators, but they are killed by MDR drugs in combination with inhibitors. The way in which inhibitors exert their action at the molecular level is still not well understood.

One of the major causes of the complications of drug therapy is drug-drug interactions. Some of the drug interactions can be explained by inhibition or induction of the transporter proteins. Several commonly used drugs are inhibitors of Pgp and thus are able to affect the pharmacokinetics of some clinically used drugs, which are substrates of Pgp [60].

There are two types of inhibitors, competitive inhibitors which compete for drug binding sites and non-competitive inhibitors which are involved in the blocking of the ATP hydrolysis process. Based on their pharmacological activity and potency to specifically inhibit Pgp, the different inhibitors can be classified into three generations [61] (**Table 3**).

Table 3. The three generations of Pgp inhibitors. Modified from C.A. McDevitt and R.Callaghan [62].

Class	Pharmaceutical name	Chemical class
1st Generation	Verapamil	Diphenylalkylamine Ca-channel blocker
	Cyclosporine A	Cyclic oligopeptide immunosuppressant
	Tamoxifen	Nonsteroideal anti-estrogen
2nd Generation	PSC833 (Valspodar)	Non-immunosuppressive derivative of Cyclosporine A
	VX-710 (Biricodar)	Derivative of FK-506-macrocyclic antibiotic
3rd Generation	GF120918 (Elacridar)	Acridonecarboximide
	LY335979(zosuquidar)	Cyclopropyldibenzosuberane
	XR9576 (Tariquidar)	Anthranilamide

Clinical trials have been performed with many Pgp blockers with the aim of suppressing Pgp in chemotherapy-resistance. The first generation of such agents did not end up in clinical practice, mostly because active concentration required to inhibit Pgp was so high that it resulted in host toxicity. Verapamil was one of the first agents used in clinical trials.

Studies with other first generation Pgp inhibitors such as tamoxifen and cyclosporine A also presented an inability to reach sufficient plasma concentrations to block Pgp and clinically significant toxicity profile [63, 64]. The limitation of the potency of this group has been addressed by the development of compounds that are less toxic and more effective as inhibitors.

Inhibitors of the second generation, including CsA analogues PSC833 and novel agents such as VX710 can be used to a concentration that can be reached in the plasma and are able to inhibit Pgp activity *in vitro*. [65, 66].

The most reliable clinical success was achieved with Pgp inhibitor, a cyclosporine A type molecule (ten times more potent than CsA) known as Valspodar (PSC833). However, this molecule is not an immunosuppressant and does not bind to cyclosphillin, the intracellular molecule to which cyclosporine A has to bind in order to initiate immunosuppression. Moreover, it is selective for Pgp (being practically inactive in other ABC transporter proteins such as MRP1). It shows acceptable systemic bioavailability. It is considered that PSC833 can achieve effective values in plasma in combination with cytotoxic agents without increasing the toxicity. Besides its use in cancer chemotherapy, PSC833 has the potential to increase exposure or to modulate the biodistribution of other chemotherapeutics, such as HIV protease inhibitors, to the brain.

However, to date, clinical trials have focused exclusively on the use of Pgp inhibitors in combination with cytotoxic drugs in treatment resistant cancer. Even though, these combinations have not proven to be successful due to pharmacokinetic and pharmacodynamic limitations [59, 67].

It has been described that the addition of Pgp inhibitors fails to improve the toxicity profiles of anticancer drugs. Although these agents inhibit multidrug resistance in tumor cells and are able to restore drug sensitivity, they could also inhibit the Pgp protection function in normal tissues (particularly for bone marrow and intestinal epithelium) where normal cells continue to grow. This phenomenon or unwanted pharmacokinetic interactions can be explained with the considerable overlap in substrate specificities with CYP3A4 [68]. A third generation of MDR modulators, have lacked the disadvantages of the previous molecules and are currently under investigation (for example; LY335979_Zosuquidar). There are some studies demonstrating

Zosuquidar as an effective pharmacological modulation of multidrug resistance.

For these reasons, structure-based strategies for new inhibitors drug design are an important field of research. The development of more specific and potent modulators of Pgp function may lead to a therapeutically useful role for Pgp inhibitors in the future.

5. Role of Pgp in immunosuppression

Transplantation is a life-saving treatment for patients with end-stage organ failure. With modern immunosuppressive drug therapy the incidence of acute rejection has been reduced significantly. Moreover, excellent short-term patient and graft survival are nowadays achieved in most transplant centers [69]. The everyday clinical use of immunosuppressive drugs is difficult and complicated by the fact that most agents display highly variable pharmacokinetics between individual patients. Second, immunosuppressive drugs have considerable toxicity and many patients suffer from side effects. The chance of an individual developing a specific adverse drug effect, however, differs markedly between patients.

Achieving therapeutic through level is very important during the initial period of transplantation, when the risk of rejection is greatest. Their low oral bioavailability is thought to result from the actions of the metabolizing enzymes CYP3A4 and 3A5 and the multidrug efflux pump Pgp. There are many immunosuppressants that share the same drug transporters and metabolizing enzymes, so Pgp and CYP3A are also responsible of many drug-drug interactions.

The immunosuppression protocols in post-transplant therapy typically include an anti-calcineurin and/or mTOR inhibitors (mTORi) and mycophenolate. As many immunosuppressants are substrates and/or inhibitors of Pgp, studies of Pgp could explain different drug exposure and a range of pharmacological interactions observed between various drugs in patients as the different associations with immunosuppressants drugs.

5.1 Calcineurin inhibitors (CNIs)

Although CNIs are potent immunosuppressive agents, the chronic nephrotoxicity of these drugs is the Achilles' heel of current immunosuppressive regimens. Other adverse effects are neurotoxicity, hypertension, hyperlipidaemia, alopecia, liver dysfunction, gastrointestinal symptoms, pancreatitis, and many more. Differences between the toxicity of CsA and tacrolimus (Tac) are well defined.

The toxic effect of the CNIs on the kidney is well known, although the mechanisms underlying it are not completely understood [70]. Data from kidney transplantation are difficult to interpret because the function of the renal allograft is influenced both by alloimmune injury and drug toxicity. CNIs nephrotoxicity has been clearly recognized after heart, liver and lung transplantation, as well as in patients with autoimmune disease [71]. Toxicity occurs in acute, potentially reversible, and chronic irreversible form. The basic mechanisms of the nephrotoxicity are closed linked to the inhibition of calcineurin and are therefore similar for CsA and Tac.

CsA and Tac are the most effective immunosuppressive drugs in the current immunosuppressive regimens, which target intracellular signaling pathways induced as a consequence of T-cell receptor activation [72]. Although they are structurally unrelated, they inhibit normal T-cell signal transduction essentially by the same mechanism. These drugs bind to an immunophilin (cyclophilin for CsA or FKBP-12 for Tac), resulting in subsequent interaction with calcineurin to block its phosphatase activity. Calcineurin-catalyzed dephosphorylation is required for movement of a component of the nuclear factor of activated T lymphocytes (NFAT) into the nucleus. NFAT, in turn, is required to induce number of cytokine genes, including that for interleukin-2 (IL-2), a prototypic T-cell growth and differentiation factor.

The effect of drugs on Pgp function and expression could be well studied in T lymphocytes. The use of native human lymphocytes instead of over expressed cell-lines is an advantage since they are the target of immunosuppression. Parasrampuria et al [73] showed the effect of CNIs in the expression and function of transporters in lymphocytes, thus changing not only the drug concentration but also the apparent efficacy of the drugs.

A. Cyclosporine A

CsA is produced by the fungus species *Beauveria nivea* and it is a lipophilic cyclic endocapeptide consisting of 11 amino acids. It inhibits the activation of the calcium/calmodulin-activated phosphatase calcineurin [74]. CsA suppresses some humoral immunity, but it is more effective against T-cell-dependent immune mechanisms such as those underlying transplant rejection [75].

CsA interacts with a wide variety of commonly used drugs. Is a well-known substrate and inhibitor of CYP3A4 and Pgp [76-78]. CsA has been reported to be among the compounds which interfere with the hepatic and intestinal uptake of some drugs via Pgp within others ABC transporters [79]. The mechanisms which CsA inhibits these transporters remains unclear.

B. Tacrolimus

Tac is a macrolide antibiotic produced by *Streptomyces tsukubaensis* [80]. Tac has much greater potency than CsA. While Tac is 30-100 times more potent than CsA *in vitro*, maximal inhibition of calcineurin phosphatase *in vivo* has been shown to be greater with CsA than with Tac [81, 82].

Since Tac is metabolized mainly by CYP3A4 and interferes with the uptake and efflux transporters almost in the same way as CsA, the potential interactions described above for CsA also apply for Tac [83]. However, not all drug-drug interactions will be of clinically importance since Tac is administrated in a lower concentration compared with CsA.

5.1.1 Pharmacokinetic properties of CNIs

CsA and Tac have similar physiochemical properties and elimination pathway. Both are substrates for the CYP family of enzymes and Pgp transport system.

The effective clinical use of the CNIs depends on the pharmacokinetics. Anglicheau et al [84] demonstrate that patients with strongly expressed MDR1 required higher tacrolimus doses to achieve similar concentrations to those patients with weak expression. These results suggested that Pgp expression plays a critical role in the ability of the gut to absorb tacrolimus.

An orally administered CNI is subject to be transported by Pgp and metabolism by CYP450 3A. Variation in the activity of these two systems results in differences between individuals in the oral bioavailability of the CNIs [85]. Furthermore, the administration of drugs or other substances that alter Pgp and CYP450 activity can change bioavailability of the CNIs [86]. CsA has a greater primary volume of distribution and clearance rate, but no significant difference in bioavailability, absorption rate, and elimination rate as compared to Tac [87]. Following absorption, the CNI is subject to further "first pass" metabolism by CYP450 in the liver before reaching the systemic circulation [88]. The resulting metabolites are primarily excreted in the bile, although a small proportion enters in the

circulation. Although the CNIs are metabolized in the liver, dose adjustment may be needed if a patient develops renal failure.

When administered intravenously, the CNI will avoid "first pass" metabolism in the intestine and liver and directly enter the systemic circulation. Consequently, the intravenous dose required to achieve any blood concentration is considerably lower than the oral dose [89]. Drug elimination from the systemic circulation is via hepatic metabolism. (**Fig. 6**).

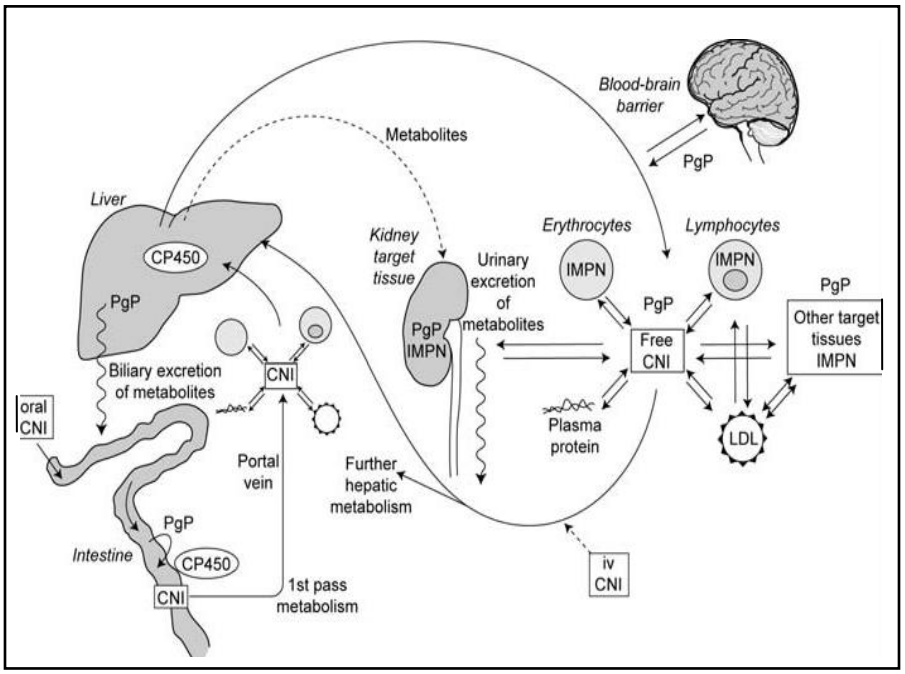

Figure 6. Pharmacokinetics of the calcineurin inhibitors. Adapted from NR.Banner et al [89].

Only a small proportion of CNI is unbound in the blood. The majority is associated with its binding proteins within red cells or bound to plasma proteins (in the case of Tac) or lipoproteins (in the case of CsA) [90].

Activity of Pgp transporter will determine the relationship of the intra to extracellular drug concentration [91].

The majority pharmacokinetic drug interactions involving the CNIs are based on either induction or inhibition of CP450 3A [89]. However, it is also seen that alterations in Pgp activity contribute to these effects. Pgp can alter the drug's immunosuppressive and toxic effect independently from the total concentration measured in blood, by altering the distribution of drug within compartments [86]. Pharmacodynamic interactions also occur where the toxicity of other drugs are additive or synergistic with those of the CNIs.

The relationship between the total concentration measured in the blood and the concentration at the site of action within the cell can be affected by the factors that influence the distribution of the drug. Drugs that affect Pgp transport system may influence this equilibrium. Thus, could alter the drug's concentration at its intracellular site of action, thereby modulating both immunosuppressive efficacy and toxicity [92]. This leads to think that it could be one of the mechanisms which explain the increase in CsA nephrotoxicity that has been observed when used with Rapa (mTORi) [93, 94].

5.2 mTOR inhibitor (mTORi)

The mammalian target of rapamycin (mTOR) has an important role in the immune response. mTOR inhibitor suppress T-cell activation and proliferation and are effective immunosuppressants. Rapamycin (Rapa) also known as Sirolimus, has been used clinically as a preventive treatment of graft rejection in renal transplantation as part of calcineurin inhibitor avoiding regimens.

Rapa is a macrolide antibiotic produced by *Streptomyces hygroscopicus* from Easter Island (Rapa Nui) [95]. It was initially proposed as an anti-fungal agent but subsequently developed as an immunosuppressive drug. mTOR inhibitors prevent progression of the cell cycle from the G1 to the S phase and thus block proliferation of T cells [96]. Rapa binds to FKBP12 to create complexes that engage and inhibit the target of Rapa but cannot inhibit calcineurin. It has been developed for use with CsA, but the combination increased nephrotoxicity, hemolytic-uremic syndrome, and hypertension [93]. It has been combined with Tac to avoid the toxicity of Rapa-CsA combinations [97]. However, it has been demonstrated that Rapa plus Tac produced more renal dysfunction and hypertension than did mycophenolate mofetil plus Tac [98]. This indicates that Rapa potentiates Tac nephrotoxicity.

Rapa is metabolized by the intestinal and hepatic CYP3A enzymes [99] without contribution of the metabolites to the pharmacological activity of the drug. It is a Pgp substrate, which may limit their intestinal absorption. The first elimination route of mTORi is through the bile. Hepatic extraction may not involve active transporters. In the kidney, Pgp is not expected to play a significant role in the pharmacokinetics of mTORs inhibitors as renal elimination is not their first disposition pathway. However, its inhibition by Rapa enhances the nephrotoxicity of CsA when the two drugs are co-administered [84]. That's why in clinical practice, the use of mTORi has not been associated with improved long-term graft survival [100]. This may be in part because they are first used in combination with drugs that inhibit their tolerogenic properties.

6. Pharmacogenetics of Pgp

During the last decade pharmacogenetic research in transplantation medicine has focused on drug-metabolizing enzymes and drug transporters [101]. In this context, proteins belonging to ABC transporter family have received considerable attention because they play an important role in the absorption, distribution, and elimination of many of the immunosuppressive drugs in use today.

6.1 Single Nucleotide Polymorphisms (SNPs) and drug interactions

In a large patient population, a medication that is proven efficacious in many patients often fails to work in some other patients. Furthermore, when it does work, it may cause serious side effects, even death, in a small number of patients. To understand the origin of individual variation in drug response is difficult. It is well known that large variability of drug efficacy and adverse drug reactions in patients is a major determinant of the clinical use, regulation, and withdrawal-from-market of clinical drugs.

It is estimated that genetics account for 20 to 95% of variability in drug disposition and effects [102]. Even though there are many non genetic factors like age, organ function, concomitant therapy, drug interactions and the nature of the disease that have an influence on the effects of medications. The most common type of genetic variation is the SNP or polymorphism from one single base. It consists of a base substitution by other in a DNA sequence that occurs in a significant proportion (more than 1%) of a large population.

At the present time, there are many examples in which inter-individual differences in drug response are due to sequence variants in genes

encoding different processes related to drug exposition as drug-metabolizing enzymes, drug transporters or drug targets [103].

Many proteins interact with the drug, affecting its transport throughout the body, absorption into tissues, metabolism into more active forms or toxic products, and excretion. If a patient has SNPs in any one or more of these proteins, they may alter the time the body is exposed to active forms of the drug or any of its toxic products.

The genome of each individual contains its own pattern of SNPs. In the future, the most appropriate drug for an individual could be determined in advance of treatment by analyzing a patient's SNP profile. The ability to target a drug to those individuals most likely to benefit it's called "personalized medicine".

6.2 Polymorphisms in the human MDR1 gene

Over the last 10 years more than 50 single-nucleotide polymorphisms (SNPs) have been identified in ABCB1 (previously known as MDR1), the gene encoding for ABCB1 [104]. MDR1 gene is located on chromosome 7 and contains 29 exons numbered from 1 to 28 with a total length of 209 Kb (**Fig. 7**). Some of the ABCB1 SNPs have been associated with altered expression of Pgp. Silent mutations or synonymous SNPs are located in introns close to exon limits and wobble positions that do not lead to amino acid changes. Nonsynonymous SNPs refer to polymorphisms that result in amino acid changes.

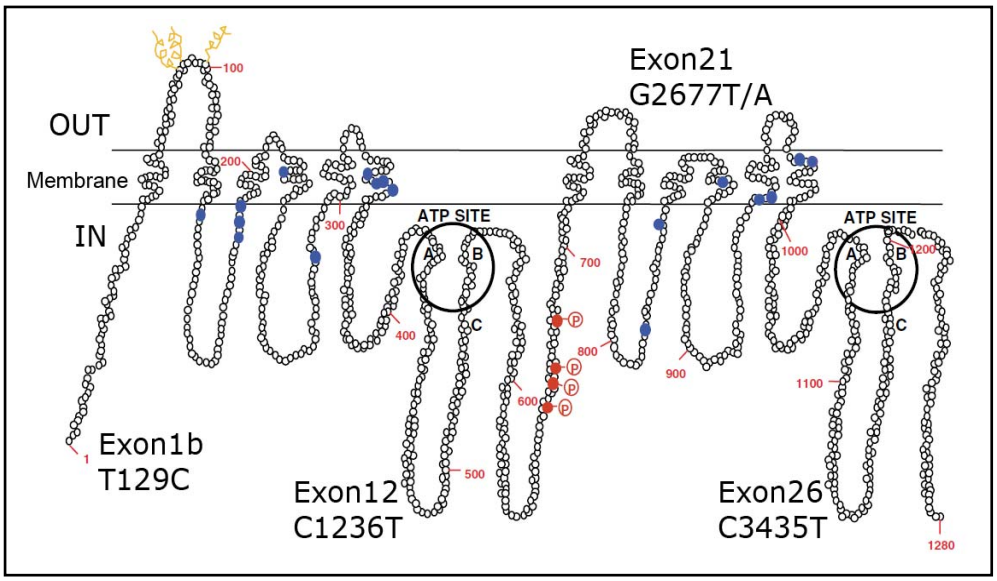

Figure 7. Schematic representation showing the distribution of MDR1 SNPs. Modified from SV.Ambdukar et al [105].

The SNPs that have been studied most widely are the C to T transition at position 3435 within exon 26, the C to T transition at position 1236 within exon 12 and the G to T/A transition at position 2677 within exon 21 [47, 106, 107]. These three SNPs are in strong linkage disequilibrium and their allelic frequency varies between different ethnic groups [108]. The first SNP reported in healthy individuals was the G2677T which was the only one that results in an amino acid substitution from Ala to Ser, whereas ABCB1 3435C>T and 1236C>T are synonymous SNPs.

The functional impact of these three SNPs is not clear *in vivo* although *in vitro* the ABCB1 3435C>T SNP has been associated with reduced mRNA expression [10] and stability [109], and more recently, with changes in substrate specificity [110]. This SNP was found to be associated with the expression of Pgp in the intestine [10, 111]. Allelic differences in individual MDR1 gene sequences may be associated with,

or even causative for different expression levels. The 3435T allele was associated with 2-fold lower levels of Pgp in the duodenum, and resulted in 50% higher plasma concentration of digoxin. The explanation for this observation is that less Pgp on the apical surface of the membrane remove less drug from the cells, resulting in increased bioavailability. Anglicheau et al postulated that these polymorphisms are associated with tacrolimus pharmacokinetic variations in renal transplant recipients [112]. The most important relation was noted for the 2677G > T/A SNP, as the Tac dose requirement was 40% higher in homozygous than in wild-type carriers. However, the impact of the 2677G > T/A SNP was validated by the haplotype analysis which included the 3435C>T, suggesting that these two SNPs may have separate roles on Pgp function. The significance of the 1236C>T on ABCB1 expression and function remains controversial and has been the topic of several recent review articles [3, 113].

Other studies have shown that SNP at exon 1b (T129C) may be also associated with altered transporter function or expression. Tanabe et al [114] has found in human placentas from patients with TT genotype in exon 1b, significantly higher Pgp levels in comparison to the group with CT genotype. Koyama et al [115] found that T129C, but not G2677T/A and C3435T, was associated with the lower expression of MDR1 mRNA in colorectal adenocarcinomas being a useful marker of prognosis. No data on the influence of this SNP (T129C) on the pharmacokinetic of immunosuppressants has been published.

6.3 Haplotype analysis of MDR1 polymorphisms

The contradictory findings of some authors [112-114] may indicate that genetic variation of *ABCB1* 3435C>T is not the causal modulator of any of the observed functional differences. Therefore, it is probably that

functional differences arise from SNPs in linkage disequilibrium with other functional polymorphisms, including the *ABCB1* 2677G>T/A SNP, suggesting that functional effects of genetic variants in the *ABCB1* gene should be considered as haplotypes rather than independent SNPs. Haplotypes are sets of single nucleotide polymorphisms along the chromosome. Theoretically haplotype analysis should increase the power to detect *cis*-interactions and associations with SNPs that have not been genotyped. Further analysis has been based on haplotypes rather than genotypes. However, haplotype analysis will only provide more power compared to single locus tests, if the functional variants occurs on the same haplotypic background and the effect of the haplotype is bigger compared to a single genotype. Linkage studies and the resulting haplotypes are powerful tools for screening large populations.

There are several studies that found strong linkage disequilibrium of the three highly frequent polymorphisms located in exon 12 (position 1236) exon 21 (position 2677), and exon 26 (position 3435). Similarly, genotypic combinations of MDR1 polymorphisms in exons 26, 21 and 12 appeared to have strong linkage disequilibrium at the three loci in more than 50% of the studied individuals [116, 117].

Individuals who are homozygous for the reference allele had approximately a 40% higher AUC value of fexofenadine, an ABCB1 substrate, compared to those who are homozygous for the variant allele. Recent work also indicates that the use of ABCB1 haplotypes is superior to un-phased SNP analysis to predict the pharmacokinetics of digoxin [117], cyclosporine [118], and fexofenadine [119]. Furthermore, it is suggested that there is a correlation between the haplotype of these three SNPs and intestinal expression of ABCB1 mRNA [111]. Assessing haplotypes in the ABCB1 gene and consideration of their interethnic

differences in future investigations will provide greater power to detect associations with functional differences [106, 118, 120, 121].

Kim et al [116] found a linkage between the two synonymous SNPs (C1236T and C3435T) and a nonsynonymous SNP (G2677T). Tang et al [120] showed high frequencies and strong linkage disequilibrium of these polymorphisms C1236T, G2677T/A and C3435T, in three ethnic groups: Chinese, Malays, and Indians. Other researchers found strong linkage disequilibrium between only two of these three coding polymorphisms [114, 117]. Significant linkage disequilibrium was also confirmed by Furuno et al [122], between the polymorphisms located in positions 2677 and 3435. A study done by Illmer et al [123] on 405 acute myeloid leukemia patients detected linkage disequilibrium of these known polymorphisms in exons 21 and 26, and found high probability of relapses in those patients carrying this haplotype.

A number of clinical haplotype studies have been performed, though many are limited by small sample size, resulting in few patients per haplotype. As such, the large sample sizes required to determine significance of the numerous possible haplotypes has limited studies analyzing any significance of haplotypes on pharmacokinetics. The effect of MDR1 polymorphisms on drug disposition may be further complicated by differences in variability and structure of haplotypes between ethnic groups. Significant differences in the frequencies of MDR1 haplotypes have been found among Caucasian, African-American, and Asian-American population as well as the identification of unique haplotypes in ethnic populations [106].

6.4 MDR1 polymorphisms as pharmacogenetic biomarkers for kidney transplantation

MDR1 genotypes are associated with alterations in the pharmacokinetics of drugs that are substrates for Pgp. In recent years evidence has accumulated that both the inter-individual variability in immunosuppressive drug pharmacokinetics, their efficacy, as well as an individual's susceptibility to drug toxicity have an important genetic basis. Studies investigated the contribution of MDR1 polymorphisms on disposition of the immunosuppressive drugs involving Tac, CsA and Rapa. However, nowadays there is still a lot of controversial results of the association.

A. Influence of the ABCB1 SNPs on CNIs

CsA and Tac display a high inter-individual variability in their pharmacokinetics, which is most marked during their absorption. The CNIs have been the subject of extensive pharmacogenetic research in transplantation medicine. ABCB1 polymorphisms may directly influence the efficacy or toxicity of calcineurin inhibitors. As a result, a large number of genetic association studies investigating the relationship between various ABCB1 SNPs and CNI disposition have been performed [112, 124-133]. Overall, the results of these studies have been disappointing as they have identified only a limited effect of ABCB1 SNPs on CsA and Tac disposition.

Other ABC transporters such as ABCC2 also have a potential role on the bioavailability of CNIs although being not clear. Despite the fact that the evidence for an important role of ABCC2 in the disposition of CNIs is limited, several investigators studied the association of ABCC2 SNPs and immunosuppressive drug pharmacokinetics [127, 134, 135].

B. Influence of the ABCB1 SNPs on mTORi

Like Tac and CsA, mTOR inhibitor, Rapa is a substrate of ABCB1 and therefore it's pharmacokinetics may also be affected by genetic variations in ABCB1 [136]. Pharmacokinetics data obtain from Rapa suggest either a large inter-individual variability or a narrow therapeutic index. Furthermore, considerable toxicity and therapeutic drug monitoring (TDM) is routinely performed [137]. Analogous to CNIs, a number of studies with the aim to investigate the role of ABCB1 SNPs in the inter-individual pharmacokinetic variability of mTOR inhibitors has been performed [138, 139]. Anglicheau et al [140] were the first to evaluate the association between ABCB1 SNPs and Rapa pharmacokinetics and no association between ABCB1 SNPs and Rapa concentration/dose ratio was found.

7. ABC transporters far beyond an efflux pump. Role of Pgp in alloimmunity.

At the present time, the role of Pgp in cellular immunity has been discussed. In recent years, some data have been accumulated on the expression and physiological relevance of ABC transporters in immune cells [31-36, 141]. Their expression on hematopoietic cells was also recently reviewed by Kock et al [142]. Although expression of these transporters on immune cells has not yet been linked to specific physiological functions in all cases, recent reports provide evidence of the involvement of these pumps in the development and functionality of T-cells and dendritic cells (DCs) [143-145] (**Fig. 8**).

Immune effector cells that belong to the innate response are NK cells, granulocytes, and APCs such as macrophages and DC. These cells are responsible for neutralization of pathogens or direct killing of pathogen-infected cells and the production of chemokines and cytokines to attract cells of the adaptive immune response such as T and B cells, which can eliminate infected cells via cell-cell contact or secreted antibodies.

Besides acting as drug pumps extrusion, the ABC transporters also play an important role in the development, differentiation, and maturation of immune cells and are involved in migration of immune effector cells to inflammation sites. The relevance of ABC transporters for immune functions raises the issue of how the combined administration of therapeutic drugs and ABC transporter antagonists would influence the survival and physiological functions of immune cells during treatment.

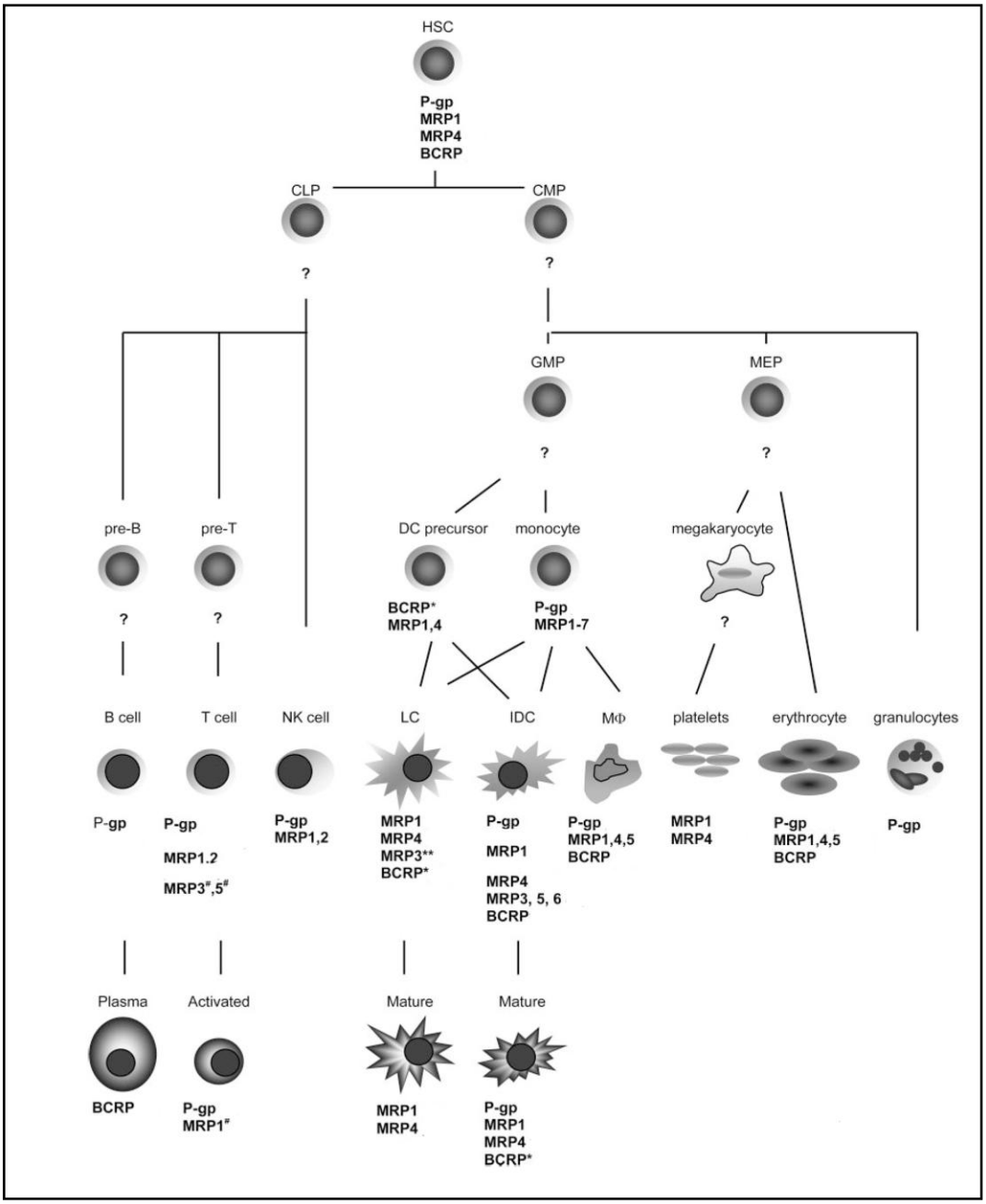

Figure 8. Schematic overview of the expression of ABC transporters on different subsets of immune effector cells. Adapted from Rienke van de Ven et al [146].

Pgp function is involved in alloimmune T-cell activation via both T-cell and antigen presenting cell-dependent mechanisms, which is relevant to the field of clinical transplantation, where Pgp has been found to be a marker of acute and chronic allograft rejection. Current *in vitro* findings raise the possibility that Pgp may represent a novel therapeutic target in the alloresponse.

7.1. Pgp expression and function in T cells

Different T cells subsets or activation states are associated with distinctive patterns of Pgp expression and activity, which could be relevant for their functions. It is known that Tregs are extremely sensitive to chemotherapy suggesting that these cells probably express low levels of drug efflux transporters [147] but the specific role of ABC transporters in these cells have still not been well reported.

Klimecki et al [148] analyzed MDR1 mRNA expression, finding Pgp highly expressed in NK cells ($CD56^+$) and suppressor cells ($CD8^+$), moderately in $CD4^+$ T cells and B cells ($CD19^+$), and at low levels in monocyte cells ($CD14^+$). Because Pgp is expressed on T lymphocytes, it could affect immune responsiveness and pharmacologic response to many drugs in the transplant setting.

These findings by multiple investigators provide a consistent picture of expression of Pgp on bone marrow-derived lymphoid progenitor cells and, to lower degrees, on differentiated resting human T-cell subsets, including $CD4^+$ and $CD8^+$ T cells. Moreover, the demonstrated presence of MDR1 mRNA in $CD14^+$ monocytes, the detection of surface Pgp expression on these cells by two distinct anti-Pgp mAbs and the observed dye efflux capacity of these cells establish that Pgp is functionally expressed in APCs.

Although Pgp has been widely studied in T cells, some apparent discrepancies were reported regarding its expression. For instance, some studies report an increase in Pgp expression upon T cell activation [149, 150], whereas other studies reported a decrease [151, 152].

Several authors have described Pgp regulation in the course of T-cell activation. Gupta et al [150] assessed the percentages of Pgp in T-cell subsets with and without polyclonal phytohemagglutinin (PHA) stimulation. From 3% of $CD4^+$ cells and 10-20% of $CD8^+$ T cells, upon PHA stimulation they found that 16-40% of $CD4^+$ and 54-100% of $CD8^+$ cells expressed Pgp.

Mu et al [153] also investigated the relationship between T-cell activation and Pgp expression. Flow cytometry showed 0.7% of lymphocytes expressing Pgp among the resting T-cell population, augmented to 3.3% expression after 24 hours of PHA-mediated mitogenic stimulation. However other studies, PHA stimulation did not significantly augment $CD4^+$ T-cellular Pgp expression at 24 hours, as assessed by flow cytometry. In addition, stimulation with allogeneic APCs did not significantly increase Pgp expression by $CD4^+$ T-cells at 24 hours. [141]. These studies indicate that Pgp is expressed by both resting and activated T cells, and that prolonged polyclonal stimulation can significantly enhance Pgp surface expression on both $CD4^+$ and $CD8^+$ human T cells. These findings have generated more interest of the role of Pgp in normal immune function.

An important event in organ transplantation is the allorecognition, which is T-cell recognition of alloantigen, in particular antigens of the major histocompatibility complex (MHC). It has been demonstrated that $CD4^+$ T-cell are essential for initiating allograft rejection. Since it is known that Pgp is implicated in different ways in T cells and APCs *in vitro*, it is

quite probably that also has an important role in direct and indirect allorecognition *in vivo* [154]. And thus, may be relevant to the processes of both acute and chronic allograft rejection.

Pgp function on peripheral lymphocytes may have important implications for the use of immunosuppressive drugs post-transplant and immunoresponsiveness after transplantation. Further research is warranted to determine the clinical impact of Pgp associated activity on transplant outcomes. High Pgp activity in T lymphocytes could result in reduced intracellular drug exposure, rendering these cells resistant to therapy in the present of adequate plasma drug concentrations.

In human T cells, Pgp function has been implicated in cytokine secretion, T cell survival and differentiation, and cytotoxic T cell effector function [155]. The Pgp inhibitors blocked IL-2 release, suggesting that Pgp functioned at the post-transcriptional level of cytokine transport and secretion. On the other hand, other studies find an anti-apoptotic role of Pgp in human T cells. Such a role of this molecule can also be seen in other cell types where Pgp has been described to confer resistance to caspase-dependent but not caspase-independent apoptosis [156, 157].

It is also being defined the role of Pgp in cytokine production as a result of alloimmune stimulation [141]. MLR (mixed lymphocyte reaction) induced T-cell proliferation. Selective blockade of Pgp in purified $CD4^+$ T-cell population prior to MLR co-culture, resulted in inhibition of IFN-Y and tumor necrosis factor (TNF-α) production, demonstrating a direct involvement of T cellular Pgp in alloimmune activation. Another mechanism by which $CD4^+$ T-cells Pgp may regulate T-cell activation and IL-2 production, relates to a function of the molecule in IFN-Y-dependent mechanisms of Th1 cell differentiation.

Pgp represents an immunomodulatory molecule and potential target for immunotherapy [36, 155, 158]. The potential role of Pgp as a regulator of in vivo alloimmunity, strongly suggested by previous *in vitro* findings, has not been investigated to date.

7.2. Pgp expression and function in dendritic cells

Expression of ABC transporters on DCs was first reported by Randolph et al [145], who detected Pgp and MRP1 expression on human (and mouse) DCs [32, 159]. Some researchers reported that Pgp is expressed and active at the plasma membrane of monocyte-derived DCs [36], others could detect Pgp expression but no functional activity [160] or found no expression or functional activity [33]. These discrepancies might be because of donor variability, isolation procedures for $CD14^+$ monocytes (for instance, plastic adherence vs. magnetic sorting), the use of different anti Pgp antibodies, Pgp antagonists or substrates or most likely a combination of these factors.

The most potent APCs are DCs, which are often considered as the bridge between the innate and adaptive response. They have the capacity to stimulate naive T cells and initiate primary immune responses. Originating from bone marrow-derived progenitor cells, immature DCs (iDC) reside in peripheral tissues, surveying their environment through capturing and processing antigens (Ag). When antigens are internalized, DC further mature, as reflected by augmented expression of distinct chemokine receptors and co-stimulatory molecules facilitating their migration to lymph nodes and T cell triggering, respectively.

Pgp and other ABC transporters such as MRP1, TAP or MRP4 have the ability to transport immune mediators across the plasma membrane of DCs. The effects of these ABC transporter substrates are very diverse;

many of them can either promote or suppress immunity, depending on the location of secretion and the activation status of the cells that are in their proximity.

Given the number of immunological substrates that can be secreted through the various ABC transporters, it is important to investigate when and where specific ABC transporters are expressed on DCs and how this potentially impacts the immune response (**Fig. 9**).

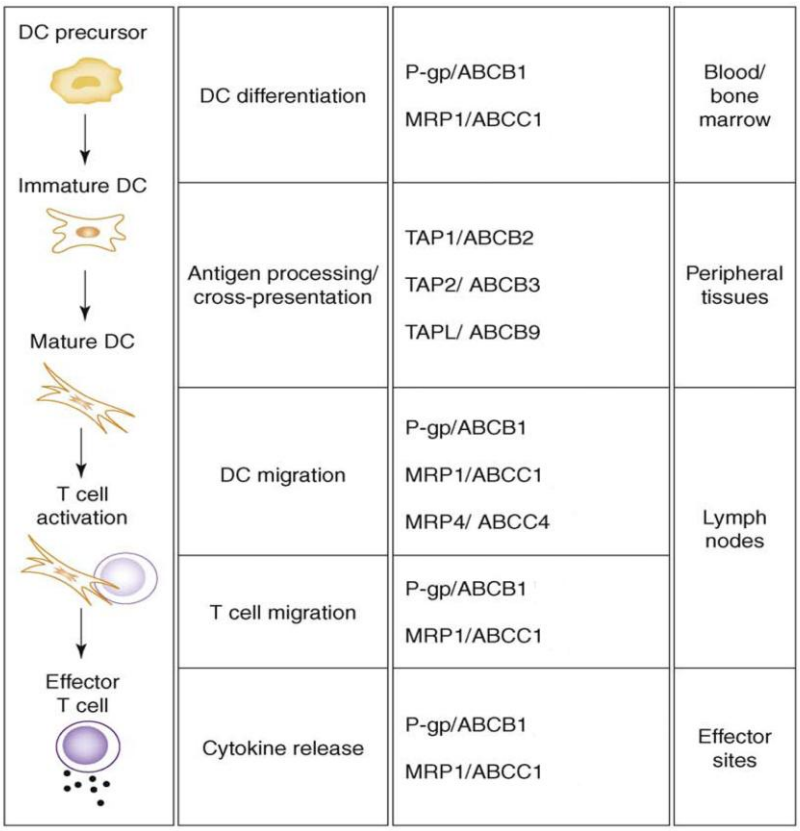

Figure 9. ABC transporters expressed on different immune cells and the functions they have for an immune response and the location of the immune response occur. Adapted from Rieneke van de Ven et al [146].

During the maturation process is given a set of phenotypic and functional changes. Dendritic cell stimulation by different stimulus (endogenous ligands or pathogens, Lipopolysaccharide (LPS), alloantigens, and hypoxia) leads to dendritic cell maturation. This makes dendritic cell acquires a great capacity for antigen processing and presentation. Also, they increased the expression of the surface markers of co-stimulatory molecules (CD80 and CD86) resulting in the activation of the immune response.

There have been further DC studies in the literature confirming a cross-talk between the hypoxic environment and DC maturation [161, 162]. Rama et al [163] proposed hypoxia as a key regulator of DC maturation in the kidney, suggesting a novel mechanism by which the lack of oxygen regulates immune responses. This work targets new investigation into the role of molecular oxygen-sensing in dendritic cell maturation and function, which may have implications in acute and chronic renal injuries in both the transplantation and non-transplantation settings.

Pendse et al [36] defined a novel role for Pgp in DC maturation, identifying this transporter as a potential novel therapeutic target in allotransplantation. Schroeijers et al [160] showed that human monocyte-derived DCs express Pgp at all maturation stages, and that they are up-regulated during DC maturation. Randolph et al [159] found that Langerhans cells express Pgp and observed that their blockade inhibited migration of these cells.

Recently, Lloberas et al [164] showed that ABC transporters could be a potential target in DC-based immunosuppressive therapies designed to abrogate innate immune response when it is activated after ischaemia or endotoxin stimulus. The cellular and molecular mechanisms underlying

the innate adaptive immune response to ischaemia-reperfusion are an active area of research with much more to tell us. These findings add more information about the specific functional role of ABC transporters as a potential therapeutic target in alloimmunity modulation.

HYPOTHESIS

AND OBJECTIVES

HYPOTHESIS

Both immunological and non immunological factors are involved in chronic allograft nephropathy such as donor specific alloreactivity, chronic inflammation and nephrotoxicity induced by anticalcineurinics. Different degrees of nephrotoxicity are related to immunosuppression and to the level of inhibition of the different transporter proteins such as MDR1, MRP1 and MRP2. Those proteins could be subjected to genetic inter-individual variability thus modifying pharmacokinetics parameters. Furthermore, optimal immunosuppressive dose could be predicted by genotype characterization of MDR1, MRP1 or MRP2 genes. Polymorphisms in these genes have been related to immunosuppressive exposure, nephrotoxicity and renal allograft rejection. Those proteins have also been associated to immunological factors. Both MDR1 and MRP1 have been involved in T cell activation and also in dendritic cells (DCs) differentiation, migration and maturation.

It has been hypothesized that ABC transporter proteins play a major role in drug efflux and also may be an underlying factor as an immunomodulator. Considering that many immunosuppressants are substrates and also inhibitors of this ABC transporter proteins, the hypothesis of this doctoral thesis is to evaluate if Pgp activity would participate in lymphocyte activation and if the polymorphisms of this protein in renal transplant recipients with different immunosuppressive regimen could have a functional role in the pharmacokinetics of the immunosuppressants.

OBJECTIVES

The main objective is to study the role of P-glycoprotein as an efflux pump and its contribution in the pharmacokinetics and pharmacogenetics of immunosuppressants. On the other hand, far from the well known role in drug exposition and nephrotoxicity, to improve the knowledge on its function in the alloimmunity responses.

Specific aims:

I. To evaluate whether Pgp expression and efflux activity by measuring rhodamine (Rho123) retention in lymphocytes stored under different conditions can influence on Pgp expression and functionality.

II. To study the association of different ABCB1 polymorphisms (C3435T, G2677T, C1236T and T129C) with Pgp activity and exposure of different immunosuppressive drugs in renal transplant patients.

III. To assess the activity of Pgp on different T-cell subsets and the effects of two calcineurin inhibitors (cyclosporine and tacrolimus) in monotherapy and associated with rapamycin. And also evaluate the antigen-specific memory T-cell responses.

RESULTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the three original contributions listed below.

I **Different storing and processing conditions of human lymphocytes do not alter P-glycoprotein rhodamine 123 efflux.** Chiva-Blanch G, Giménez-Bonafe P, Llaudó I, Torras J, Cruzado JM, Petriz J, Castaño E, Franquesa Ml, Herrero-Fresneda I, Tortosa A, Rama I, Bestard O, Grinyó JM, Lloberas N. J Pharm Pharm Sci. 2009;12(3):357-66.

II **Do drug transporter (ABCB1) SNPs and P-glycoprotein function influence cyclosporine and macrolides exposure in renal transplant patients? Results of the pharmacogenomic substudy within the Symphony study.** Llaudó I, Colom H, Giménez-Bonafé P, Torras J, Caldés A, Sarrias M, Cruzado JM, Oppenheimer F, Sánchez-Plumed J, Gentil MA, Ekberg H, Grinyó JM, Lloberas N. Transpl Int. 2012 Dec 7.

III **Impact of small molecules immunosuppressants on P-glycoprotein activity and T-cell function.** Llaudó I, Cassis L, Torras J, Bestard O, Franquesa Ml, Cruzado JM, Cerezo G, Castaño E, Pétriz J, Herrero-Fresneda I, Grinyó JM, Lloberas N. J Pharm Pharm Sci. 2012 May;15(3):407-19

LIST OF ORIGINAL PUBLICATIONS NOT INCLUDED IN THE THESIS (Annex 2)

I. **Dendritic cells phenotype fitting under hypoxia or lipopolysaccharide; adenosine 5_-triphosphate-binding cassette transporters far beyond an efflux pump.** Lloberas N, Rama I, Llaudó I, Torras J, Cerezo G, Cassis L, Franquesa M, Merino A, Benitez-Ribas D, Cruzado JM, Herrero-Fresneda I, Bestard O, Grinyó JM. Clin Exp Immunol. 2013 Jun;172(3):444-54.

II. **Pretransplant Immediately Early-1-Specific T Cell Responses Provide Protection For CMV Infection After Kidney Transplantation.** Bestard O, Lucia M, Crespo E, Van Liempt B, Palacio D, Melilli E, Torras J, Llaudó I, Cerezo G, Taco O, Gil-Vernet S, Grinyó JM, Cruzado JM. Am J Transplant. 2013 Jul;13(7):1793-805.

Study I

Different storing and processing conditions of human lymphocytes do not alter P-glycoprotein Rhodamine 123 efflux

Chiva-Blanch G, Giménez-Bonafe P, Llaudó I, Torras J, Cruzado JM, Petriz J, Castaño E, Franquesa Ml, Herrero-Fresneda I, Tortosa A, Rama I, Bestard O, Grinyó JM and Lloberas N.

Journal of Pharmacy and Pharmaceutical Sciences 2009;12(3):357-66.

STUDY I. Different storing and processing conditions of human lymphocytes do not alter P-glycoprotein rhodamine 123 efflux. J Pharm Pharm Sci. 2009;12(3):357-66.

In this study we compared Pgp expression and efflux activity by measuring Rho123 retention in lymphocytes stored under different conditions to evaluate the potential influence of any of the storing conditions on Pgp expression and functionality. To improve Pgp studies, especially multicentric ones, sampling strategies should be considered in order to optimize the sensitivity and reproducibility of efflux assays. There is no data which compares Pgp activity measured from fresh lymphocytes with cryo-preserved lymphocytes. Moreover, most authors do not specify under which conditions cells are preserved and stored before measuring Pgp activity. Here, we isolated lymphocytes from fresh venous blood from 12 healthy volunteers. Four storage conditions of lymphocytes were used:

1) Fresh lymphocytes (Fresh/Non-frozen) (F/NFr);
2) Lymphocytes frozen immediately after the extraction (Fresh/Frozen) (F/Fr);
3) Lymphocytes isolated within 24 hours after the extraction (Non-fresh/Non-frozen) (NF/NFr);
4) And lymphocytes isolated within 24 hours after the extraction and immediately frozen (Non-fresh/Frozen) (NF/Fr).

Results

- First, we evaluated Pgp lymphocyte expression by two different techniques, Western Blot and quantitative Real Time PCR (figure 1 and 2 in the article). We compared the four different storage conditions but no statistical differences were found in the protein levels neither in Pgp mRNA expression ($p>0.05$).

- We studied the viable $CD3^+$ T lymphocyte population by Rho123 retention experiments excluding the early and late apoptotic, and necrotic subpopulations which could interfere in the results. We used two different Pgp inhibitors (Verapamil and PSC833). When Pgp is blocked by one of these two inhibitors, the four storage conditions of lymphocytes retained double amount of Rho123 (measured by mean fluorescence intensity; MFI) than without inhibitors. We found no significant differences in Rho123 retention among the four conditions and the two inhibitors used ($p=ns$) (table 1 in the article). We observed by flow cytometry that the four storage conditions of lymphocytes showed similar patterns of retention of Rho123 both in the absence and presence of the inhibitor (figure 3 in the article). Therefore, we conclude that Pgp activity was not modified by storing conditions or sample manipulation.

- We also showed no inter-individual differences in none of the storing groups in $CD3^+$ viable cells (table 2 in the article). In further assays (data not shown in the article), we also analyzed the differences of Pgp activity in in $CD3^+$ viable cells within the same individual in the four different conditions; and also no significant differences were found ($p>0.05$).

- Pgp activity was higher in viable $CD3^+$ T cells than apoptotic population ($p<0,05$). We evaluated the apoptotic population separately. Pgp activity was not statistically different when comparing the four conditions, taking the F/NFr condition as a reference (figure 4A in the article). However, within intra-individuals, high variability was found in the Pgp activity comparing the four conditions (figure 4B in the article).

- We worked with a $CD3^+$ cell viability of about 90% in F/NFr and NF/NFr, and 80% in F/Fr and NF/Fr conditions. The apoptotic and necrotic cells represented a minority of the lymphocyte population (figure 5A in the article).

J Pharm Pharmaceut Sci (www.cspsCanada.org) 12(3) 357 - 366, 2009

Different Storing and Processing Conditions of Human Lymphocytes do not Alter P-Glycoprotein Rhodamine 123 Efflux

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ABSTRACT

P-glycoprotein (Pgp), a protein codified by Multi Drug Resistance (MDR1) gene, has a detoxifying function and might influence the toxicity and pharmacokinetics and pharmacodynamics of drugs. Sampling strategies to improve Pgp studies could be useful to optimize the sensitivity and the reproducibility of efflux assays. This study aimed to compare Pgp expression and efflux activity by measuring Rhodamine123 (Rh123) retention in lymphocytes stored under different conditions, in order to evaluate the potential utility of any of the storing conditions in Pgp functionality. Our results show no change in protein expression of Pgp by confocal studies and Western blotting, nor changes at the mRNA level (qRT-PCR). No differences in Rh123 efflux by Pgp activity assays were found between fresh and frozen lymphocytes after 24 hours of blood extraction, using either of the two Pgp specific inhibitors (VP and PSC833). Different working conditions in the 24 hours post blood extraction do not affect Rh123 efflux. These results allow standardization of Pgp activity measurement in different individuals with different timing of blood sampling and in different geographic areas.

INTRODUCTION

P-glycoprotein (Pgp) is a multridug transporter that belongs to the ATP-Binding Cassette (ABC) family, and it is the product of the Multidrug Resistance-1 (MDR-1) gene. Pgp acts as a drug efflux pump extruding a range of different hydrophobic drugs from cells, contributing to drug disposition in humans and reducing the bioavailability of many orally administered medications. Pgp confers the multidrug resistant phenotype by maintaining reduced intracellular concentrations of anticancer drugs by virtue of its ability to act as a primary active transporter (1-3). As well, Pgp plays a role in the cells of the immune system, particularly in dendritic cell maturation and function (4,5). This multifaceted involvement in drug disposition, cancer drug resistance and regulation of the immune response makes Pgp an attractive target to deepen the optimization of different strategies and to foster a better understanding of its function in cells and tissues.

Pgp works as a transporter molecule pumping a wide variety of endogenous substances and drugs from the cytoplasm to the exterior of the cell (6). Many widely used clinical agents are substrates, inhibitors or inducers of Pgp, including immunossupressive drugs (7). Therefore, Pgp plays a very important role in drug disposition (absorption, distribution, and excretion processes) (8,9). It is well known that Pgp is expressed constitutively at relatively high levels in many normal tissues such as intestinal epithelial cells, placenta, biliary canalicular cells, kidney, blood-brain barrier and immune system cells such as lymphocytes (10-12). Regarding the wide tissue distribution and the effect on membrane transport of Pgp, the expression and activity of this protein could be essential in highlighting drug interactions.

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ABBREVIATIONS

Rhodamine123 (Rh123), P-glycoprotein (Pgp), ATP-Binding Cassette (ABC), Multidrug Resistance-1 (MDR-1), Annexin-V PE (AnnV), 7-Aminoactinomycin D (7-AAD), Binding Buffer (BB), Phosphate Buffered Saline (PBS), Foetal bovine serum (FBS), fresh non-frozen lymphocytes (F/NFr), fresh frozen lymphocytes (F/Fr), non-fresh non-frozen lymphocytes (NF/NFr), non-fresh frozen lymphocytes (NF/Fr), Mean fluorescence intensity (MFI), Verapamil (VP) and Flow analysis cell sorter (FACS).

Measuring the protein level of Pgp is not always an indicator of the efflux activity of the protein. The MDR-1 activity controlling Pgp-dependent drug transport depends on two factors: 1) the level of expression of the MDR-1 gene that controls the amount of protein synthesized in the cells, where several single nucleotide polymorphisms in the MDR-1 gene have been identified, and 2) the functionality of the MDR-1-encoded Pgp (13).

Several methods have been developed to measure Pgp activity in blood samples (14-19). In all of them lymphocyte isolation and incubation with substrates (such as Rh123) in the presence or absence of specific Pgp inhibitors followed by FACS analysis are performed. Some authors carry out Pgp activity assays using lymphocytes isolated from fresh blood, which could be a limitation. Despite some uncontrolled factors such as co-medication (9,20), age (21,22) and inter-individual differences in Pgp activity considered for result analysis (23), some methodological factors ought to be taken into account. There are no data comparing Pgp activity measured from fresh lymphocytes with cryo-preserved lymphocytes. Furthermore, most authors do not specify under which conditions cells are preserved and stored before measuring Pgp activity. The published studies do not specify whether they use lymphocytes isolated from fresh drawn blood, lymphocytes isolated within the 24 hours after the drawing, frozen lymphocytes isolated from fresh drawn blood, or lymphocytes isolated from blood drawn and frozen within 24 hours of the extraction and then thawed prior the assays. In view of the fact that Pgp is a labile protein and its activity could be lost depending on the temperature, these methodological factors should be considered because they determine the interval of time among blood collection, lymphocyte isolation and Pgp activity assays.

Therefore, this present study aimed to compare Pgp expression and Rhodamine123 (Rh123) retention in lymphocytes stored under different conditions to evaluate the potential influence of any of the storing conditions on Pgp expression and functionality.

MATERIALS AND METHODS

Drugs and Reagents

The following drugs and reagents were used for the activity assays: Annexin-V PE (AnnV) (BD Pharmingen, CA, USA), 7-Aminoactinomycin D (7-AAD) (Sigma-Aldrich, Madrid, Spain), Binding Buffer (BB) (Medical and Biological Laboratories, Japan), Phosphate Buffered Saline (PBS) (PAA, Cambridge, UK), RPMI (Biological Industries, Israel), Rhodamine 123 (Molecular Probes Inc., USA), and PSC833 (kindly provided by Novartis, Basel, Switzerland). To select T lymphocytes from the whole lymphocyte population, CD3-APC conjugated Mouse anti-Human monoclonal antibody (BD Pharmingen, CA, USA) was used. To detect P-glycoprotein in lymphocytes by Western blot, the antibody C-219 was used (Calbiochem, Germany).

Cell lines

Two cell lines were used as a positive control for both the expression and the function of P-glycoprotein. The cells lines used were SW1573/2R160, a Pgp-overexpressing cell line, and its counterpart SW1573 parental cell line. Both cell lines were cultured on DMEM supplemented with 10% FBS, 1% L-Glutamine, and 1% penicillin-streptomycin (Biological Industries, Israel), at 37ºC and 5% CO_2 atmosphere. The overexpression of Pgp in the SW1573/2R160 cell line was maintained by adding 160 nM of doxorubicin to the culture media every two to three passages. Both cell lines were a kind gift from Dr. G. Scheffer and Dr. R. Skepper of the Free University Medical Center, Amsterdam, The Netherlands.

Human lymphocytes

Informed consent was obtained from each healthy volunteer after the nature and possible consequences of the study were fully explained.

Written informed consent was obtained from all healthy volunteers in accordance with the Hospital Universitari de Bellvitge Ethic Committee.

Lymphocyte isolation

Lymphocytes were obtained from fresh venous blood from healthy volunteers, collected into EDTA tubes and kept at room temperature, and separated by standard Ficoll-Paque (Amersham Biosciences, UK) gradient centrifugation. After isolation, $25x10^6$ lymphocytes were frozen in freezing media lymphocyte consisting of RPMI, 20% DMSO and 20% FBS, 1:1, with FBS. Before activity assay, lymphocytes were rapidly thawed, washed twice with culture medium to free them of DMSO, and incubated for 2 hours at 37ºC and 5% CO_2 in culture medium (RPMI supplemented with 15% FBS and 1% L- Glutamine). In the non-frozen group (F/NFr and NF/NFr), lymphocytes were incubated with culture medium for 2 hours before the assay.

Blood sampling was performed in healthy volunteers (n=12, 6 men/ 6 women) ranging from 23 to 53 years old. Four storage conditions of lymphocytes were used: fresh lymphocytes (F/NFr) (n=12), lymphocytes frozen immediately after the extraction (F/Fr) (n=12), lymphocytes isolated within 24 hours after the extraction (NF/NFr) (n=12) and lymphocytes isolated within 24 hours after the extraction and immediately frozen (NF/Fr) (n=12).

P-glycoprotein expression: Western Blot

Membrane protein extracts were obtained by homogenizing lymphocytes in 100-300µl of lysis buffer (10mMTrisCl, 1.5mMMgCl$_2$, 10mMKCl and 0.5% SDS) containing 4% protease inhibitor cocktail (Roche, Switzerland) and 2% PMSF. The extracts were stained on ice for 45 minutes and sonicated four times during that time. Then they were centrifuged for 10 minutes at 4ºC at 400 g, and the supernatant was recovered and ultra-centrifuged for 1 hour at 4ºC at 18,000 g. The pellet was resuspended in 200µl of lysis buffer containing 4% protease inhibitor cocktail and 2% PMSF. Quantification of membrane proteins was done using the BCA Assay (Pierce, IL, USA), following the instructions of the manufacturer. 80 µg of protein lysates were separated on to an 8% SDS−polyacrylamide gel and transferred to PVDF membranes (BioRad, CA, USA). The membranes were blocked in 5% (w v^{-1}) non-fat dry milk in Tris-buffered saline, pH 7.4, containing 0.1% Tween 20 (TBST) (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% Tween-20) at room temperature for 1 h. Blocked membranes were incubated with primary mouse antibodies specific to Pgp, C219 (1:100) (Calbiochem-Novabiochem, San Diego, CA, U.S.A.) in TBST containing 5% BSA at 4°C overnight as described previously (24). After incubation, immunoblots were washed with TBST and incubated at room temperature for 1 h with the horseradish peroxidase conjugated antimouse secondary antibody (1:5000, Dako, Denmark) in TBST containing 5% milk. ECL detection kit (Pierce, IL, USA) was used to detect the protein bands. Pgp levels were expressed as a percentage of respective control groups. β-Actin (Cell Signaling Technology, Danvers, MA) was used as a comparator using antibody detection on stripped blots to confirm equal protein loading and blotting.

P-glycoprotein mRNA level by quantitative real-time PCR (qRT-PCR)

Total RNA was obtained following the Trizol manufacturer's instructions (Invitrogen, Carlsbad). The RNA used for the study had a 28s/18s ratio between 1.8 and 2.0. Total RNA was reverse-transcribed as follows: 2µg of RNA was incubated with 1µl of 50µM random hexamers followed by RNA denaturalization. Then, 5x reaction buffer was added, 0.4µl of 100mM dNTP mix, and MMLV retrotranscriptase 200u/µl (Ecogen, Langhorne, PA, USA) was added for a final volume of 20µl. The reaction was incubated for 5 min at 25ºC, followed by 30 min at 42ºC and 85ºC for 5 min to stop the reaction. Real-time PCR was performed using 5µl of cDNA, primers and the TaqMan probe for MDR1 (Hs-00184500_m1, Applied Biosystems, CA, USA), and the ABI Prism 7700 Sequence Detector. The level of target gene expression was determined using the $\Delta\Delta$Ct method as described previously (25), normalized to the GAPDH (Hs-99999905_m1) control (Applied Biosystems, CA, USA). Results were expressed as many fold Pgp. Duplicates were carried out in each experiment.

P-glycoprotein function in lymphocytes: Rh123 retention assay

500 µl of the cell suspension (10^6 cells/ tube) in serum-free RPMI was added to 3 test tubes: 1 tube, to evaluate cell auto-fluorescence: efflux

control (EC); 1 tube, to evaluate Rh123 efflux (E0), and the last tube, to evaluate the effect of PSC833 on Rh123 efflux (EV). 400 µl of Binding Buffer (BB) was added to the EC tube. PSC833 was added to the EV tube at a final concentration of 10µM. Then, 2.1 µl of Rh123 at 500 µM (0.525 µM final) dissolved in methanol was added to the E0 and EV tubes. All the tubes were incubated for 30 min at 37°C, avoiding light exposure. At the end of incubation, the tubes were kept on ice a few minutes to stop Pgp efflux activity. Cells were centrifuged for 10 min at 450 g at 4°C and washed again with 2 ml of BB. After washing, the cells were diluted in 50 µl of BB containing 2 µl of CD3-APC, except for the EC tube, and incubated on ice for 30 min. 250 µl of BB containing 2µl AnnV (to detect apoptotic cells) and 2µl of 7-AAD (to detect cells with membrane damage) were added in all tubes except the EC and incubated for 15 min on ice avoiding light exposure, prior to flow cytometry analysis. The samples were acquired on a FACSCalibur flow cytometer (Becton and Dikinson, BD) with four-color analysis. 10,000 CD3+ events were acquired and analyzed using CellProQuest software (BD). The instrument settings were established first and compensation was adjusted by acquiring cells stained with each fluorochrome. FSC and SSC were collected using linear scales and fluorescence signals were collected using logarithmic scales. All the data were collected in list mode. Results were expressed as the mean fluorescence intensity (MFI). Efflux activity was calculated as (MFI of Rh123 (FL1) (EV) - MFI of Rh123 (E0))/ ($\sqrt{sd^2}$ (EV)+ sd^2 (EO)), while retention ratio was calculated as (MFI of Rh123 (EV)/ MFI of Rh123 (EO)). Each experiment was performed three times.

The same protocol was performed with the first generation Pgp inhibitor Verapamil, using a range of different concentrations (10 to 50µM) to establish the best functional and non-toxic concentration, at 20 µM for lymphocytes and 40 µM for the SW1573 cell lines.

Statistical Analysis

Rh123 retention of the four conditions of lymphocytes was measured in n=12 healthy volunteers. The activity was measured both in AnnV negative and 7-ADD negative population, and apoptotic (AnnV positive population) lymphocytes. Non-parametric statistical analysis was performed, using the non-parametric Mann-Whitney test to compare the activity of F/Fr, NF/NFr and NF/Fr CD3+ cells to the activity of F/NFr CD3+ lymphocytes. The analysis was performed both within the viable and the apoptotic lymphocytes, and also between viable and apoptotic lymphocytes.

RESULTS

1. Pgp lymphocyte expression under four different storage conditions

Western Blot. Pgp expression in lymphocytes was measured and compared in all storage conditions. The four conditions showed no differences in the protein levels (Fig 1). Pgp is a protein that is glycosylated in several positions, adopting different molecular weights, as can be seen in the overexpressing Pgp cell line SW1573/2R160 used as a positive control.

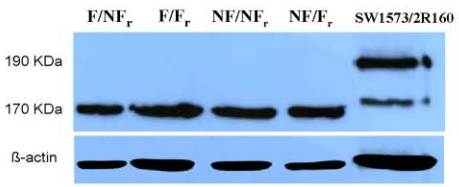


Figure 1. Pgp expression in $CD3^+$ lymphocytes by Western blot analysis. Membrane protein extracts (80µg) from each of the lymphocyte conditions were separated in a SDS-polyacrylamide gel, and Pgp was detected with a specific antibody (C219). A positive cell line control that over-expresses Pgp was used (SW1573/2R160). All conditions showed a similar band in the170 KDa molecular weight. The different mobility observed in the positive control is due to different degrees of post-transductional modifications (glycosilation) of the protein.

Quantitative Real Time PCR. The expression of Pgp mRNA was measured to look for evidence of differences among the four storage conditions with the healthy volunteers. No statistical differences were found at the expression level of the transporter in the four conditions (F/NFr = 1.0±0, F/Fr = 0.9±0.33, NF/NFr= 0.74±0.12 and NF/Fr = 0.68±0.51), suggesting that freezing lymphocytes does not affect Pgp mRNA expression ($p>0.05$) (Fig. 2).

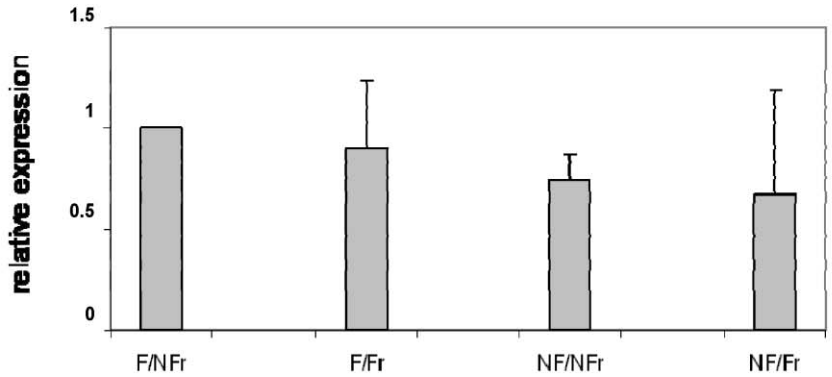


Figure 2. Pgp expression in $CD3^+$ lymphocytes at the mRNA level by qRT-PCR. The expression of Pgp was measured by quantitative real-time PCR in the four conditions established in 12 healthy human volunteers. No significant differences were found related to F/NFr control group.

2. Flow cytometry analysis of Rh123 retention in lymphocytes

To assess which CD3+ lymphocytes were the optimal for studying Pgp activity, retention experiments using Rh123 were performed using viable CD3+ T lymphocytes ($AnnV^-$ and $7ADD^+$), excluding the early apoptotic ($AnnV^+$ and $7ADD^-$), late apoptotic or necrotic ($AnnV^+$ and $7ADD^+$) populations in different lymphocytes conditions. The four conditions of viable lymphocytes had a similar retention of Rh123 in the presence or absence of any of the inhibitors, showing a retention ratio of 2.05±0.10 for VP and 1.36±0.07 for PSC833 (Table 1) as an average value of the four conditions.

To assess whether freezing lymphocytes could modify Pgp activity, retention experiments in healthy volunteer lymphocytes were done. Rh123 retention in the CD3+ T viable cells was analyzed by FACS in the presence or absence of the inhibitors. Results were expressed in the four conditions by subtracting the mean channel of Rh123 MFI from the mean channel number of Rh123 MFI in the presence of VP or PSC833. As Figure 3 shows, the four conditions of lymphocyte demonstrated similar patterns of retention of Rh123 both in the absence and presence of the inhibitor. Pgp activity in the presence of the inhibitors in the four conditions resulted in an increase of approximately one logarithm of MFI compared with Rh123 alone. Therefore, no significant differences in Pgp activity were found. Results showed no inter-individual differences in F/NFr vs NF/NFr, F/Fr or NF/Fr in $CD3^+$ viable cells (Table 2). Additionally, we analyzed the differences of Pgp activity within the same individual in the four conditions in viable cells; no significant differences were found ($p=0.183$) (data not shown).

3. Rh123 retention assay in viable versus early apoptotic lymphocytes

Pgp activity in the early apoptotic $CD3^+$ cell population ($AnnV^+/7\text{-}ADD^-$) was not statistically different when comparing the four conditions (F/Fr ($p=0.66$), NF/NFr ($p=0.71$) and NF/Fr ($p=0.87$)), taking the F/NFr condition as a reference (Fig 4A). The Pgp activity observed in the apoptotic population was lower ($p<0.05$) than the activity in viable lymphocytes, probably due to the loss of functionality of the cell, and there was higher variability among different individuals compared to viable cells (Fig 4B). It should be mentioned that the apoptotic population represents a low percentage of the total $CD3^+$ lymphocytes (4.5%). Therefore it was not considered in the Pgp activity analysis.

When measuring cell viability in the four conditions, $CD3^+$ population was viable between 90% (F/NFr, NF/NFr) and 80% (F/Fr, NF/Fr). The early and late apoptotic and necrotic cells represented a minority of the lymphocyte population (Fig 5A). These results indicate that freezing lymphocytes partially modifies cell viability (Fig 5B).

Table 1. Rh123 retention in the four conditions of viable $CD3^+$ using two Pgp inhibitors: VP and PSC833.

	VP((MFI)	Non VP(MFI)	Retention ratio	PSC833(MFI)	Non PSC833 (MFI)	Retention ratio
F/NFr	1232.09±117.40	609.87±150.02	2.02±0.06	1576.37±117.47	1237.76±91.37	1.27±0.05
F/Fr	1629.97±164.18	744.07±113.23	2.19±0.11	2279.03±146.67	1657.29±118.64	1.34±0.07
NF/NFr	822.57±108.2	408.18±78.56	2.02±0.09	1598.53±120.20	1154.34±97.38	1.38±0.06
NF/Fr	1520.5±149.12	777.19±95.48	1.96±0.12	2262.00±120.77	1574.07±98.65	1.43±0.09
mean±sd			2.05±0.10			1.36±0.07

MFI of Rh123 was measured after 30 min exposure in the presence and absence of the Pgp inhibitors VP and PSC833. When Pgp was blocked by VP, the four conditions of lymphocytes retained double the amount of Rh123 MFI without inhibitor (2.05±0.10); when using PSC833, the retention ratio was 1.36±0.07. Results are expressed as the mean of MFI of Rh123 of 12 healthy volunteers. No significant differences in Rh123 retention were seen among the four conditions studied with the two inhibitors used (p=NS).

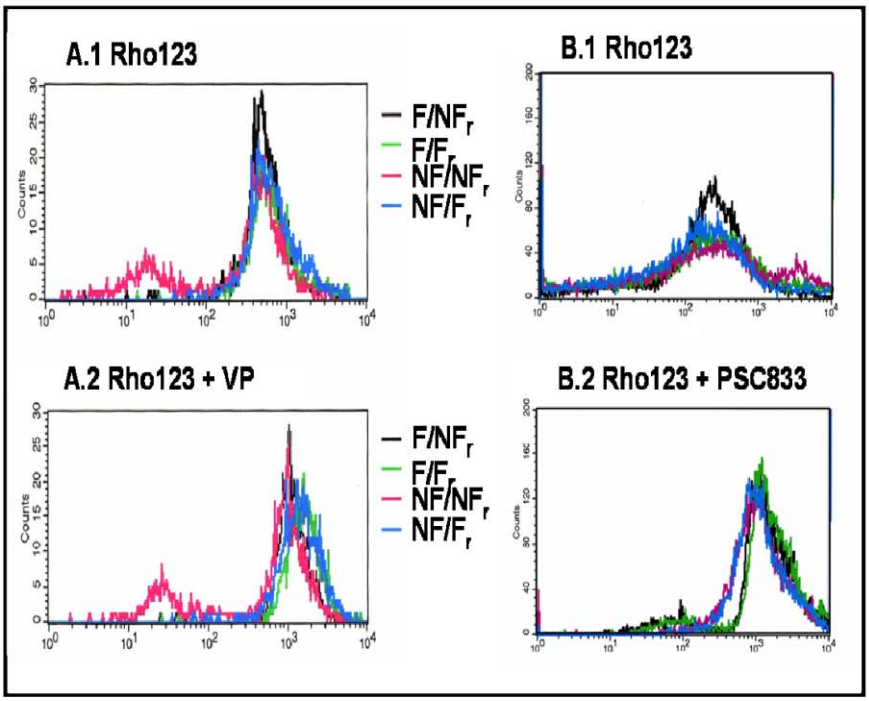
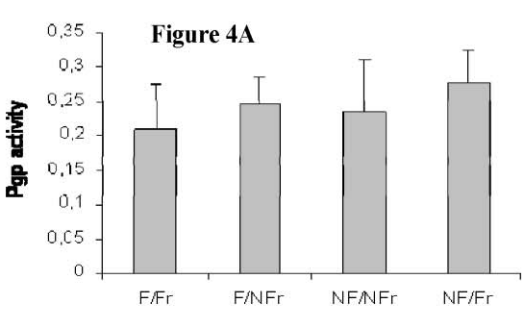


Figure 3. Pgp retention ratio of Rh123 in human lymphocytes measured by Flow cytometry analysis (FACs).
Pgp retention ratio in the four conditions was measured in viable $CD3^+$ lymphocytes ($AnnexinV^-/7ADD^-$) by Rh123 retention measurements detected by FACS. Retention was measured as mean fluorescence intensity (MFI) of Rh123 in a logarithmic scale. Retention of Rh123 by Pgp was estimated as a logarithm increase of MFI of Rh123 when a Pgp inhibitor is added. A.1 The Pgp retention of the Rh123 plot of the four conditions overlapped, meaning that Pgp activity was not modified due to the storing and sample manipulation. A.2. When VP was used to block Pgp, an increase in one logarithm of MFI of Rh123 was observed in all four conditions where the plots also overlapped. B.1 and B.2. The same results were observed when PSC 833 was used to inhibit Pgp activity.



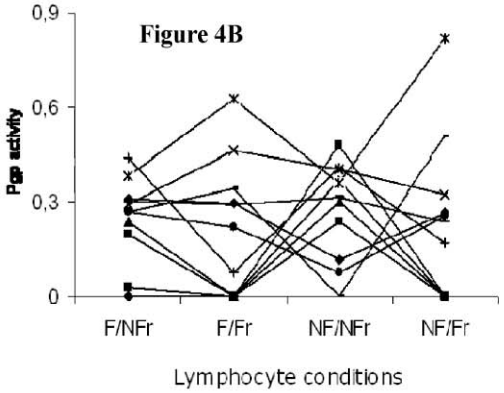


Figure 4. Pgp activity in apoptotic lymphocytes. Pgp activity was measured in the T cells' early apoptotic population ($CD3^+/AnnV^+/7\text{-}ADD^-$) by Rh123 retention assays. A. Mean (n=12) of the activity measured in the four conditions of apoptotic lymphocytes. B. High variability was found in the activity measured within intra-individuals comparing the four conditions.

Table 2. Effect of storage conditions in the Pgp activity in $CD3^+$ viable cells.

	VP	PSC833
F/NFr	0.121± 0.061	0.247± 0.138
F/Fr	0.184± 0.071	0.326± 0.136
NF/NFr	0.134± 0.058	0.331± 0.097
NF/Fr	0.215± 0.095	0.337± 0.202
p	NS	NS

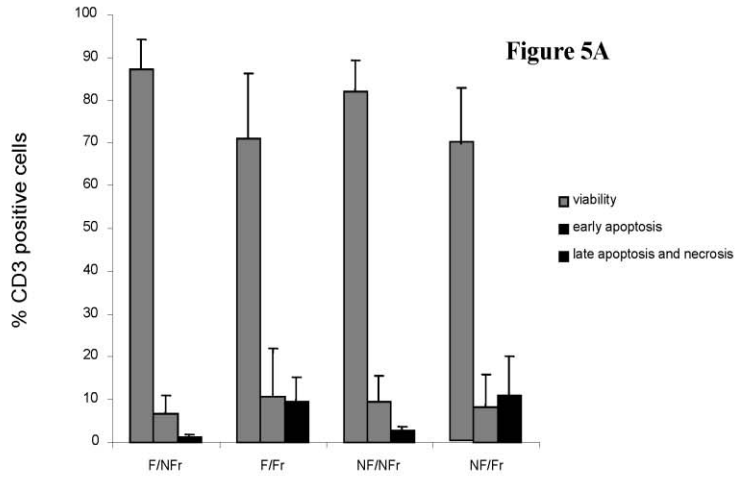
Pgp activity, calculated as (MFI of Rh123 in the presence of inhibitor (EV) - MFI of Rh123 in the absence of inhibitor (E0))/($\sqrt{sd^2}$ (EV)+ sd^2 (EO)), was expressed as mean ± sd of 12 healthy volunteers (p= NS).

DISCUSSION

In this study, the expression of Pgp and Rh123 retention in lymphocytes handled and stored under four different conditions was measured, and our results showed no significant differences among the storage conditions. To date, studies by other groups have not specified the processing and storing conditions of the cells before analysis. Some groups performed Rh123 retention assays directly from fresh whole blood (26), and others from fresh isolated lymphocytes (27), but the majority of the studies have not indicated whether the lymphocytes were isolated from freshly drawn blood or blood stored at room temperature for a short period of time (28). The main interest of the present study is to show that Pgp expression and activity measured through Rh123 retention are not modified when blood is kept at room temperature for 24 hours nor when the lymphocyte isolation is carried out immediately after the blood is drawn.

Peripheral mononuclear cells are known to express ABC efflux transporters. Hence, isolated cells could be useful to study drug-transporters in the in vivo/ex vivo monitoring of the potential effects of drugs on the expression and activity of these transporters. There is clear evidence that normal blood cells, particularly natural killer (NK) cells and T lymphocytes, express Pgp (29), although the level of Pgp expression on lymphocytes does not imply a direct correlation with Pgp activity (30). This lack of correlation between Pgp expression and activity may be explained, in part, by the fact that functional analysis based on Pgp substrate dyes such as Rh123 is much more sensitive in the detection of low-level multidrug resistance than Pgp expression analysis (31,32). Although NK cells play an important role in Pgp expression and function, this subpopulation represents a low percentage of the PBMC population. Therefore, considering that the CD3+ cells represent a high percentage of the PBMC, as they show functional Pgp expression and play a predominant role in immune response, this population was the most suitable for the purpose of the present study.

It has to be considered that Pgp is a fragile protein and could be inactivated by freeze/thawing cycles (33). Therefore manipulation of lymphocytes could be extremely critical in this regard.



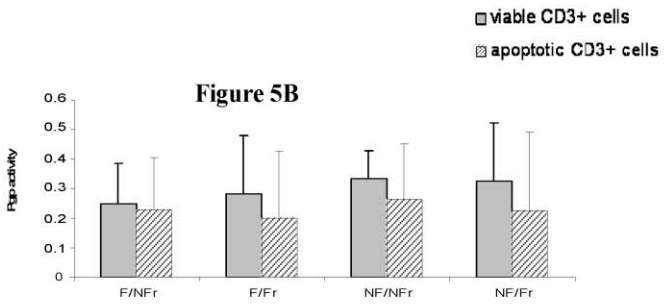


Figure 5. Cell viability in the four conditions of lymphocytes. A. In each lymphocyte condition cell viability was measured, distinguishing among viable ($CD3^+/AnnV^-/7\text{-}ADD^-$), early apoptotic ($CD3^+/AnnV^+/7\text{-}ADD^-$), late apoptotic and necrotic ($CD3^+/AnnV^+/7\text{-}ADD^+$) cells. In all four conditions viable cells were the vast majority. B. Pgp activity in viable and apoptotic $CD3^+$ lymphocytes.

The manner of drawing, isolating and storing lymphocytes is a relevant key toward obtaining good reproducibility for the Pgp activity studies. For multicentre studies, where blood is collected from different centers and Pgp substrate retention assays have to be performed from lymphocytes isolated and/or stored under different conditions, it is critical to guarantee uniformity of the procedure conditions for all the samples. In the present study, lymphocytes were frozen and no significant changes in the expression or the activity of the Pgp protein were found.

One approach to measuring Pgp activity was the retention of Rh123 within the cells, in the presence and absence of Pgp inhibitors. The two efflux pump inhibitors used were the Ca^{+2} channel blocker VP and PSC833, a Pgp selective inhibitor, also known as Valspodar. The two inhibitors showed no differences in the Rh123 retention among the four conditions of the lymphocytes studied. Nevertheless, PSC833 is a more specific and potent inhibitor of Pgp than VP, as the concentration used to inhibit Ppg efflux was half the concentration of VP.

Within the $CD3^+$ population, three cell subsets were compared in terms of Pgp efflux activity calculated by Rh123 retention measurements: viable, early apoptotic and late apoptotic lymphocytes. Although early apoptotic lymphocytes prove Pgp efflux activity, this population represents only 4.5% of the total lymphocytes purified from the blood samples.

Therefore, not measuring the activity of the early apoptotic lymphocytes does not alter the results of activity obtained from viable population. In contrast, late apoptotic and necrotic cells should be considered separately or excluded because they always show background activity that interferes in the analysis of the viable lymphocyte Rh123 retention measurements. We established that in order to obtain the most accurate results, the apoptotic population should be considered separately and necrotic cells should be excluded.

CONCLUSIONS

Our results show that for the 24 hours post blood extraction, Pgp activity measured by Rh123 retention was not affected by the 4 different handling processes. They also confirm that in order to assess global Pgp activity, Pgp should be analyzed in viable cells, removing the necrotic population. These parameters could contribute to improve Pgp activity studies in individuals with different blood sampling timing and/or different geographic areas.

ACKNOWLEDGEMENTS

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Study II

Do drug transporter (ABCB1) SNPs and P-glycoprotein function influence cyclosporine and macrolides exposure in renal transplant patients? Results of the pharmacogenomic substudy within the Symphony study.

Llaudó I, Colom H, Giménez-Bonafé P, Torras J, Caldés A, Sarrias M, Cruzado JM, Oppenheimer F, Sánchez-Plumed J, Gentil MA, Ekberg H, Grinyó JM and Lloberas N.

Transplant International 2012 Dec 7

<u>STUDY II</u>. Do drug transporter (ABCB1) SNPs and P-glycoprotein function influence cyclosporine and macrolides exposure in renal transplant patients? Results of the pharmacogenomic substudy within the Symphony study. Transpl Int. 2013 Feb;26(2):177-86

The objective of this study was to investigate the association of different ABCB1 polymorphisms (C3435T, G2677T, C1236T and T129C) with Pgp activity and exposure of different immunosuppressive drugs in renal transplant patients from eight different centers. This substudy of pharmacogenomics was part of the Symphony study. The main objective of the Symphony trail was to establish the differences on MPA and its metabolites exposure among the four immunosuppressive groups: MMF in combination with normal or low doses of CsA, low doses of Tac and low doses of SRL. The study was evaluated in seventy patients: CsA (n=30), tacrolimus (n=13) and sirolimus (n=23). For the pharmacokinetic analysis, the AUC_{0-12} of all immunosuppressants was compared at each time along the follow-up. For this purpose, 11 blood samples were collected for each point: before the first MMF administration of the day and up to 12h post-dose. To perform the study, patients were genotyped for SNPs in ABCB1 gene and moreover, Pgp activity was evaluated in PBMCs using the Rho123 efflux assay.

Results

1. Descriptive analysis.

Nor demographic variables (sex and age) neither biochemical parameters (creatinine and albumin) revealed differences among groups of patients according to treatment (ns) (table 2 in the article).

2. Haplotype analysis.

First, linkage disequilibrium (as measured by r^2) was computed for each pair of SNPs within each gene and we found a positive correlation among the three SNPs of ABCB1 gene: C3435T-G2677T (r^2=0,91 and P=0,001), C3435T-C1236T (r^2=0,96 and P=0,0001), and G2677T-C1236T (r^2=1,0 and P=0,0001).

Then, in the haplotype-based analysis we observed in the additive model which was not significant (P=0,09) for TTT but was significant for the recessive model (P=0,026), with similar results to those in the genotype analysis. Pgp activity value in individuals with TTT haplotype (39% frequency) was 16 units less than Pgp activity in wild type individuals (table 3 in the article).

3. Influence of the ABCB1 SNPs on Pgp activity and immunosuppressive agents:

A. <u>ABCB1 SNPs and Ppg activity</u>

This table shows the prevalence of each SNP.

	CC/GG	CT/GT	TT
C3435T (rs1045642)	24,07 %	59,26 %	16,67 %
G2677T (rs2032582)	35,9 %	52,8 %	11,3 %
C1236T (rs1128503)	34 %	50 %	16 %
T129C (rs3213619)	-	10%	90%

- Pgp activity was influenced by the first three ABCB1 polymorphisms but not by T129C SNP. We did not find any significant differences between genotypes (figure 1 in the article).

- We observed that TT carrier patients on C3435T, G2677T and C1236T SNPs (also described as Pgp-low pumpers) showed lower Pgp activity than non carriers. The ABCB1 C3435T genotype showed higher Pgp activity in the CC and CT subjects compared with homozygous T carriers patients (CC: 48,33±4,69; CT: 45,90±3,18; TT: 31,62±3,37; P=0,02) (figure 1 in the article). Pgp values are means (MESF) units ±SD. Similar results were found for both G2677T and C1236T SNPs.

B. Immunosuppressive agents and Pgp activity

Variations of Pgp activity depending on immunosuppressive therapy were found. Pgp activity was evaluated in isolated PBMCs obtained from patients at the steady state of immunosuppression treatment at 3 months after renal transplantation.

- We found lower Pgp activity in patients with CsA (n=28) compared with macrolides (n=26) (P=0,04) (figure 2 in the article). Then, we also analyzed macrolides groups to study possible differences in the Pgp

activity. We analyzed individually the effect of Tac and SRL. Our results showed that CsA patients had lower Pgp activity than Tac patients (39,31±2,72 vs 55,06±8,57 P=0,02; respectively) and SRL patients (ns) (figure 2 in the article). Additionally, patients treated with Tac showed higher Pgp activity than SRL patients but this difference did not reach statistically significant levels.

- Taking in to consideration our previous study where Pgp activity values in healthy volunteers was 45±4,98. We demonstrated that transplant patients treated with macrolides showed similar Pgp activity than healthy volunteers. Meanwhile, patients treated with CsA showed a significant decrease in Pgp activity (P<0,05).

C. ABCB1 SNPs and immunosuppressive agents on Pgp activity

- Genotypes were sorted into high pumpers (CT and CC) and low pumpers (TT) correlating with Pgp function. Independently of immunosuppressor treatment, as we expected, we found that high pumpers patients showed more Pgp activity than low pumpers (figure 3 in the article).

- In C3435T SNP, high pumper patients treated with CsA showed lower values of Pgp activity than the macrolides group (P<0,05).

- In C3435T SNP, both CsA and macrolides high pumper patients showed higher Pgp function than lower pumpers (P<0,05) (figure 3 in the article). The same results in Pgp activity profile were observed for G2677T and C1236T SNPs (data not shown).

- In contrast, high pumper patients with T129 SNP were the TT heterozygotes and displayed the opposite behavior in Pgp expression compared with the other three SNPs. Any statistical difference was observed (data not shown).

4. Correlation between pharmacokinetic parameters of CsA (AUC and Cmin) and Pgp activity.

Considering the risk group those that showed a high pumper genotype and low doses of CsA, we evaluate this sub-group independently. We found a negative correlation between CsA AUC and Cmin and Pgp activity at 1 month post-transplant (figure 4 in the article). The same results were observed after 7 days, 3 and 12 months post-transplant (data not shown) by any of SNPs.

- No correlation was seen in low pumper patients exposed to high doses of CsA, probably because of the low number of patients.

- Results did not show any correlation between the macrolides treated patients (Tac and SRL) AUC or Cmin and Pgp activity neither at 7 days, first month post-transplant neither at 3 or 12 months.

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ORIGINAL ARTICLE

Do drug transporter (ABCB1) SNPs and P-glycoprotein function influence cyclosporine and macrolides exposure in renal transplant patients? Results of the pharmacogenomic substudy within the symphony study

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Authors declare that they have had no involvements that might raise the question of bias in the work reported or in the conclusions, implications, or opinions stated.

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Summary

The function of the efflux pump P-glycoprotein (Pgp) and ABCB1 single nucleotide polymorphisms (SNPs) should be considered as important tools to deepen knowledge of drug nephrotoxicity and disposition mechanisms. The aim of this study is to investigate the association of C3435T, G2677T, C1236T, and T129C ABCB1 SNPs with Pgp activity and exposure to different immunosuppressive drugs in renal transplant patients. Patients included in the Symphony Pharmacogenomic substudy were genotyped for ABCB1 SNPs. According to the design, patients were randomized into four immunosuppressive regimens: low and standard dose of cyclosporine (n = 30), tacrolimus (n = 13), and sirolimus (n = 23) concomitantly with mycophenolate and steroids. Pgp activity was evaluated in PBMC using the Rhodamine 123 efflux assay. TT carrier patients on C3435T, G2677T, and C1236T SNPs (Pgp-low pumpers) showed lower Pgp activity than noncarriers. Pgp-high pumpers treated with cyclosporine showed lower values of Pgp function than macrolides. There was a negative correlation between cyclosporine AUC and Pgp activity at 3 months. Results did not show any correlation between tacrolimus and sirolimus AUC and Pgp activity at 3 months. We found an important role of the ABCB1 SNPs Pgp function in $CD3^+$ peripheral blood lymphocytes from renal transplant recipients. Pgp activity was influenced by cyclosporine but not macrolides exposure.

Introduction

The human multidrug-resistance MDR1 gene, also known as ABCB1, encodes for P-glycoprotein (Pgp), a 170 kDa membrane protein first described in drug-resistant cells. Pgp has been extensively studied both for its role in normal physiology and for its potential role in clinical drug resistance [1]. This protein acts as a primary active transporter, playing

an important role in protecting tissues from toxic xenobiotics and endogenous metabolites. Pgp effectively modulates the absorption, cellular metabolism, and toxicity of pharmacological agents [2,3]. As a drug-efflux pump, it extrudes a range of hydrophobic drugs from cells contributing to drug disposition in humans and reducing the bioavailability of many oral medications [4–6]. Pgp expression could be an important component of a complex detoxifying system in kidney against xenobiotics or in regulating the traffic of metabolites responsible for the nephrotoxicity against different drugs [7]. Pgp also modulates cells of the immune system, particularly interfering with dendritic cell maturation and T-cell activation [8–10]. This multifaceted involvement in drug disposition, cancer drug resistance, and regulation of the immune response makes Pgp an attractive target for further investigation to increase understanding of its function in drug pharmacokinetic strategies in cells and tissues.

The key proteins involved in biotransformation and transport of tacrolimus (Tac) and cyclosporine (CsA) include cytochrome P450IIIA (CYP3A) and ABCB1. There are several clinical agents broadly used as substrates, inhibitors, or inducers of the Pgp, including immunosuppressive drugs [11]. Therefore, the degree of expression and function of ABCB1 could directly influence the therapeutic effectiveness of such agents, affecting their bioavailability. Single nucleotide polymorphisms (SNPs) in ABC drug-efflux pumps may play a role in response to drug therapy and disease susceptibility. The effect of various genotypes and haplotypes on the expression and function of these proteins is not yet clear, and their real impact remains controversial [12,13].

In the past ten years, more than 50 SNPs have been identified in the gene encoding for ABCB1. The SNPs that have been most widely studied are the C-T transition at position 3435 within exon 26, the C-T transition at position 1236 within exon 12, and the G-T/A transition at position 2677 within exon 21 [14–16]. These SNPs have also been the ones most frequently described as associated with Pgp function on Tac and CsA absorption in renal transplant recipients. Anglicheau *et al.* [17] demonstrated that SNPs in ABCB1 gene were associated with the Tac requirements, and therefore transplant population with Tac treatment should be genotyped for ABCB1 SNP. Moreover, ABCB1 SNPs are also associated with CsA exposure in the first post-transplant week [18]. Furthermore, authors suggest that the nephrotoxicity related to Tac may also be enhanced by donor and recipient ABCB1 polymorphisms [19]. The inter-individual differences in the pharmacokinetics of calcineurin inhibitors have been related to inter-individual heterogenicity in enzymatic activity of Pgp. There are also several clinical studies on ABCB1 genotype related to Pgp expression and function but the results are quite controversial [20–26].

Considering that ABCB1 polymorphisms may partly explain the large inter-individual variations in the pharmacokinetics of the immunosuppressors, we investigated the effect of the four SNPs in the ABCB1 gene (exon 1b T-129C, exon 12 1236C>T, exon 21 2677G>T, and the exon 26 3435C>T).

Materials and methods

This pharmacogenomic study is within the framework of the Symphony trial. It was conducted in accordance with the Declaration of Helsinki and the International Conference on Harmonization-Good Clinical Practice Guidelines (ICH-GCP), and with local ethical committee or institutional review board approval at each center. All patients provided written informed consent before inclusion in the pharmacogenomic substudy. The main objective of the Symphony trial was to establish the differences on MPA and its metabolites exposure among the four immunosuppressive groups: MMF in combination with full or reduced doses of CsA, Tac, or sirolimus. A secondary objective was a pharmacokinetic population analysis and the pharmacogenetic evaluation of genes involved in immunosuppressors exposure.

Reagents

Annexin-V PE (AnnV) (BD Pharmingen, San José, CA, USA), 7-aminoactinomycin D (7-AAD) (Sigma-Aldrich, Madrid, Spain), binding buffer (BB) (Medical and Biological Laboratories, Nagoya, Japan), phosphate buffered saline (PBS) (PAA, Cambridge, UK), Biotarget-1 (Biological Industries, Israel), Rhodamine 123 (Molecular Probes Inc., Eugene, OR, USA). CD3-APC conjugated Mouse anti-Human monoclonal antibody (BD Pharmingen). PSC833 (kindly provided by Novartis, Basel, Switzerland).

Subjects

Seventy renal transplant recipients (58.6% men; mean age: 47.9 ± 11.8 years) from eight Spanish centers of the Symphony study were included in the Pharmacogenomic substudy. Four patients were withdrawn because of logistic pitfalls. Patients were originally randomized to four branches of immunosuppressive regimen, all of them consisting of daclizumab induction, mycophenolate mofetil and corticosteroids potentiated by either low dose of CsA, standard dose of CsA, Tac, or sirolimus (SRL). Low and standard dose of CsA were combined into one group (CsA) for the statistical analyses, yielding three treatment groups according to immunosuppressive regimen: CsA (n = 30), Tac (n = 13), and sirolimus (n = 23).

Immunosuppressive regimes

The MMF daily dose range across the branches of immunosuppressive regimen was 1680–1946 mg over the first 3 months of treatment. CsA mean daily dose on Day 7 was 425 mg decreased to 222 mg (±81 mg) and 183 mg (±79 mg), respectively, by Month 3. These doses corresponded to median trough levels by Day 7, Month 1 and 3 of 292, 218, and 164 ng/ml for the standard-dose group, and 75.5, 109, and 80.5 ng/ml for the low-dose group, respectively. During the first 3 months, the Tac and SRL range daily doses were 4.3–5.8 mg and 2.9–3.5 mg, respectively (median trough levels by Day 7, Month 1, and Month 3: 8.1, 7.7 and 7.1 ng/ml, and 4.6, 7.5 and 7.8 ng/ml, respectively). The mean exposure (AUC_{0-12}) to CsA, Tac, or SRL as appropriate during the first 3 months in the high-dose CsA, low-dose CsA, Tac, and SRL groups were 4842.3–9230.7 ng h/ml, 2796.2–3601.6 ng h/ml, 129.3–152.3 μg h/ml, and 134.1–160.9 μg h/ml, respectively.

Pharmacokinetic analysis

The AUC_{0-12} of MPA and its metabolites between treatment groups was compared at each time along the follow-up. Pharmacokinetic data were collected on Day 7 and on Months 1 and 3 post-transplant. For this purpose, at each visit, 11 blood samples were collected: before the first MMF administration of the day [predose (Time 0)] and up to 12 h postdose (at 20, 40, 75 min and 2, 3, 4, 6, 8, 10, and 12 h postdose). Pharmacokinetic analysis of MPA was carried out with a standard no compartmental model using WinNonlin. All AUC results were dose-corrected at 2 g/day to obtain the right correlation in the pharmacokinetic dates.

For the analysis of pharmacokinetic interactions between drugs, C_{max} and AUC_{0-12} values for MPA and its metabolites, CsA, Tac, and SRL were normalized by the dosage of the medication taken prior to blood sampling [27].

Isolation of peripheral blood lymphocytes

Peripheral blood mononuclear cells (PBMCs) were isolated from blood samples by Ficoll-Paque Plus density gradient centrifugation according to the manufacturer's instructions. Cells were then washed and resuspended in Biotarget-1 medium supplemented with 1% L-glutamine, 1% sodium pyruvate, and 1% pen-streptomycin. Cell viability is always greater than 90%. Lymphocytes were frozen in lymphocyte freezing media as described previously [28].

Genotyping of ABCB1 polymorphisms

For genotyping we obtained DNA from 66 renal transplant patients. Patients were genotyped for SNPs in ABCB1 gene; exon 1b:T-129C (rs3213619), exon 12:1236C>T (rs1128503), exon 21:2677 G>T (rs2032582), and the exon 26:3435 C>T (rs1045642). DNA was extracted from a peripheral whole blood sample using the Wizard® Genomic DNA Purification Kit (Promega Corporation, Sydney, Australia) and was stored at −80 °C. Genotyping procedures were performed with the MassARRAY™ SNP genotyping system (Sequenom Inc., San Diego, CA, USA). The method involves multiplex PCR and single base extension assays, designed by the AssayDesigner software (Sequenom Inc.), and followed by mass spectrometry analysis with the Bruker Autoflex MALDI-TOF mass spectrometer (Bruker Daltonics, Billerica, MA, USA). Spectral output was analyzed and checked using MassARRAY™ Typer 3.4 software (Sequenom Inc.). The primers and the iPlex assay are listed in Table 1.

Measurement of Pgp function in PBMCs

Intracellular Rho123 uptake

Human PBMCs (1×10^6 cells) were incubated with Rho123 at a final concentration of 200 ng/ml for 30 min at 37 °C 5% CO_2, avoiding light exposure, in the presence or absence of the specific inhibitor PSC833 (10 μM). At the end of the Rho123 uptake, cells were kept on ice to stop Pgp activity and then washed twice with ice-cold PBS without Ca^{2+} and Mg^{2+} to remove extra cellular Rho123. PSC833 was also maintained during the washes to avoid periods of noninhibition. Cells were diluted in 50 μl of BB containing 2 μl of CD3-APC and incubated on ice for 20 min. After washing, 250 μl of BB containing 2 μl AnnV (to detect apoptotic cells) was added to all samples and incubated for 15 min on ice, avoiding light exposure. A total of 2 μl of 7-AAD (to detect cells with membrane damage) was added prior to flow cytometry analysis.

Table 1. Primer properties in a 4-SNP iPlex assay for sequenom SNP genotyping.

SNP	Exon	rs	2nd-PCRP	1st-PCRP	UEP_MASS
C3435T	26	1045642	ACGTTGGATGACTGCAGCATTGCTGAGAAC	ACGTTGGATGTATGTTGGCCTCCTTTGCTG	5048.3
G2677T	21	2032582	ACGTTGGATGTGCAATAGCAGGAGTTGTTG	ACGTTGGATGCATATTTAGTTTGACTCACC	6027.9
C1236T	12	1128503	ACGTTGGATGGTTTTTTTCTCACTCGTCCTG	ACGTTGGATGCACAGCCACTGTTTCCAACC	6646.3
T129C	1b	3213619	ACGTTGGATGCTTCGCTCTCTTTGCCACAG	ACGTTGGATGCCTCTGCTTCTTTGAGCTTG	7070.6

Flow cytometry analysis

Flow cytometry was performed on a FACSCalibur flow cytometer (Becton, Dickinson and Company, BD, Franklin Lakes, NJ, USA) with four-color analysis. Gating was based on forward scatter and side scatter dot plots by encircling populations with amorphous regions and then excluding dead cells (life gate) by 7-ADD counterstaining and Annexin-V. We monitored the linearity of the flow cytometer measurements daily using calibration standard beads (K0110; Dako, Glostrup, Denmark). These quantitative measurements included mean fluorescence intensity in linear values, which were transformed into calibrated values of molecules of equivalent soluble fluorochrome (MESF). A calibration curve ($\log = a \times \log + b$) was constructed to convert fluorescence measurements to MESF units. Data were analyzed using Cell Quest software. Each experiment was performed in duplicate.

Statistical analysis

Demographic variables, baseline characteristics, and transplantation-related data were described by frequencies and mean. Nonparametric statistics (Kruskal–Wallis, and chi-squared test) were applied to study differences in baseline data according to immunosuppressive regimens. Allele and genotype frequencies for the various SNP were assessed for deviation from Hardy–Weinberg equilibrium. Frequencies of genotypes and alleles were given with their 95% confidence intervals (95% CI). Pgp activity was confirmed to be normally distributed by the Kolmogorov-Smirnov method. Differences between the groups were assessed using an analysis of variance, followed by *post hoc* Fisher's test. P values were corrected for the number of variables compared according to the Bonferroni method. Low and standard dose of CsA, were combined into one group, and also Tac and SRL group were gathered as macrolides in some analysis. Linear regression analysis was used to analyze the impact of immunosuppressors pharmacokinetic parameters in Pgp activity. All statistical studies were performed with SPSS 12.0 K for Windows (12.0.1; SPSS Inc; Chicago, IL, USA).

Results

Distribution of ABCB1 SNPs and Pgp activity related to age and sex

Data from renal transplant recipients were collected. Descriptive analysis of the sample is shown in Table 2. Demographic variables (sex and age) did not reveal differences among groups of patients according to treatment. No significant differences for biochemical parameters (creatinine or albumin) at baseline were found.

We found a positive correlation among the three SNPs of ABCB1 gene: C3435T-G2677T ($r^2 = 0.91$, $P = 0.001$), C3435T-C1236T ($r^2 = 0.96$, $P = 0.0001$), and G2677T-C1236T ($r^2 = 1.0$, $P = 0.0001$). The genotype distribution was in Hardy-Weinberg equilibrium. The analysis of haplotypes in the additive model was not significant ($P = 0.09$) for TTT but was significant for the recessive model ($P = 0.026$), with similar results to those in the genotype analysis. The Pgp activity value in individuals with TTT haplotype (39% frequency) was 16 units less than Pgp activity in wild-type individuals (Table 3).

Influence of the ABCB1 SNP on Pgp activity

The prevalence of the ABCB1 SNP C3435T (rs1045642): CC, CT, and TT genotypes was 24.07%, 59.26%, and 16.67% of the recipients, respectively. ABCB1 G2677T (rs2032582): GG, GT, and TT genotypes were found in 35.9%, 52.8%, and 11.3%, respectively. For ABCB1 C1236T (rs1128503) SNP prevalence, CC, CT, and TT genotypes were detected in 34%, 50%, and 16% of recipients, respectively, and T129C (rs3213619) SNP with CT, and TT genotypes were identified in 10% and 90% of the recipients.

Pgp activity was influenced by the different ABCB1 polymorphisms analyzed. Pgp activity was higher in the carriers of 3435 CC genotype and 3435 CT heterozygous patients,

Table 2. Sample description at baseline ($n = 66$).

	CsA* ($n = 30$)	Tac† ($n = 13$)	SRL‡ ($n = 23$)	*P*-value
Age, mean, (SD)§	48.18 (10.18)	52.36 (13.43)	47.41 (12.03)	$P = 0.444$
Gender male, (%)¶	65.5	72.7	41.2	$P = 0.165$
Creatinine (mg/dl), mean (SD)§	8.52 (2.81)	8.17 (2.58)	6.89 (2.93)	$P = 0.289$
Albumin (g/dl), mean (SD)§	4.25 (0.45)	4.01 (0.55)	4.18 (0.80)	$P = 0.633$

SD, standard deviation.

*Standard immunosuppression with normal and lose dose of cyclosporine, mycophenolate mofetil (MMF), and corticosteroids (CS).

†Low dose of tacrolimus with daclizumab induction, MMF and CS.

‡Low dose of sirolimus with daclizumab induction, MMF and CS.

§Kruskal–Wallis test.

¶Chi-squared test.

Table 3. SNPStats, haplotypes analysis. Analysis of haplotypes in the recessive model ($P = 0.026$).

Coefficients	Coef	se	t.stat	*P*-value
(Intercept)	46.647	2.317	20.130	0.000
hap. TTT	−16.162	7.079	−2.283	0.026
hap. rare	−14.630	NA	NA	NA
Haplotypes	**loc.1**	**loc.2**	**loc.3**	**hap.freq**
haplo.base	C	G	C	0.50893
hap. TTT	T	T	T	0.39286
hap. rare	–	–	–	0.09821

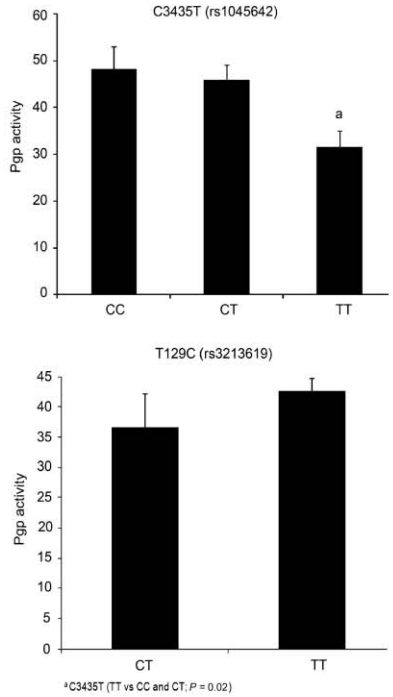

Figure 1 Pgp activity by flow cytometry analysis measured with MESF units of Rho123 fluorescence in $CD3^+$ T-cell population and the influence of two ABCB1 polymorphisms: C3435T (rs1045642) and T129C (rs3213619). Values are means (MESF) ± SD; $n = 54$ patients for C3435T (CC = 13; CT = 32 and TT = 9, $P = 0.02$), and $n = 51$ patients for T129C (CT = 5 and TT = 46, NS).

whereas the lowest activity was found in the carriers of 3435 TT genotype (CC: 48.33 ± 4.69; CT: 45.90 ± 3.18; TT: 31.62 ± 3.37; $P = 0.02$) (Fig. 1).

Similar results were found in the G2677T and C1236T SNPs. The ABCB1 G2677T genotype showed higher Pgp activity in the GG and GT subjects compared with homozygous T carriers patients (GG: 45.96 ± 3.79; GT: 45.71 ± 3.38; TT: 30.47 ± 4.52; $P = 0.04$). Carriers of 1236 CC genotype showed higher Pgp activity than CT heterozygous, which showed intermediate values, and TT homozygous which displayed the lowest activity (CC: 46.11 ± 4.11; CT: 43.71 ± 2.68; TT: 30.90 ± 3,33; $P = 0.02$). Pgp activity was not influenced by the T129C SNP. No significant differences were observed between genotypes (Fig. 1). All values are represented as means (MESF) ± SD.

Variations of Pgp activity depending on immunosuppressor therapy

Pgp activity was evaluated in isolated PBMCs obtained from patients in the Cmin of the pharmacokinetic study, at the steady state of immunosuppression treatment at 3 months after renal transplantation.

Results showed lower Pgp activity in patients with CsA ($n = 28$) compared with macrolides ($n = 26$) ($P = 0.04$). Considering that the two macrolides showed different effects on Pgp function, we further analyzed this group. CsA patients showed lower Pgp activity than Tac patients (39.31 ± 2.72 vs. 55.06 ± 8.57 $P = 0.02$, respectively) and SRL patients (39.31 ± 2.72 vs. 46.19 NS) (Fig. 2). In addition, Tac was also higher than SRL patients but this difference did not reach statistically significant levels. All patients received CsA, Tac, or SRL with steroids and MMF at fixed daily doses. Considering that the Pgp activity values in healthy volunteers in our area was 45 ± 4.98 [29], transplant patients with macrolides showed similar Pgp activity. Patients treated with CsA showed a significant decrease in Pgp activity ($P < 0.05$).

Influence of ABCB1 SNPs and immunosuppressor therapy on Pgp activity

Considering the immunosupressor treatment, the individual contribution of each SNP on Pgp activity increase was analyzed. Genotypes were sorted into high pumpers (CT and CC) and low pumpers (TT) correlating with Pgp function. High pumper patients showed more Pgp activity than low pumpers, independently of immunosuppressor treatment.

Pgp activity in high pumper patients treated with CsA in the C3435T SNP showed lower values of Pgp activity than the macrolides group ($P < 0.05$). However, in CsA low pumper Pgp showed a similar trend but there were no significant differences. Both CsA and macrolides high pumpers showed higher Pgp function than low pumpers ($P < 0.05$) (Fig. 3). The same results in Pgp activity profile were observed for G2677T and C1236T SNPs. In contrast, the high pumpers patients with T129 SNP were the TT heterozygote and displayed the opposite behavior in Pgp expression compared with the other three SNPs and any statistical difference was observed (data not shown).

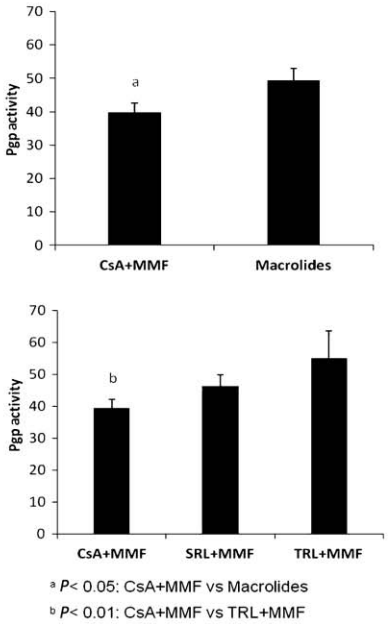

Figure 2 Variations of Pgp activity depending on immunosuppressor therapy; $n = 54$ patients for each SNP: gathered in CsA, $n = 28$ and macrolides, $n = 26$ ($n = 17$ for SRL and $n = 9$ for Tac).

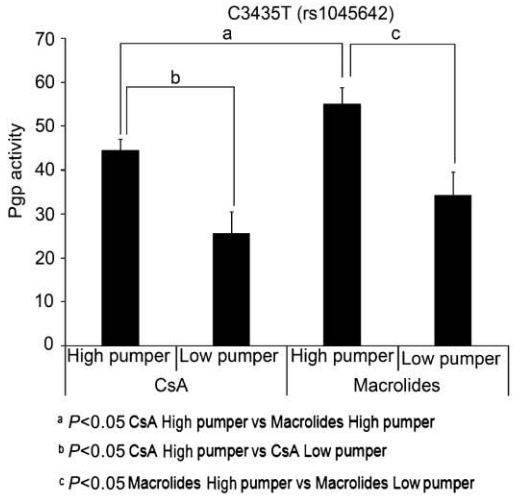

Figure 3 Influence of ABCB1 SNPs and immunosuppressor therapy on Pgp activity; $n = 54$ patients for the C3435T SNP (CsA, $n = 28$ and macrolides, $n = 26$).

Influence of Pgp activity and ABCB1 SNP in the CsA AUC and Cmin

We analyzed the correlation between different immunosuppressor pharmacokinetic parameters and Pgp activity. We focused on the sub-group of high pumpers and low CsA doses because these patients should have less cellular CsA concentration because of increased drug extrusion and lesser lymphocyte exposure. A negative correlation between CsA AUC and Cmin and Pgp activity was seen at 1 month (Fig. 4) with regression analysis between Pgp activity and AUC (R sq linear = 0.365; $P = 0.05$) and Pgp activity and Cmin (R sq linear = 0.44; $P = 0.01$). When low pumpers exposed to high doses of CsA were evaluated, no correlation was seen, probably because of the low number of patients. In addition, we did not find any correlation between the macrolides (Tac and SRL) AUC or Cmin and Pgp activity at 1 month.

Discussion

The bioavailability and metabolism of CsA and Tac are primarily controlled by efflux pumps belonging to the ABC transporter family and members of the cytochrome P-450 isoenzyme system. Several *in vitro* studies have identified immunosuppressors as substrates and/or inhibitors of Pgp [30–34]. As such they could alter the bioavailability of many concomitant drugs, causing potentially important drug interactions. So far, several SNPs have been identified in the ABCB1/MDR1 gene that might alter Pgp expression and function in humans, leading to clinical interest in the pharmacokinetics of immunosuppressors to improve their dosing in individuals [35]. Despite the association of SNPs with Pgp protein expression and function, indicating that Pgp is a major determinant in the absorption and disposition of drugs, there is still controversy as to whether the pharmacokinetics and pharmacodynamics of drugs could be modified by MDR1 genotypes/haplotypes [17,36–41].

Our results confirm a significant correlation between ABCB1 genotyping and Pgp activity in PBMCs from renal transplant recipients. Homozygous 3435 TT carrier subjects showed the lowest Pgp activity compared with 3435 CT and CC carriers. Although our results on CC $\geq$ CT > TT in relation to Pgp function corroborate the data reported by others [13,42–45], there are some discrepancies among authors, and the functionality of 3435 CT ABCB1 SNP remains a matter of controversy [7,46–50]. In our renal transplant population, we described the influence of ABCB1 SNPs on Pgp function in PBMCs, supporting results reported in other tissues where the functional effect of Pgp polymorphism was also demonstrated [35,42]. Blood samples were obtained from patients from different centers and all measurements were performed with frozen cells. In a previous study [28] we demonstrated that different working conditions did not affect Pgp activity. Measurement of rhodamine efflux from peripheral blood cells

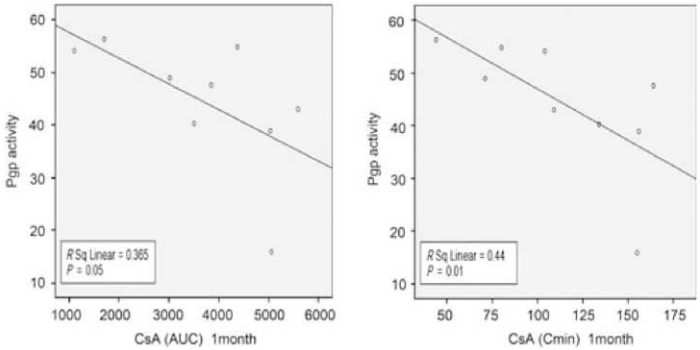

Figure 4 Influence of the Pgp activity and ABCB1 SNP in the CsA AUC and Cmin in high pumper low-dose renal transplant patients at 1 Month. Regression analysis between Pgp activity and AUC (R sq linear = 0.365; $P = 0.05$ and coef: -, 650) and Pgp activity and Cmin (R sq linear = 0.44; $P = 0.01$, and coef: -, 767); $n = 9$ patients high pumper and low doses of CsA with Pgp function data.

allows assessment of the polymorphisms effect not only on Pgp expression but also on Pgp activity.

Current immunosuppressive drugs are characterized by a narrow therapeutic index and large intra- and inter-individual variability both in pharmacokinetics and in pharmacodynamics. Pgp is an important component of a detoxifying system, and ABC transporters affect the bioavailability of their substrate drugs [7,51]. CsA was the first immunosuppressor shown to modulate Pgp activity in laboratory models and clinical trials, and intestinal Pgp has been shown to determine oral clearance of CsA [52]. Tac is both a substrate and an inhibitor for Pgp, less active than CsA. SRL is a Pgp substrate which may limit its intestinal absorption, and Pgp is also probably involved in its excretion [51]. Our group has shown an *in vitro* inhibitory effect of Pgp activity in T-cell subpopulations incubated with immunosuppressors, particularly CsA either alone or associated with SRL [29]. In the present study, patients with CsA showed lower Pgp activity compared with macrolides, both Tac and SRL. Interestingly, macrolides did not affect Pgp function, achieving similar values as those found in healthy volunteers. Thus, the results observed in patients replicated data observed in *in vitro* T-cell studies.

In view of the pharmacokinetic variability of immunosuppressors, pharmacogenomic research could help to improve drug dosage. Numerous studies have addressed the relevance of the MDR1 polymorphism in dose requirement, blood concentrations, chronic rejection, and nephrotoxicity in renal transplant patients treated with CsA and Tac. Studies supported that the dose requirements are influenced by ABCB1 polymorphism, although the use of ABCB1 genotype as a helpful marker in clinical practice remains unclear [17,37,45,53,54].

Some ABCB1 SNPs have been correlated with the *in vivo* activity of Pgp and should therefore be considered in renal transplant recipients treated with Tac [55,56]. So far, no authors have described an association of Pgp activity, MDR1 polymorphisms, and exposure to three immunosuppressors at 7 days, and 3, 6, and 12 months in renal transplantation. The present results demonstrate for the first time the existence of this correlation in a multicenter clinical study in which CsA, Rapa, and Tac exposure were well defined and co-medication drugs were specified. Our results show that patients on CsA and macrolides therapy display the same correlation pattern between Pgp activity depending on the genotypes of the three ABCB1 SNPs studied as observed in the general population (CC ≥ CT > TT). The values of Pgp activity in patients with CsA were lower than those for macrolides in the SNPs studied. Our results also confirm the inhibitory effect of CsA described by other authors [57–59].

The CsA is a Pgp modulator, and the variability of its absorption and disposition has been attributed to intestinal Pgp expression and activity. The movement of a drug through membranes is an essential step in absorption. Delays or loss of drug during absorption can introduce large variability in drug response. In this study, we found a negative correlation between Pgp activity in lymphocytes and CsA AUC and Cmin at steady state conditions in those patients with high activity of Pgp and low CsA doses. Regarding the correlation between exposure of CsA and Pgp activity we should consider that CsA is a potent Pgp inhibitor, so an increase in CsA exposure should lead to a decrease in Pgp activation. However, we must not overlook the intestinal effect. In the enterocyte, CsA is absorbed and excreted back, in part, to the intestinal lumen through specific proteins belonging to the ABC family. Therefore, the small intestine emerges as the first bottleneck in the entry of CsA into the organism. In addition, metabolism in the liver cannot be underestimated before the distribution to tissues, where ABC proteins are also present. All these processes influence the distribution and subsequent elimination of the drug from the organism. As a result of this complex interplay between enzyme activities and efflux transporters, the concentrations of CsA in plasma could also be affected. C3435T SNP correlates with lower intestinal

Pgp expression, and this in turn does directly affect the oral bioavailability of Pgp substrates. Thus, in individuals with lower intestinal Pgp concentrations the extent of drug absorption from the gastrointestinal tract should be higher. Consequently, an increase in the plasma levels in comparison to the remainder of the population would be observed.

We may conclude that in patients with a CT or CC nucleotide exchange in exon 26 (C3435T) with high Pgp activity on the apical surface of intestinal enterocytes, more CsA is removed from the cells, resulting in decreased bioavailability. In this context, high pumpers treated with low doses of CsA would show less drug exposure and this could affect the therapeutic response. In our study, we found a negative correlation between CsA exposure (AUC and Cmin) in plasma and Pgp activity, which might reflect the increasing CsA bioavailability because of enhanced Pgp blocking in the intestinal enterocytes. However, these results should be interpreted with caution because of the small sample size. Additional studies with larger sample sizes will be required to confirm the results. Genotyping research in this field may yield more refined immunosuppressive drug therapy while further exploring the role of ABC transporters as biomarkers in the measurement of immunosuppressive drug concentration.

Authorship

NL and JMG: designed research/study. NL and IL: performed research/study. HC, PGB, AC, MS, JMC, FO, JSP, MÁG and HE: contributed new reagents or analytic tools. NL, IL and JT: analyzed data. NL, IL and JT: wrote or contributed to the writing of the manuscript.

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Study III

Impact of small molecules immunosuppressants on P-glycoprotein activity and T-cell function.

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STUDY III. Impact of small molecules immunosuppressants on P-glycoprotein activity and T-cell function. J Pharm Pharm Sci. 2012 May;15(3):407-19.

Considering earlier studies in our group which describes the *in vivo* impact of the association of Rapa (mTORi) with two calcineurin inhibitors (CsA and Tac) on Pgp function and the involvement of Pgp in this immunosupressor-related renal toxicity, here, we analyzed the activity of Pgp on different T-cell subsets and we studied the effects of the same immunosuppressants in monotherapy and associated with rapamycin. Rho123 uptake, efflux and kinetic of extrusion in $CD4^+$ and $CD8^+$ subsets of peripheral blood mononuclear cells isolated from eight healthy volunteers were measured. We also studied the antigen-specific memory T-cell responses by measuring T-cell proliferation and cytokine secretion using an allogeneic mixed lymphocyte reaction.

Results

Pgp function was measured by flow cytometry using the following parameters:

- Intracellular Rho123 uptake:

- Groups treated with CsA and CsA+Rapa significantly decreased the intracellular Rho123 uptake compared to non-treated group and to the other immunossuppressants (Tac, Rapa, Tac+Rapa) both in $CD4^+$ and in $CD8^+$ T cells subsets (figure 2 in the article).

- Interestingly, we observed that $CD8^+$ T cell showed higher Rho123 uptake than $CD4^+$ subset.

- Kinetic extrusion:

- Different time points (0, 30, 60, 90 and 120min) were chosen to evaluate the kinetics of Rho123 extrusion according to each immunosuppressive treatment in T-cell population. We observed a noticeable slower efflux in CsA and CsA+Rapa compared to the others immunosuppressants (Tac, Rapa, Tac+Rapa) at 60, 90 and 120 minutes ($p<0,001$).

- In contrast, macrolides-treated T cells (Tac, Rapa, Ta+Rapa) showed an extrusion speed similar to the non-treated group (figure 3 in the article).

- In figure 4 in the article we showed a representative histogram of the kinetic profile of Rho123 extrusion that we observed. $CD4^+$ T cells displayed slower extrusion rate than $CD8^+$ T cells. After two hours of

efflux, there was still more than 50% of the Rho123 in the $CD4^+$ T cells whereas in $CD8^+$ T cells 70-80% of Rho123 had already been extruded.

-After that, we expressed Rho123 extrusion at different time-points in comparison with minute 0 (100%). These results were expressed as normalized values (table 1 in the article) and data were in percentages as mean±SE. We observed only for CD8 T cells significant differences in Rho123 extrusion between the non-treated group and CsA alone and associated with Rapa.

- Pgp activity:

- For $CD8^+$ T cells, Pgp activity values were significantly lower in groups treated with CsA than macrolides alone (Rapa, Tac) and associated groups (Tac+Rapa) versus non-treated group. In CD4 subset no significant differences were observed between groups (figure 5 in the article).

- t_{50} (Average rate of drug efflux):

- The average rate of drug efflux displayed a similar kinetic pattern than Pgp activity measured by Rho123 extrusion. T_{50} of $CD8^+$ T cells was faster than $CD4^+$ (figure 6 in the article).

- Both T-cell subpopulations showed longer time to efflux 50% of the drug in the CsA groups than the other treatments and the non-treated group confirming the inhibitory effect of CsA.

Additionally, we evaluate antigen-specific memory T-cell proliferation:

- Both specific Pgp inhibitor (PSC833) as well as all immunosuppressants diminished T-cell proliferation similarly.

- If we focused on $CD3^+$ subset, we observed between 5-15% of proliferation in all treated groups compared the 30-35% proliferation in non-treated stimulated group ($p<0,05$) (figure 7 in the article). Inhibition of T-cell proliferation was especially more evident in CD8 than in CD4 T cells ($p<0,05$).

- Both groups associated with Rapa (CsA+Rapa and Tac+Rapa) showed greater inhibition compared with these immunosuppressants in monotherapy (CsA and Tac).

Finally, we analyzed the cytokine secretion into supernatants by PBMCs in mixed lymphocyte reaction (MLR);

- We found that Th1 pro-inflammatory cytokines like IFN-Y and TNF-α were significantly reduced in all immunosuppressive agents and PSC833-treated groups than in those non-treated ($p<0,05$).

-Concerning Th2 cytokines, we observed that PSC833 and the immunosuppressive agents, except for CsA, diminished IL-6 secretion compared to the non-treated stimulated group. For IL-10, we found an important decrease in this cytokine secretion in groups treated with Rapa compared to the other groups, reflecting its important role in the modulation of IL-10 (figure 8 in the article).

Impact of Small Molecules Immunosuppressants on P-Glycoprotein Activity and T-cell Function

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ABSTRACT - Purpose. P-glycoprotein (Pgp) is a member of the ABC-transporter family that transports substances across cellular membranes acting as an efflux pump extruding drugs out of the cells. Pgp plays a key role on the pharmacokinetics of several drugs. Herein, we have studied the effects of immunosuppressants on Pgp function, assessing rhodamine-123 (Rho123) uptake and efflux in different T-cell subsets. **Methods.** Different immunosuppressants such as Cyclosporine (CsA), Rapamycin (Rapa) and Tacrolimus (Tac) were used to assess the *in vitro* effect on Pgp function of main T-cell subsets among healthy volunteers. We measured Rho123 uptake, efflux and kinetic of extrusion in $CD4^+$ and $CD8^+$ subsets by flow cytometry. Antigen-specific memory T-cell responses were assessed by measuring T-cell proliferation and cytokine secretion using an allogeneic mixed lymphocyte reaction. **Results.** Rho123 uptake in groups treated with CsA and CsA+Rapa was significantly decreased compared to non-treated group and the other immunosupressants in both T cells subsets. Pgp activity was also reduced in CsA and CsA+Rapa compared to the other immunosupressants but it was only significant in the CsA group for $CD8^+$ subset. Kinetic extrusion of Rho123 by Pgp in all groups was faster in $CD8^+$ T cells. All immunosuppressants and the specific Pgp inhibitor PSC833 diminished antigen-primed T-cell proliferation, especially $CD8^+$ T-cell subset. **Conclusions.** Our data indicate that small molecules immunosuppressants, especially CsA, inhibit Pgp activity and T-cell function being the $CD8^+$ T cells more susceptible to this effect. These findings support the importance of Pgp when designing combined immunosuppressive regimens.

INTRODUCTION

The MDR1 gene product P-glycoprotein (Pgp) is the most studied membrane protein of the large mammalian ABC transporter family (1). These plasma membrane proteins play a major role in determining drug uptake, distribution and excretion processes, and they play a key role in drug pharmacokinetics (2). Pgp has been described as a possible mediator to explain variability in toxicity patterns associated with immunosuppressive therapies (3). There is an increasing interest in the precise role of Pgp in T lymphocyte activation and antigen presenting cell function. Pgp actively extrudes a wide variety of structurally diverse cytotoxic compounds out of the cell. It is widely expressed in the epithelial cells of the kidney, liver and intestine, and in the endothelial cells of brain and placenta (4). Also, its expression has been described in lymphoid cell populations from human bone marrow and peripheral blood (5). Specifically, it has been shown on the membrane of pluripotent stem cells, monocytes, dendritic cells, $CD4^+$ and $CD8^+$ T lymphocytes, NK cells, and B lymphocytes, suggesting that Pgp may influence cell-mediated immune responses (6-9).

ABC transporters may be therapeutic targets in organ transplantation, but the importance of Pgp in immune function still remains unclear (10-13). Up-regulation of Pgp in T-cell populations could result in decreasing intracellular drug concentrations rendering these cells resistant to immunosuppressive therapy despite appropriate plasma drug exposure (14).

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Pgp and cytochrome P450 3A4 (CYP3A4) are determinants of the bioavailability of widely used immunosuppressants such as cyclosporine A (CsA), tacrolimus (FK506/Tac) and sirolimus (Rapa) (15). These immunosuppressants themselves, as substrates and/or inhibitors of Pgp, could alter the bioavailability of many concomitantly drugs being potentially important in drug interactions (16,17). CsA, remains as an important immunosuppressive drug in solid organ transplantation and has been described as a potent Pgp inhibitor (18). Conversely, the role of Tac and Rapa on Pgp function still remains undefined. Tac is 10-100 times more potent than CsA as an immunosuppressive agent (19) with effective concentrations of 0.003-0.019μM compared to 0.083-0.208 μM for CsA (20, 21). Rapa is structurally similar to Tac that modulates the immune response at a different level to calcineurin inhibitors (CNIs). The combination of CNIs, either CsA or Tac, with mTOR inhibitor (mTORi) exert a potent immunosuppressive effect, but potentiates CNI-related nephrotoxicity (18, 22).

In a previous study (23) we investigated the impact of the association of Rapa with Tac or CsA on Pgp expression in a rat nephrotoxicity model, showing a renal over-expression related to CsA exposition. The aim of the current study was to evaluate the effect of several CNIs and mTORi on Pgp function evaluated by Rho123 uptake and efflux measurements in $CD4^+$ and $CD8^+$ T-cell subsets.

Therefore, CsA in monotherapy and associated with Rapa decreases Pgp activity in $CD8^+$ T-cells. Immunosuppressive drugs and Pgp inhibitor significantly reduced the $CD8^+$ T-cell proliferation and cytokine release. The Pgp inhibition could be a novel therapeutic target to avoid allograft rejection in solid organ transplantation.

MATERIAL AND METHODS

Reagents and antibodies

The following monoclonal antibodies were used to characterize cell subpopulations: mouse anti-human CD4-APC, mouse anti-human CD3-APC and mouse anti-human CD8-PE were purchased from eBioscience (San Diego, CA, USA). PSC833 was kindly provided by Novartis (Basel, Switzerland). FK506 (Tacrolimus) was a gift from Astellas Pharma Inc. Cyclosporine and rapamycin were purchased from Sigma-Aldrich (Madrid, Spain) as 7-Aminoactinomycin D (7-AAD). Appropriate stock solutions of drugs were prepared in culture medium RPMI 1640 (Biological Industries, Israel) and diluted in 100% dehydrated ethanol. Phosphate Buffered Saline (PBS) was obtained from PAA (Cambridge, UK). Biotarget-1 is a specific culture medium for lymphocytes which was used; it was purchased from Biological Industries. Rho123 was purchased from Invitrogen, Molecular Probes Inc., USA as 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE). Cytometric Bead Array (CBA) Human Th1/Th2/Th17 kit was purchased from BD.Biosciences Pharmingen (San Diego, CA).

Isolation of peripheral blood lymphocytes

Peripheral blood mononuclear cells (PBMCs) were isolated from healthy volunteers (n=8) by Ficoll-Paque Plus (GE Healthcare Bio Sciences AB, Uppsala, Sweden) density gradient centrifugation according to the manufacturer's instructions. Cells were then washed and resuspended in Biotarget-1 medium supplemented with 1% L-Glutamine, 1% Sodium Pyruvate and 1% Pen-Streptomycin. Cell viability was confirmed by trypan blue exclusion and was always greater than 90%. Lymphocytes were frozen in freezing medium as described previously (24).

The study groups were defined according to the different immunosuppressive drugs used: cyclosporine (CsA 2.5μM), rapamycin (Rapa 0.25μM), tacrolimus (Tac 0.1μM) and two more groups with Rapa associated with CNIs. A non-stimulated, non-treated (negative control) and non-treated stimulated (positive control) were added (n=8 for group). Stimulated cells are exposed to allogeneic T-cell depleted lymphocytes. Drug concentrations were obtained from Pawarode et al. choosing those in the upper level (25).

Informed consent was signed from each healthy volunteer after the nature and possible consequences of the study were fully explained. Written informed consent was obtained in accordance with the Hospital Universitari de Bellvitge Ethic Committee.

Flow cytometry analysis

Flow cytometry was performed on a FACSCalibur flow cytometer (Becton, Dickinson and Company, BD) with four-color analysis. The four optical filters on the FACS Calibur were Fl 1 at 530±15 nm (for Rho123 uptake), Fl 2 at 585nm (for CD8-PE), Fl 3 at 670 nm (for 7-AAD) and Fl

4 at 660 nm (for CD4-APC). Acquisition was stopped when 10,000 gated events were collected in the fluorescence cell count histogram. Gating was based on forward scatter and side scatter dot plots by encircling populations with amorphous regions and then excluding dead cells (life gate) by 7-ADD counterstaining (under dye-compatibility conditions). We monitored the linearity of the flow cytometer measurements daily to show the results in accurate standard fluorescence units by using calibration standard beads (K0110, Dako, Glostrup, Denmark) to decrease the variation in flow results and to provide the reference dye fluorescence for quantitative measurements. These quantitative measurements included mean fluorescence intensity (MFI) in linear values, which were transformed, according to the manufacturer's instructions, into calibrated values of molecules of equivalent soluble fluorochrome (MESF). A manual calibration curve (log = a × log + b) was constructed to convert fluorescence measurements to MESF units. Data were analyzed using Cell Quest software.

Measurement of Pgp function by flow cytometry

Pgp function was estimated using three parameters: 1) considering that ABC transporters such as ABCB1 may handle not only the efflux but also the influx of their substrates, the intracellular uptake of Rho123 was the first evaluated; 2) Cells pump out Rho123 by passive diffusion through Pgp (26), so Rho123 efflux assays were used to evaluate Pgp activity; and 3) The time needed to efflux 50% of Rho123 out the cells ($t_{50;}$ average rate of drug efflux) was used to define the efflux kinetic parameter (Fig. 1).

Intracellular Rho123 uptake

The intracellular Rho123 uptake was established after 30 min of co-incubation of cells with Rho123. Human PBMCs ($1x10^6$ cells) were suspended in 1ml of supplemented Biotarget-1 and incubated o/n at 37ºC 5%CO_2 with the different immunosuppressive drugs. Finally, cells were washed with PBS and resuspended in 1mL Biotarget-1. To allow Rho123 uptake, cell suspension was incubated with Rho123 at a final concentration of 200ng/mL for 30 min at 37ºC 5%CO_2, avoiding light exposure, in the presence or absence of PSC833 (10µM). PSC833 was used as a Pgp-specific competitive inhibitor to estimate the amount of Rho123 accumulated after Pgp inhibition. At the end of the uptake period, cells were kept on ice to stop Pgp activity and then washed with ice-cold PBS without Ca^{2+} and Mg^{2+} to remove extra cellular Rho123. PSC833 was also maintained during the washes to avoid periods of non-inhibition.

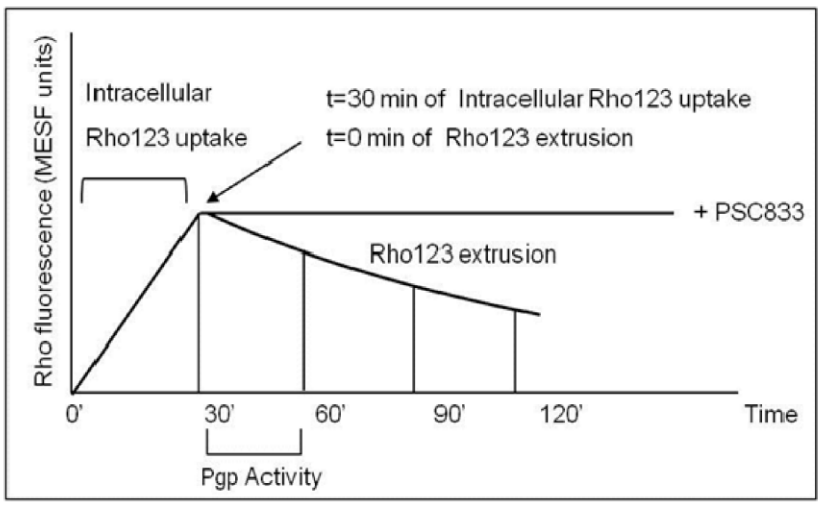

Figure 1. Pgp function measured by flow cytometry. 1) Intracellular Rho123 uptake (30 min). 2) Pgp activity (Rho123 uptake) was measured after the first 30 min. 3) Kinetic profile of Rho123 extrusion ($t_{50;}$ average rate of drug efflux) was evaluated at different time points (0, 30, 60, 90 and 120 min) according to each immunosuppressive treatment.

After washing, $0.5x10^6$ cells of each treatment were stained for phenotypic expression of surface $CD4^+$ and $CD8^+$. After 20 min incubation on ice, cells were washed with PBS supplemented with 2% Fetal Bovine Serum and immediately acquired on the flow cytometer. The remaining half of the cells was resuspended in 1ml Biotarget-1 without Rho123 to allow efflux of the dye out of the cell, for 120 min at 37ºC 5%CO_2. At the end of the efflux incubation, cells were kept on ice to prevent further reaction and stained for phenotypic expression of surface $CD4^+$ and $CD8^+$ and then immediately acquired on the cytometer. To detect cells with membrane damage (necrotic cells) 7-AAD was added to all samples and incubated for 15 min on ice, avoiding light exposure prior to flow cytometry analysis in order to exclude dead cells from the analysis.

Determination of Pgp activity

Basal Pgp activity was estimated by Rho123 fluorescence at 30 min after an established loading period considered enough time to achieve the intracellular steady-state level but not so long as to induce *de novo* activity (27). In our experiments, Pgp activity was estimated on the kinetic of Rho123 uptake and extrusion using Rho123 fluorescence (MESF units).

Kinetic of Rho123 extrusion

Different time points (0, 30, 60, 90 and 120 min) were chosen to evaluate the early, the linear and the late phases of the efflux for $CD4^+$ and $CD8^+$ T-cell subsets. For kinetic measurements we calculated the time needed to efflux 50% of Rho123 out of cells ($t_{50;}$ average rate of drug efflux).

Antigen-specific memory T-cell proliferation assay by flow cytometry

To asses the effect of the different immunosupressive agents and the specific Pgp inhibitor (PSC833) on memory/effector T-cell responses an alloantigen-specific memory T-cell co-culture was done. For this purpose a short term antigen-specific memory T-cell line was produced by mixing $2x10^5$ PBMC from a responder subject with $2x10^5$ stimulator T-cell depleted (Rosette Sep Human CD3 Depletion Cocktail Kit; Stem Cell Technologies, Grenoble, France) and irradiated (40 Gy) PBMCs obtained from an allogeneic subject. Cells were incubated in a final volume of 200µL in a complete medium consisted of RPMI-1640 supplemented with 10% heat-inactivated FBS, 1% L-Glutamine and 1% Pen-Streptomycin in round-bottom 96-well cell culture plates (Costar, Cambridge, MA). The plates were then placed in a humidified 37ºC, 5%CO_2 incubator for 6 days. Then, cells were harvested and incubated o/n with the different immunosuppressants and PSC833 at the same concentrations than Pgp function assay. $2x10^5$ of the resulting primed responder PBMCs for each treatment were labeled with succinimidyl ester of carboxyfluorescein diacetate (CFSE 5µM) as described (28) and were plated again in duplicate wells with the same stimulator cells in a ratio of 1:1 for 5 days in the same conditions. Culture supernatants were harvested after this period of time, snap frozen and stored at -80ºC until analysis. As positive responder control, PBMCs were stimulated with 1 µg/ml phytohemagglutinin (PHA). Proliferation of responder cells was evaluated by FACS analysis of T-cells subsets using monoclonal antibodies specific for CD3, CD4 and CD8. Experiments are representative of four independent assay.

Cell cytokine analysis by Flow Cytometry

Cytokine secretion into supernatants by PBMCs was analyzed by flow cytometry with a fluorescent bead assay (CBA Human Th1/Th2/Th17 kit) after staining according to the manufacturer's instructions. Cytokines quantification was performed on non-treated stimulated and treated with immunosuppressants and the specific Pgp inhibitor (PSC833) in PBMCs after MLR. Experiments are representative of three independent assays.

STATISTICAL ANALYSIS

All data are presented as mean±SE. Group means were compared with analysis of variance (ANOVA) for parametric values, followed by post hoc Fisher's test. All *p*-values were two-tailed and a *p*-value of less than 0.05 was considered statistically significant.

RESULTS

Intracellular Rho123 uptake of $CD4^+$ and $CD8^+$ T-cell subsets

Intracellular Rho123 uptake in CsA groups (CsA and CsA+Rapa) was significantly decreased compared to non-treated group and the other immunosupressants in $CD4^+$ and $CD8^+$ T cells. Considering the intracellular Rho123 uptake after 30 minutes of incubation as the baseline

intracellular value, $CD8^+$ T cells showed higher Rho123 uptake than $CD4^+$ (Fig. 2).

Pgp activity of $CD4^+$ and $CD8^+$ T-cell subsets

The study of the kinetics of Rho123 extrusion in T-cell population displayed a noticeable slower efflux in CsA and CsA+Rapa compared to the others immunosupressants at 60, 90 and 120 minutes ($p<0.001$). In contrast, macrolides-treated T cells showed a similar extrusion speed to the non-treated group (Fig. 3).

Thereafter, we further analysed the kinetic pattern of Rho123 efflux on $CD4^+$ and $CD8^+$ T-cell subsets. As shown in figure 4, $CD4^+$ T cells displayed slower extrusion rate than $CD8^+$ T cells, showing a similar kinetic than Rho 123 uptake. After two hours of efflux, there was still more than 50% of the Rho123 in the $CD4^+$ T cells, whereas in $CD8^+$ T cells 70-80% of Rho123 had already been extruded. In both T-cell subsets additional treatments with PSC833 increased fluorescence (data not shown) corroborating the role of Pgp on T cells. Normalized values of Rho123 extrusion at different time-points expressed in percentages are depicted in table 1.

We observed significant differences in Rho123 extrusion between non-treated group and CsA alone and CsA+Rapa for CD8 T-cell subset. On the other hand, in CD8 T cells, Pgp activity in CsA group was significantly lower than macrolides alone and associated groups (Rapa,Tac and Tac+Rapa). In $CD4^+$ T cells, CsA groups showed less Pgp function compared with all groups but no significant differences were found between any group (Fig. 5).

Pgp activity calculated as t_{50} (average rate of drug efflux) on $CD4^+$ and $CD8^+$ T-cell subsets displayed a similar kinetic pattern than Pgp activity measured by Rho123 extrusion. Rho123 t_{50} corroborated faster efflux time in $CD8^+$ subset than in $CD4^+$ (Fig. 6). Both $CD4^+$ and $CD8^+$ T cells showed longer time to efflux 50% of the drug in the CsA groups (alone and associated with Rapa) than the other treatments and the non-treated group confirming the inhibitory effect of CsA.

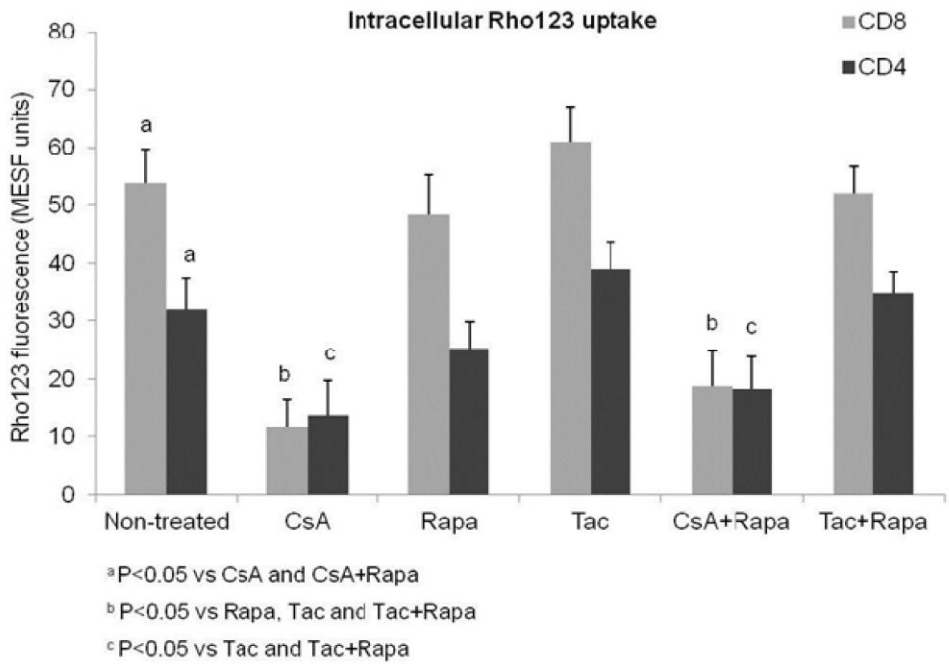

Figure 2. Intracellular Rho123 uptake by flow cytometry analysis in $CD4^+$ and $CD8^+$ subsets. Human PBMCs ($1x10^6$ cells) were pre-incubated at 37ºC 5%CO_2 o/n with the different immunosuppressive drugs, and Rho123 uptake was measured after 30 min of co-incubation of cells with Rho123. Values are means (MESF)±SE. N=8 different subjects for each group.

Table 1. Rho123 fluorescence in $CD8^+/CD4^+$ T cells from each immunosuppressive group after 30, 60, 90 and 120 minutes efflux in comparison with minute 0 (100%). The results are presented as normalized values. Data are in percentage as mean±SE. Each experiment was performed from blood of healthy volunteers. N=8 different subjects for each group.

	Non-treated	Rapa	CsA	Tac	CsA+Rapa	Tac+Rapa
$CD8^+$						
0 min	100	100	100	100	100	100
30 min	74.79± 4.5	70.87± 5.2	85.81± 6.2 [a]	66.16± 5.2	89.64± 4.1 [a]	64.46± 6.4
60 min	56.19± 6.1	54.36± 5.6	82.62± 2.5 [a]	50.8± 3.5	84.94± 6.4 [a]	48.76± 3.2
90 min	44.79± 5.2	44.15± 6.1	76.09± 3.5 [a]	39.42± 4.1	79.89± 3.6 [a]	41.52± 2.7
120 min	37.85± 3.6	34.1± 4.1	69.34± 4.1 [a]	32.06± 6.3	73.69± 2.8 [a]	34.95± 1.4
$CD4^+$						
0 min	100	100	100	100	100	100
30 min	90.83± 5.6	88.36± 2.1	92.15± 7.1	88.35± 4.5	95.4± 5.6	85.88± 2.6
60 min	86.22± 2.3	83.43± 7.8	91.47± 7.4	81.47± 2.6	94.09± 5.1 [b]	78.59± 4.9
90 min	80.08± 6.8	78.05± 4.8	88.69± 5.6	75.98± 3.9	91.64± 6.3 [c]	76.86± 3.7
120 min	72.18± 2.1	68.48± 3.4	85.21± 6.3 [a]	67.89± 1.8	89.67± 2.7 [c]	71.11± 2.5

[a] P<0.05 vs Non-treated, Rapa, Tac and Tac+Rapa
[b] P<0.05 vs Tac and Tac+Rapa
[c] P<0.05 vs Non-treated, Tac and Tac+Rapa

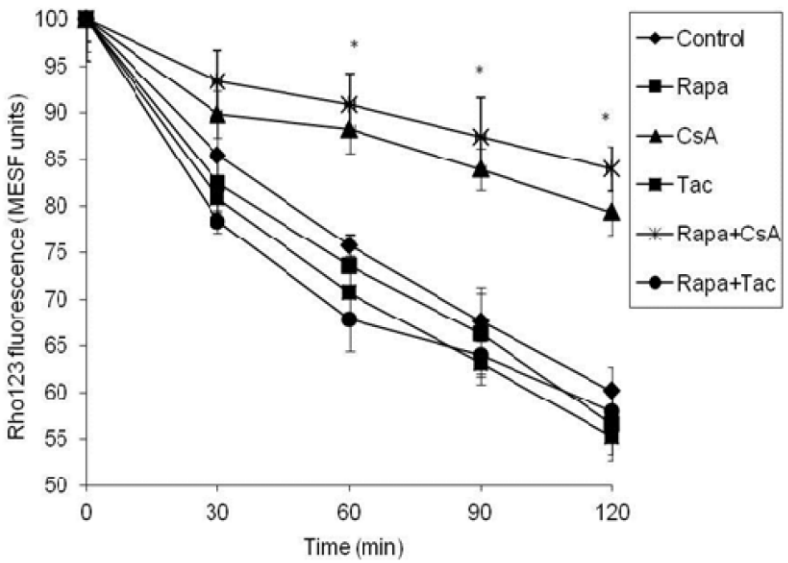

Figure 3. T-cell population kinetic profile. Drug-efflux curves were obtained after Rho123 uptake. Cells were incubated for up to 120 min at 37ºC 5%CO_2. Kinetic profile of Rho123 extrusion was evaluated using different time points (0, 30, 60, 90 and 120 min) according to each immunosuppressive treatment. Arbitrary fluorescence units were transformed to calibrated MESF values and normalized (*p<0.001).

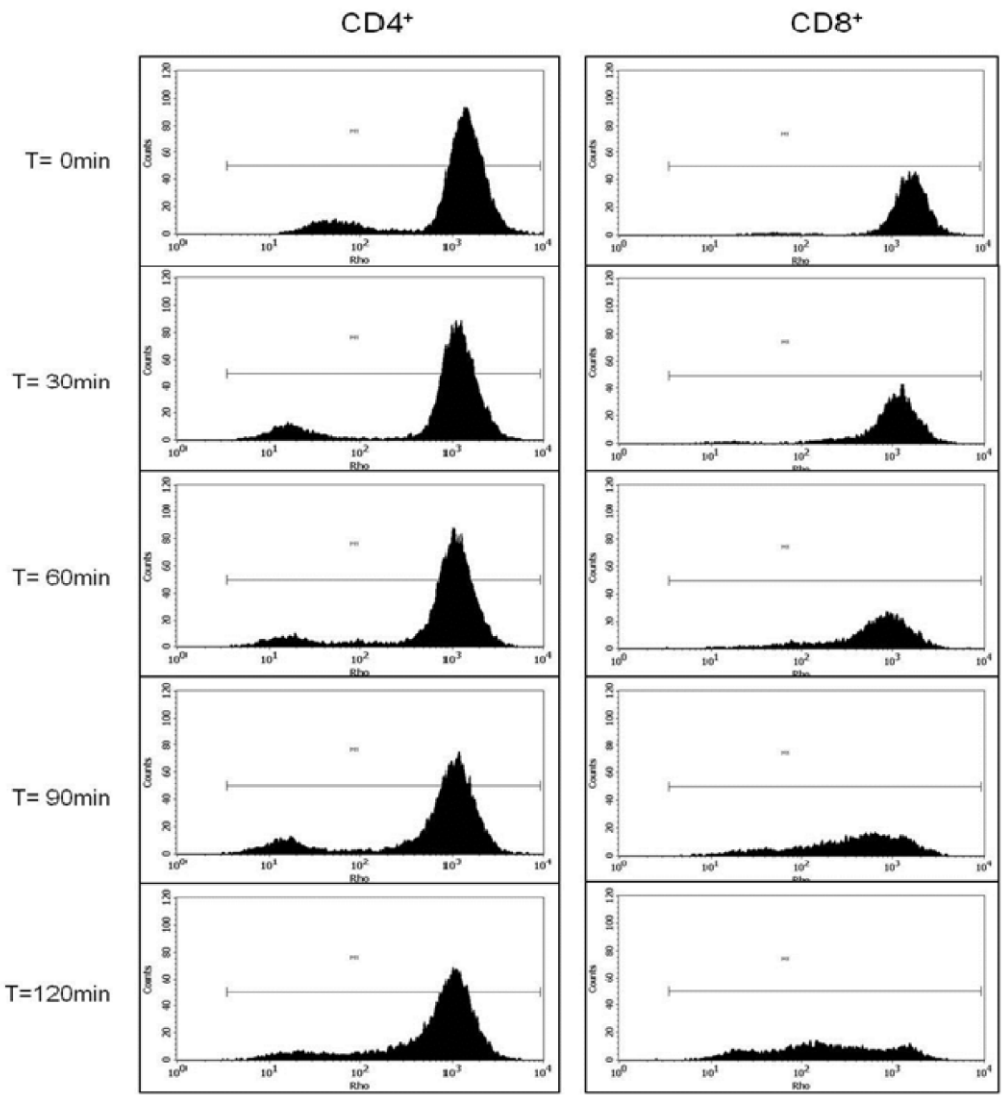

Figure 4. Representative histograms of the kinetic profile of Rho123 extrusion in $CD4^+$ and $CD8^+$ subsets. Ten thousand events of PBMCs were acquired for each assay. N=8 different subjects for each subsets and immunosuppressants groups.

Antigen-specific memory T-cell proliferation

Pgp blockade using the specific inhibitor PSC833 showed a similar inhibition profile on lymphocyte proliferation as all the immunosupressants (Fig. 7). Proliferation of antigen-specific memory $CD3^+$ T cells was significantly abbrogated in groups treated with PSC833 and immunosuppressants compared to non-treated groups (5-15% vs 30% of T-cell proliferation respectively, $p<0.05$). All immunosupressants in monotherapy inhibited T-cell proliferation in a similar degree as PSC833. Interestingly, both associations with Rapa (CsA+Rapa and Tac+Rapa) showed a further proliferation reduction compared with these immunosupressants in monotherapy.

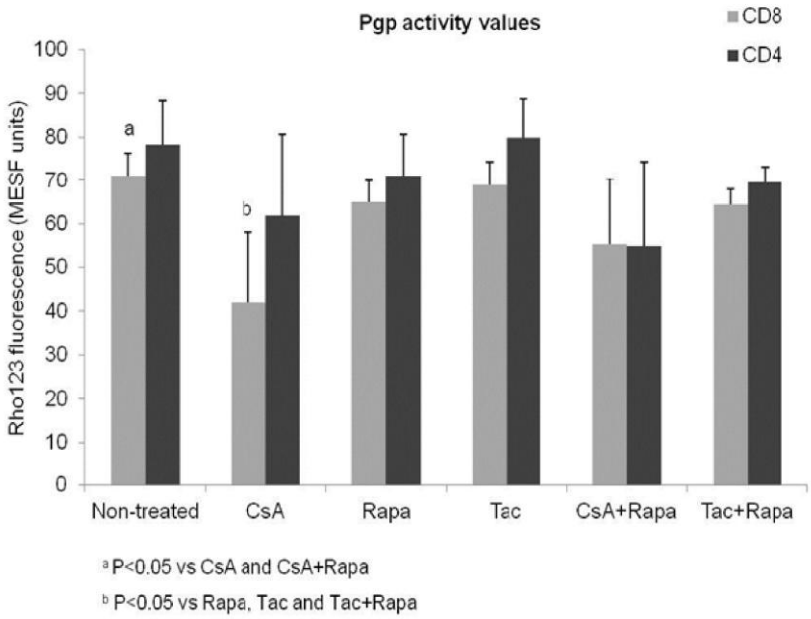

Figure 5. Pgp activity by flow cytometry analysis measured with MESF units of Rho123 fluorescence in $CD4^+$ and $CD8^+$ T-cell subsets. N=8 different subjects for each group. Any difference was observed among treatment groups in $CD4^+$ T-cell subset.

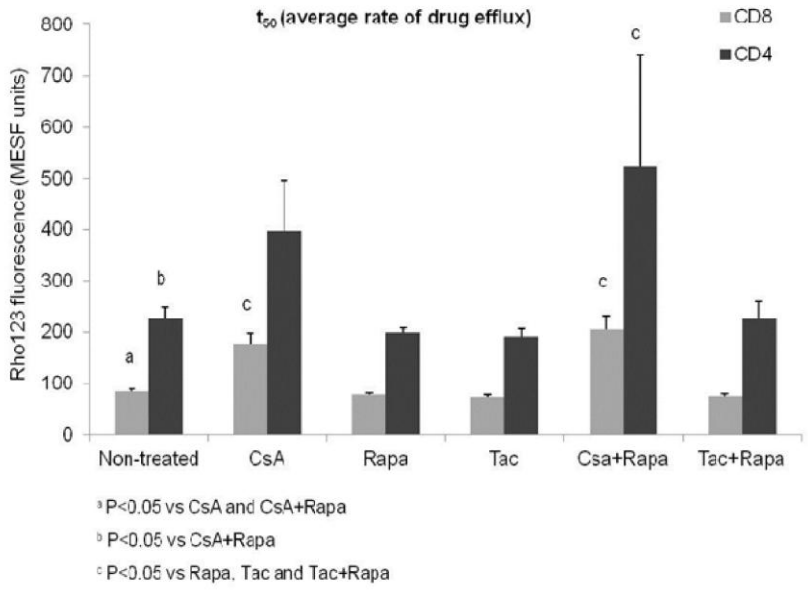

Figure 6. Pgp kinetic profile ($t_{50;}$ average rate of drug efflux) by flow cytometry analysis in $CD4^+$ and $CD8^+$ T-cell subsets. Arbitrary fluorescence units were transformed to calibrated MESF values. N=8 different subjects for each group.

In $CD4^+$ and $CD8^+$ T-cell subsets all immunosuppressant therapies and PSC833 diminished lymphocyte proliferation, and this effect was especially more evident in CD8 than CD4 T cells (25% vs 15% of T-cell proliferation inhibition respectively, $p<0.05$). However, despite T-cell proliferation decreased in PSC833 group in CD4 and CD8 T cells, there was only significant difference for CD8. Furthermore, in the $CD4^+$ subset, both associations with Rapa showed a reduced proliferation compared with these immunosupressants in monotherapy, in a similar pattern as CD3 T cells, but in this case only Tac+Rapa achieved statistical differences ($p<0.05$).

Cytokine secretion in MLR

Th1 pro-inflammatory cytokines such as IFN-γ and TNF-α, were significantly reduced in all immunosuppressive therapies and PSC833-treated groups than in those non-treated ($p<0.05$). However, CsA group did not reach statistical significance in the IFN-γ secretion, even it was significantly higher than all the other immunosuppressants and PSC833 ($p<0.05$). On the other hand, only Tac showed significantly lower TNF-α release compared to the other treatment groups ($p<0.05$) (Fig. 8). Regarding the Th2 cytokine profile, PSC833 and the immunosuppressive therapies, except for CsA, diminished IL-6 secretion compared to the non-treated stimulated group. Furthermore, the two macrolides in monotheray showed a significant decrease compared with both drugs associated ($p<0.05$). Concerning IL-10, rapamycin groups showed an important decrease in this cytokine secretion compared with the other groups, reflecting that Rapa plays an important role in the modulation of IL-10 (Fig. 8).

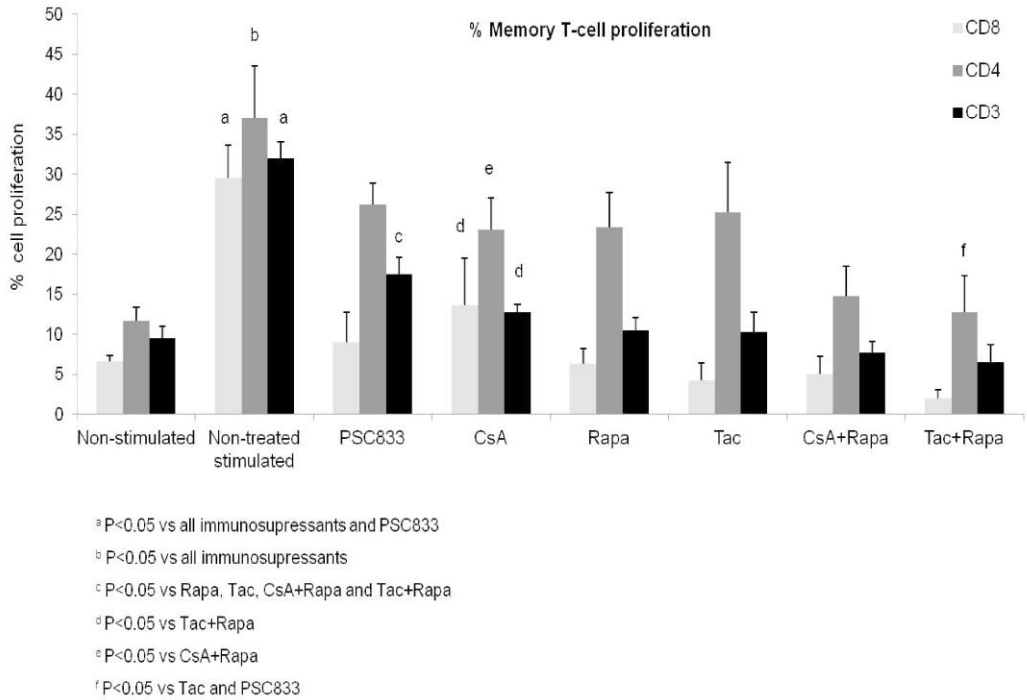

Figure 7. Antigen-specific memory T-cell proliferation under different immunosupressants and Pgp specific inhibitor (PSC833) in different T-cell subsets. Values are means (MESF)±SE. Experiments are representative of four independent assay.

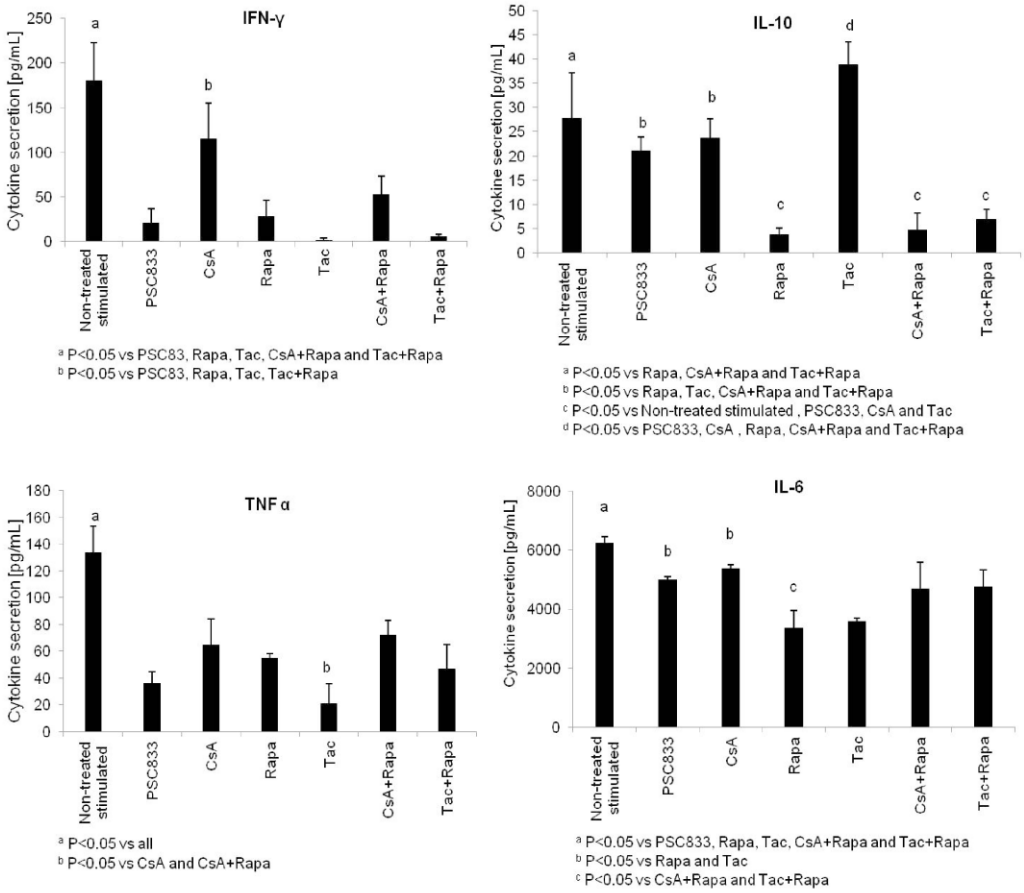

Figure 8. Cell cytokine analysis by flow cytometry into supernatants by PBMCs in MLR. Fluorescent bead assay (CBA Human Th1/Th2/Th17 kit). Experiments are representative of three independent assays.

DISCUSSION

Pgp is a functionally active efflux pump physiologically expressed in several tissues and peripheral blood mononuclear cells. Its role in pumping drugs out of the cells is widely known but its function in immunologic mechanisms is complex and still not fully understood. The crucial role of ABC transporters in lymphocyte survival and antigen presenting cell differentiation has been described (13). Pendse et al underlined the important immunoregulatory effects of P-glycoprotein-inhibiting agents, suggesting a promising novel Pgp therapeutic target for immune modulation in acute and chronic allograft rejection, and cell-mediated autoimmune disorders (29).

In our study, we analyzed the influence of immunosuppressive drugs on intracellular Rho123 uptake and the kinetic profile of Pgp activity in different lymphocyte subsets. Immunosuppressive agents are Pgp substrates and/or inhibitors that may influence the functional response of T cells (30). Our results demonstrated a significant decrease in Rho123 uptake in T lymphocyte subsets treated with CsA, suggesting the role of Pgp not only in the efflux but also in the influx of Pgp substrates inside the cells. In addition, our results illustrate the inhibitory effect of CsA in blocking Pgp function not only in the

efflux but also in the influx of Pgp substrates. On the other hand, Pgp activity in T cells treated with both macrolides showed a similar profile to cells without immunosuppressive drugs; therefore, at the concentrations used, Rapa and Tac did not modify Pgp activity.

When the efflux of Rho123 was analyzed, T cells treated with CsA with or without Rapa presented a slower kinetic profile compared to Tac and Rapa alone or associated, confirming the potent inhibitory effect of CsA on Pgp compared to the other immunosuppressors. Cells treated with CsA spent 2-fold minutes to efflux 50% of the Rho123 as compared to the macrolide groups. Furthermore, the time required to extrude Rho123 was longer in T cells incubated with CsA plus Rapa than CsA alone, suggesting an additive inhibitory effect of rapamycin on Pgp when combined with CsA. The association, Tac and Rapa, and both immunosupressants separately, lasted the same time to efflux 50% of the Rho123 as took the non-treated non-stimulated group**.** In fact, rapamycin and tacrolimus affected Pgp activity but they did not modulate Pgp function at pre-established doses. Similarly to what was previously reported by Pawarode and collegues (25) here, we show that CsA seems to be a broad-spectrum MDR modulator impairing drug transport at the clinically achievable concentration of 2.5 µM, tacrolimus enhanced cellular drug uptake at 1 µM, but not at its clinically achievable concentration (0.1 µM). Rapamycin exerts the optimal effect of enhancing cellular drug uptake at 2.5 µM, but it was not effective at its clinically achievable concentration of 0.25 µM.

The specific role of Pgp modulators and their kinetic profile is thus essential to fully characterize and approach the broad properties of immunosuppressive drugs (31), Laurent G et al (32) and Gupta et al (33) clearly showed that the expression of Pgp was higher in $CD4^+$ than in $CD8^+$ T cells. Functionally, Pgp was more efficient in the $CD8^+$ subpopulation. In our study we found higher Rho123 uptake and efflux in $CD8^+$ T cells than in $CD4^+$ T cells, suggesting that both influx and efflux of Pgp substrates showed a different modulation depending on the T-cell subset studied. Furthermore and, in line with previously published results (33), the kinetic of Rho123 extrusion was also remarkebly faster in $CD8^+$ than in $CD4^+$ subsets suggesting a physiologic role of Pgp within the cytotoxic T lymphocyte subset. Pgp function was modified after T-cell pre-tretament with immunosuppressors showing slower Rho extrussion in both CsA groups compared with macrolide groups. The Pgp kinetic was also faster in $CD8^+$ T cells than $CD4^+$ T cells.

Nowadays the interest of the role of ABC transporters is increasing. Their extruding function and/or their inhibitory functions could be an interesting new pharmacodynamic target. Recently, Brennan et al examined a new molecule (LIM-0705) designed to activate specific ABC transporters in the kidney and liver, facilitating the export of Tac out of vulnerable cells in order to reduce toxicity while preserving its immunosuppressive effect. The increase in the intra-lymphocyte drug concentration is associated with more effective inhibition of lymphocyte proliferation by CsA *in vitro*, these results could translate *in vivo* into more effective protection against graft rejection (34). Upon secondary exposure to alloantigen, both $CD4^+$ and $CD8^+$ memory T cells rapidly proliferate and differentiate into effector cells. It is well known, that CNIs are the most robust drugs to suppress alloreactive effector/memory T cells (35). Therefore, we studied whether both Pgp blocker and the different immunosuppressants showed an efficient inhibitory effect on alloreactive memory T cells. Our results proved that Pgp blockade and immmunosuppressants resulted mainly in $CD8^+$ lymphocyte inhibition diminishing lymphocyte proliferation as $CD3^+$ subset. In contrast in $CD4^+$ T-cell proliferation was slightly inhibited by PSC833 and all immunosuppressants. Considering that antigen-specific memory T cells have less sensibility to classical immunosuppressive agents being the main responsible of acute cellular rejection episodes after transplantation these results have relevance in clinical renal transplantation.

Based on its known function as a transmembrane transporter (36), Pgp has been proposed to facilitate the membrane transport and/or release of cytokines. In our study we measured cytokine secretion in MLR with different immunosuppressants and specific Pgp inhibitor. Our data showed that pro-inflammatory Th1 cytokines secretion, IFN-γ and TNF-α, were significantly reduced in all treatment groups, pointing up the maximum response after Tac exposition. On the other hand, Th2 cytokine response by IL-6 was minimized in all immunosupressants except for CsA and PSC833 compared with non-treated stimulated group. On the other hand, rapamycin in monotherapy or associated was the only immunosupressant able to

reduce IL-10 secretion, reflecting the important role of Rapa in IL-10 modulation.

In summary, Pgp activity appears to be modulated depending on the immunosupressor treatment with faster Pgp kinetic in $CD8^+$ than $CD4^+$ T-cell subsets. Our data indicate that CsA inhibit Pgp activity and T-cell function being the $CD8^+$ T cells more susceptible to this effect than $CD4^+$ T cells. On the other hand, immunosuppressants significantly reduced T-cell proliferation of $CD4^+$ and $CD8^+$ subsets. Our results underline the importance of the immunoregulatory effects of Pgp in T-cell activation and in immunosuppressive regimens. Thus, looking for new strategies to target this T-cell subset population is necessary to further delve into potential mechanisms of Pgp in T-cell activation. The inhibition of this protein could be a novel therapeutic target in order to avoid allograft rejection in solid organ transplantation.

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DISCUSSION

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In chronic allograft nephropathy immunological and non immunological factors are involved such as donor specific alloreactivity, chronic inflammation and nephrotoxicity induced by anticalcineurinics. Different degrees of this nephrotoxicity are related to immunosuppressive associations and to inhibition degrees of the different transporter proteins such as MDR1, MRP1 and MRP2 which can be subjected to genetic inter-individual variability modifying pharmacokinetics parameters. Thus, optimal immunosuppressive dose could be predicted by genotype characterization of MDR1, MRP1 or MRP2 genes as well as Pgp and MRPs protein functions. Those proteins have also been related to immunological factors.

Over the last decade, enough evidence has emerged emphasizing critical functional roles for MDR related ABC transporters in immune effector cells. This knowledge should be considered for the development of new strategies in auto and alloimmune diseases. It may be beneficial to decrease the expression or functionality of specific transporters in specific subsets of immune effector cells, as this could attenuate the generation of an alloimmune response interfering on their differentiation and functionality. Pgp contributes in several distinct functions in the initiation of primary immune responses.

Based on these considerations, our first goal was the investigation of the effect of different storage conditions on Pgp expression, functionality and efflux activity in T lymphocytes. The second study was to evaluate the association of different ABCB1 polymorphisms (C3435T, G2677T, C1236T and T129C) with Pgp activity and exposure to different immunosuppressive drugs in renal transplant patients from a multi-centric study. Because Pgp is expressed on T lymphocytes, it could affect immune and pharmacologic responsiveness to many drugs. In the third

study, we assessed the activity of Pgp on $CD4^+$ and $CD8^+$ T-cell subsets and we studied the effects of two calcineurin inhibitors (CsA and Tac) in monotherapy and associated with rapamycin in healthy volunteers. We also studied the antigen-specific memory T-cell responses by measuring T-cell proliferation and cytokine secretion in an allogeneic mixed lymphocyte reaction.

Pgp is an efflux pump with an activity that can be measured by the efflux/retention of Rh123 inside the cell. In the first study, we compared Pgp expression and Rho123 retention in lymphocytes from blood samples either stored at room temperature for 24 hours or carried out immediately after blood drawn. The expression and activity of Pgp was measured in different lymphocyte storage conditions. This study was performed to define sampling strategies to optimize the sensitivity and reproducibility of the retention and efflux assays. It has to be considered that Pgp is an unstable protein and could be inactivated by freeze/thawing cycles [165]. Most of the publications did not specify the cells processing and storing conditions before analysis and it is fundamental to obtain good reproducibility mainly for future multi-centric studies to guarantee the uniformity of the conditions. There are no data comparing Pgp activity measured from fresh lymphocytes with cryo-preserved lymphocytes. Even though, we found few groups which performed Rho123 retention assays directly from fresh whole blood [166], and others from fresh isolated lymphocytes [167], but there is almost no literature defining how lymphocytes were isolated.

In the present study we showed no significant differences neither in the expression nor the activity of the Pgp protein between fresh and frozen lymphocytes. There are different functional techniques described in the literature to analyze Pgp activity [168-170]. Our experiments were

determined by flow cytometry and Rho123, a non-toxic fluorescent dye used as Pgp substrate.

Peripheral mononuclear cells are known to express ABC efflux transporters. Hence, isolated cells could be useful to study drug transporters in the *in vivo*/*ex vivo* monitoring of the potential effects of drugs on the expression and activity of these transporters. As reported in the literature, all lymphocyte populations show potent transport activity, mediating both efflux and import. This finding is corroborated in our studies, in which we observed Pgp activity in $CD3^+$, $CD4^+$ and $CD8^+$ subpopulations. Moreover, the efficiency with which Pgp transports specific drugs is cell type dependent and does not imply a direct correlation between Pgp function and expression. This lack of correlation may be explained, in part, by the fact that functional analysis, using Pgp substrate dyes such as Rho123, are much more sensitive in the detection of low level resistance than Pgp expression analysis [171, 172]. Although NK cells play an important role in Pgp expression and function, this subpopulation represents a low percentage of the PBMC population. Therefore, considering that the $CD3^+$ cells represent a high percentage of the PBMCs, as they show functional Pgp expression and play a predominant role in immune response, this population was the most suitable for the purpose of the present study.

To date, Rho123 has been used [173-176] to measure Pgp activity, in the presence or absence of Pgp inhibitors. The two efflux pump inhibitors used were the Ca^{+2} channel blocker Verapamil (VP) and PSC833, a Pgp selective inhibitor also known as Valspodar. The two inhibitors showed no differences in the Rh123 retention among the four conditions of the lymphocytes studied. Nevertheless, PSC833 is a more specific and potent inhibitor of Pgp than VP, as the concentration used to inhibit Pgp efflux was half of the concentration of VP.

We studied Pgp efflux activity in three different cell subsets: viable (70-90%), early apoptotic (4-5%) and late apoptotic (3%) of the total lymphocytes ($CD3^+$) purified from the blood samples. Interestingly, the results of activity from viable cell population were not affected whether considering or not the activity of the early apoptotic lymphocytes. In contrast, late apoptotic and necrotic cells should be considered separately or excluded because they always show background activity that interferes in the analysis of the viable lymphocyte Rh123 retention measurements. We established that in order to obtain the most accurate results, the apoptotic population should be considered separately and necrotic cells should be excluded.

Several *in vitro* studies have identified immunosuppressants as substrates and/or inhibitors of Pgp [77, 177-180]. Their bioavailability is mainly controlled by ABC transporter efflux pumps and cytochrome P450 enzymatic system. Polymorphisms in the MDR1 gene that might alter Pgp expression and function in humans, leads to a clinical interest in the pharmacokinetics of immunosuppressants to improve their dosing in individuals [60]**.** Those polymorphisms could affect the absorption and tissue concentrations of several substrates of Pgp and have an important role in the inter-individual variability in drug disposition and pharmacological response. However, there is still controversy as to whether the pharmacokinetics and pharmacodynamics of drugs could be modified by MDR1 genotypes/haplotypes [111, 112, 139, 181-183].

In our pharmacogenomic study, the results confirmed a significant correlation between ABCB1 genotyped and Pgp activity in PBMCs from renal transplant recipients. Homozygous patients carrying the T-allele for 3435 SNP showed the lowest Pgp activity compared with 3435 CT and CC carriers. Our results on CC≥CT>TT in relation to Pgp function corroborates the data reported by others [10, 184-187]. Even though, there are still some discrepancies among authors, and the functionality of

3435 CT ABCB1 SNP remains a matter of controversy [188-193]. Several experimental studies have associated the homozygous ABCB1 3435 TT variant genotype with lowered intestinal Pgp expression or activity *in vivo* [10, 114, 185, 194]. This hypothesis is supported by the fact that these patients have an inefficient export of drugs out of the cells, leading to increase absorption of the drugs across the intestine, and higher systemic and intracellular drug concentrations. However, other studies have demonstrated the opposite association [116, 195, 196] or no association [111, 197, 198] *in vitro* and *in vivo*. The same results were observed in patients with C1236T and G2677T polymorphisms where TT or GG homozygotes, respectively, showed the lowest Pgp activity.

After the results obtained in the first study which underline the importance of the whole parameters related with Pgp activity, measurements of Pgp by Rho123 efflux were performed in frozen PBMC from blood samples of renal transplant recipients from different centers. Here, we described the influence of ABCB1 SNPs of a renal transplant population on Pgp function in PBMCs, supporting results reported in other tissues where the functional effect of Pgp polymorphism was also demonstrated [60, 185].

Current immunosuppressive drugs are characterized by a narrow therapeutic index and large intra- and inter-individual variability both in pharmacokinetics and in pharmacodynamics. Pgp is an important component of a detoxifying system, and ABC transporters affect the bioavailability of their substrate drugs [136, 190]. CsA was the first immunosuppressor shown to modulate Pgp activity in laboratory models and clinical trials, and intestinal Pgp has been shown to determine oral clearance of CsA [199]**.** Tac is both a substrate and an inhibitor for Pgp, less active than CsA. SRL is a Pgp substrate that may limit its intestinal absorption, and Pgp is also probably involved in its excretion [136]**.** In

the present study, patients with CsA showed lower Pgp activity compared with macrolides, both Tac and SRL. With the immunosuppression concentrations that works in the clinic, CsA affects Pgp activity, whereas macrolides (TAC and SRL) don't modify its function.

Many studies have addressed the importance of MDR1 polymorphism in dose requirement, blood concentrations, chronic rejection, and nephrotoxicity in renal transplant patients treated with CsA and Tac. In this regard, studies supported the idea that dose requirements are influenced by ABCB1 polymorphism. It would be expected that patients carrying genetic polymorphisms resulting in greater expression and/or enzymatic activity of Pgp and CYP3A would show greater drug metabolism and therefore require larger doses to obtain adequate target blood drug concentrations [200-202]. Also, there are studies that show the dose requirements influenced by MDR1 polymorphism, although the use of MDR1 genotype as a helpful marker in clinical practice remains unclear [112, 132, 181, 187, 203].

Some ABCB1 SNPs have been correlated with the *in vivo* activity of Pgp and should therefore be considered in renal transplant recipients treated with Tac [204, 205]. Up to now, no authors have described an association of Pgp activity, MDR1 polymorphisms, and exposure to CsA, Tac and Rapa at different timing, 7 days, and 1, 3, 6 and 12 months in renal transplantation. Our results were analyzed in a multi-center clinical study where patients with CsA, Rapa and Tac exposure had well defined AUCs and follow-up during one year, and co-medication drugs were specified.

We observed the same correlation pattern between Pgp activity depending on the genotypes of the three ABCB1 SNPs studied (C1236T,

C3435T and G2677T) as observed in the general population (CC≥CT>TT) on both groups of patients, the ones who are treated with CsA and the ones with macrolides. The values of Pgp activity in patients treated with CsA were lower than those for macrolides in the SNPs studied. These results compared with tacrolimus and rapamycin data, may be explained by the fact that CsA is a potent inhibitor of the Pgp function [118, 206]. CsA is a Pgp modulator, and the variability of its absorption and disposition has been attributed to intestinal Pgp expression and activity. The movement of a drug through membranes is an essential step in absorption. Delays or loss of drug during absorption can introduce large variability in drug response.

In this study, we found a negative correlation between Pgp activity in lymphocytes and CsA AUC and Cmin at steady state conditions in those patients with high activity of Pgp and low CsA doses. Regarding the correlation between exposure of CsA and Pgp activity we should consider that CsA is a potent Pgp inhibitor, so an increase in CsA exposure should lead to a decrease in Pgp activation. However, we must not overlook the intestinal effect. In the enterocyte, CsA is absorbed and excreted back, in part, to the intestinal lumen through specific proteins belonging to the ABC family. Therefore, the small intestine emerges as the first bottleneck in the entry of CsA into the organism. In addition, metabolism in the liver cannot be underestimated before the distribution to tissues, where ABC proteins are also present. All these processes influence the distribution and subsequent elimination of the drug from the organism. As a result of this complex interplay between enzyme activities and efflux transporters, the concentrations of CsA in plasma could also be affected. C3435T SNP correlates with lower intestinal Pgp expression, and this in turn does directly affect the oral bioavailability of Pgp substrates. Thus, in individuals with lower intestinal Pgp concentrations the extent of drug absorption from the gastrointestinal tract should be higher.

Consequently, an increase in the plasma levels in comparison to the remainder of the population would be observed.

From all this, we may conclude that patients with CT or CC genotype in exon 26 (C3435T) with high Pgp activity on the apical surface of enterocytes, takes more CsA outside cells resulting in decreased bioavailability. In our study, high pumpers treated with low doses of CsA would show less drug exposure and this could directly affect the therapeutic response. On the other hand, the negative correlation between CsA exposure (AUC and Cmin) in plasma and Pgp activity might reflect the increasing CsA bioavailability because of the enhanced Pgp blocking in the intestinal enterocytes. However, we must not overlook the small sample size of this group. To better interpret the results, additional studies with larger sample sizes will be required. The study of polymorphisms related to immunosuppressive drugs and the role of ABC transporters could improve drug dose tailoring.

As the role of Pgp in pumping drugs out of the cells is already widely known, its function in immunologic mechanisms is complex and still not fully understood. The main role of ABC transporters in lymphocyte survival and antigen presenting cell differentiation has been described [33]. Pendse et al underlined the important immunoregulatory effects of Pgp inhibiting agents, suggesting a promising novel Pgp therapeutic target for immune modulation in acute and chronic allograft rejection, and cell-mediated autoimmune disorders [155].

In our study, we evaluated the role of Pgp on the functional response of T cells analyzing the influence of immunosuppressive drugs (CsA, Tac and Rapa) on intracellular Rho123 uptake and the kinetic profile of Pgp activity in different lymphocyte subsets. Our first results demonstrated a significant decrease in Rho123 uptake in T cells subsets treated with CsA

suggesting the role of Pgp not only in the efflux but also in the influx of Pgp substrates inside the cells. Data from the Rho123 retention/efflux assays demonstrate that Pgp in both $CD4^+$ and $CD8^+$ T cells is functional, even though this data do not provide a quantitative measure of Pgp expression in these cells [73]. In addition, our results illustrate the inhibitory effect of CsA in blockade of Pgp. On the other hand, Pgp activity in T cells treated with macrolides showed a similar profile to cells without immunosuppressive drugs; therefore, at the concentrations used, Rapa and Tac did not modify Pgp activity. These results are in accordance with those obtained in an experimental nephrotoxicity model in rats in which the authors explored the role of Pgp in the association of Rapa (mTORi) with CsA and Tac [207]. It is well documented that CsA induces the overexpression of Pgp *in vitro* and *in vivo* [177], in a reversible and dose-dependent manner [208].

The literature provides contradictory data bearing on the hypothesis that the expression of the MDR1 gene product Pgp represents a mechanism of therapy-resistant rejection in solid organ transplant patients. Gotzl et al measured Pgp mRNA expression level in peripheral blood from renal transplant patients [209]. They concluded that the lack of MDR expression may coincide with prolonged graft survival. In renal transplant patients, there was no difference in Pgp expression in PBMCs from patients with stable grafts versus those with chronic rejection [210] or in CsA or Tac sensitive versus CsA or Tac resistant patients [172]. In contrast, reports in heart [211] and lung [212] transplantation indicate that increase of Pgp expression in PBMC or mononuclear cells from lung biopsy specimens, respectively, were associated with rejection.

Here, we measured Pgp function rather than expression on defined T-cell subsets. When we evaluated Rho123 efflux, T cells treated with CsA either with or without Rapa presented a slower kinetic profile compared

to the macrolides alone or associated with Rapa, confirming the potent inhibitory effect of CsA on Pgp compared to the other immunosuppressants. Furthermore, Pgp activity was also calculated as average rate of drug efflux (T_{50}). Our results showed that cells treated with CsA spent 2-fold minutes to efflux 50% of the Rho123 as compared to the macrolide groups. It means that the time required to extrude Rho123 was longer in T cells incubated with CsA plus Rapa than CsA alone, suggesting an additive inhibitory effect of Rapa on Pgp when combined with CsA. The potentiation of CNI-induced nephrotoxicity by rapamycin has been confirmed in animal models [213-215]. On the other hand, the association Tac and Rapa, and both immmunosupressants separately, lasted the same time to efflux 50% of the Rho123 as reached by the non-treated non-stimulated group. Actually, Rapa and Tac affected Pgp activity but they did not modulate Pgp function at pre-established doses. The majority of the macrolides not only inhibit Rho123 export through Pgp, but also display the ATPase activation curve characteristic for quickly transported substrates with the possibility to bind to at least two binding sites in Pgp; they activate ATPase at lower concentrations and inhibit at higher [216]. This suggests that macrolides inhibit Rho123 export by competing for the transport through the same transporter.

Similarly to what was previously reported by Pawarode and collegues [217], we show that CsA seems to be a broadspectrum MDR modulator impairing drug transport at the clinically achievable concentration of 2.5 μM, Tac enhanced cellular drug uptake at 1 μM, but not at its clinically achievable concentration (0.1 μM). Rapamycin exerts the optimal effect of enhancing cellular drug uptake at 2.5 μM, but it was not effective at its clinically achievable concentration of 0.25 μM.

To characterize the broad properties of immunosuppressive drugs we studied the specific role of Pgp inhibitors and their kinetic profile in $CD4^+$

and $CD8^+$ T cells. Functional studies indicated significantly higher functionality of Pgp in $CD8^+$ T cells, although the expression of Pgp in $CD4^+$ T cells is higher than the $CD8^+$ T cells [73]. Other authors showed that naïve $CD8^+$ T cells exhibited higher Pgp activity than CD8 memory T cells [149]. Although Pgp has been widely studied in T cells, some apparent discrepancies were still reported regarding its expression and function between CD8 and CD4 subsets. Here, we found higher Rho123 uptake and efflux in $CD8^+$ T cells than in $CD4^+$, suggesting that both influx and efflux of Pgp substrates showed a different modulation depending on the T cell subset studied. According to previously published results [150], the kinetic of Rho123 extrusion was also remarkably faster in $CD8^+$ than in $CD4^+$ subsets suggesting a physiologic role of Pgp within the cytotoxic T lymphocyte subset. Pgp function was modified after T-cell pre-treatment with immunosupressants. We also observed slower Rho extrusion in both CsA groups compared with macrolide groups in different T-cell subsets.

Nowadays, the interest in the role of ABC transporters is increasing. Their extruding and/or inhibitory function could be an interesting new pharmacodynamic target [164]. Recently, Brennan et al examined a new molecule (LIM-0705) designed to activate specific ABC transporters in kidney and liver, facilitating the export of Tac out of vulnerable cells in order to reduce toxicity while preserving its immunosuppressive effect. The increase in the intra-lymphocyte drug concentration is associated with more effective inhibition of lymphocyte proliferation by CsA *in vitro*. These results could translate *in vivo* into more effective protection against graft rejection [212]. Upon secondary exposure to alloantigen, both $CD4^+$ and $CD8^+$ memory T cells rapidly proliferate and differentiate into effector cells. It is well known, that CNIs are the most robust drugs to suppress alloreactive effector/memory T cells [171]. Here, we studied whether both Pgp blocker and different immunosuppressants have an

inhibitory effect on alloreactive memory T cells. We observed a stronger inhibition of lymphocyte proliferation in $CD8^+$, compared with $CD4^+$ T cell proliferation after blocking Pgp with both PSC833 and all immmunosuppressants. These results could have an impact in clinical renal transplantation considering that antigen-specific memory T cells have less sensibility to classical immunosuppressive agents being these cells responsible of acute cellular rejection episodes.

Pgp facilitates membrane transport and release of cytokines. In our experiments we measured cytokine secretion in MLR with different immunosuppressants and specific Pgp inhibitor. Our findings showed that pro-inflammatory Th1 cytokine secretion, IFN-γ and TNF-α, were significantly reduced in all treatment groups, pointing up the maximum response after Tac exposition. IL-6, a Th2 cytokine, was minimized in all immunosuppressant treatments except for CsA and PSC833 compared with non-treated stimulated group. On the other hand, Rapa in monotherapy or associated with CsA or Tac, was the only immunosuppressant able to reduce IL-10 secretion, suggesting the important role of Rapa in IL-10 modulation. Both Th1 and Th2 cytokines have marked effects on the expression of the transporters like CYP, or here Pgp.

In conclusion, these results demonstrate the importance of Pgp as a transporter protein of many immunosuppressants and it is exposition, and also the role of Pgp in T lymphocytes. Furthermore, the study of polymorphisms of the gene encoding the Pgp, allow adjusting the immunosuppressants exposure avoiding erratic doses and increasing their safety and efficacy, minimizing undesirable sub-therapeutic levels. These studies are a step towards personalized medicine.

CONCLUSIONS

CONCLUSIONS

1. Pgp activity measured by Rho123 retention was not modified by any of the different working conditions, room temperature or frozen samples or the 24 hours of post blood extraction. The Pgp activity may be considered in viable cell population and necrotic cells should be excluded.

2. The use of these parameters is a key tool to standardize Pgp activity studies in individuals with different timing of blood extraction or from different geographic areas and to optimize the sensitivity and the reproducibility of efflux assays.

3. The polymorphisms of the human multidrug resistance gene MDR1 (C3435T, C1236T and G2677T) were influenced by Pgp function in $CD3^+$ T-cell subsets isolated from renal transplant recipients. Pgp activity was higher in patients carrying the C-allele for 3435 SNP whereas the lowest activity was found in patients with TT genotype. Similar results were obtained in the G2677T and C1236T SNPs. Pgp activity was not influenced by the T129C SNP.

4. Variations of Pgp activity depend on the immunosuppressive therapy. Patients treated with CsA showed lower Pgp activity compared with macrolides treatment (tacrolimus or rapamycin). Patients treated with low dose of CsA with a high pumper genotype in ABCB1 SNPs should have less cellular CsA concentration because of increased drug extrusion. This group would reflect less drug exposure modifying the therapeutic response.

5. A negative correlation between high pumpers exposed to low doses of CsA (AUC and Cmin) and Pgp activity was observed. Any correlation was observed in low pumpers with high doses of CsA. In addition, no correlation was found between the macrolides (Tac and SRL) AUC or Cmin and Pgp activity at 7 days, 1, 3 and 12 months post-transplant.

6. Pgp activity measured as the average rate of drug efflux displayed a similar kinetic pattern than Pgp activity measured by Rho123 extrusion. The kinetic of Rho123 extrusion was remarkably faster in $CD8^+$ than in $CD4^+$ subsets.

7. CsA inhbit Pgp activity and T-cell function being the $CD8^+$ T-cell subset more susceptible to this effect than $CD4^+$ T cells.

8. All immunosuppressants significantly reduced the proliferation of $CD4^+$ and $CD8^+$ subsets. Inhibition of Pgp could be a novel therapeutic target in order to avoid allograft rejection in solid organ transplantation.

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ANNEX 1

SUMMARY

RESUM

En la nefropatia crònica de l'aloinjert (NCT) s'hi involucren tant els factors immunològics com els no immunològics. Alguns desencadenants de l'aparició de la NCT són l'aloreactivitat donant específica, la inflamació crònica i la nefrotoxicitat induïda per anticalcineurínics. Se sap que diferents graus de nefrotoxicitat es poden relacionar amb diverses associacions de fàrmacs immunosupressors i amb la inhibició de determinades proteïnes transportadores com la Pgp, MRP1 i MRP2, que són les responsables d'afluïr el fàrmac cap a l'exterior de les cèl·lules. Considerant la importància del paper que tenen aquestes proteïnes transportadores, les variants genètiques dels gens que les codifiquen i la seva funció, aquests transportadors poden afectar la varietat inter-individual modificant la farmacocinètica de molts fàrmacs. Des d'aquest punt de vista, tant la monitorització del genotip d'aquests gens (MDR1, MRP1 i MRP2), com també la seva funció, podria predïr la capacitat d'extrusió dels immunosupressors i la seva biodisponibilitat així com també la dosi inicial que necessita cada pacient per obtenir la immunosupressió adient.

Així doncs, els polimorfismes d'aquests gens estan relacionats amb l'exposició al fàrmac, la nefrotoxicitat i el rebuig. En els darrers anys, aquestes proteïnes també s'han associat a factors immunològics. Tant MDR1 com MRP1 juguen un paper important en l'activació de la cèl·lula T i també en la diferenciació i maduració de la cèl·lula dendrítica. S'ha vist doncs, que el paper d'aquestes proteïnes transportadores va més enllà de ser una bomba d'eflux ja que es coneix la seva participació en la resposta immune siguent una nova diana terapèutica per a la immunosupressió.

El principal objectiu que ens vam plantejar va ser estudiar el rol de la Pgp com una bomba transportadora de fàrmacs i la seva contribució en la farmacocinètica i la farmacogenètica dels immunosupressors. Per altre banda, estudiar el seu paper en la resposta alloimmune.

1er TREBALL

Different storing and processing conditions of human lymphocytes do not alter P-glycoprotein rhodamine 123 efflux. J Pharm Pharm Sci. 2009;12(3):357-66.

Objectius

En aquest estudi es va comparar l'expressió de Pgp i l'activitat d'eflux mitjançant l'assaig de Rho123 en limfòcits de voluntaris sans processats i emmagatzemats en diferents condicions per avaluar la influència que podien tenir aquestes condicions sobre l'expressió i la funcionalitat de Pgp. Per els estudis d'activitat de Pgp, principalment per estudis multi-cèntrics, s'han de considerar estratègies per optimitzar al màxim la sensibilitat i la reproduïbilitat dels assaigs. La majoria d'autors no especifiquen les condicions amb les quals les cèl·lules es conserven i emmagatzemen abans de mesurar l'activitat.

Es va posar a punt un mètode per a la mesura de l'activitat de Pgp en limfòcits T, per poder ser aplicada en mostres de limfòcits de pacients transplantats renals. Es van aïllar els limfòcits de la sang de 12 voluntaris i es va avaluar les diferències d'expressió de Pgp a nivell de RNA i proteïna en limfòcits processats i guardats en diferents condicions. S'analitzaren les diferències en l'activitat de Pgp mesurada per l'assaig de Rho123 en els diferents grups:

1. Limfòcits de sang acabada d'extreure (Frescos no congelats; F/NFr).

2. Limfòcits de sang acabada d'extreure i immediatament congelats (Frescos congelats; F/Fr).

3. Sang extreta 24h abans de la realització de l'assaig i mantinguda a temperatura ambient (No frescos i no congelats; NF/NFr).

4. Sang extreta 24h abans de la congelació dels limfòcits i mantinguda a temperatura ambient durant les 24h (No frescos i congelats; NF/Fr).

Resultats

-Anàlisis de l'expressió de Pgp per Western Blott i Real Time PCR quantitativa (figura 1 i 2 a l'article). Es van comparar les quatre condicions de processament i emmagatzematge i no es van trobar diferències significatives ni a nivell d'expressió de mRNA ni a nivell de proteïna ($p>0.05$).

-Mitjançant l'assaig de Rho123 es va estudiar la població de limfos $CD3^+$ excluïnt la població de limfos apoptòtica i necròtica ja que es va veure que podia interferir en els resultats. Quan la Pgp es bloquejava per qualsevol dels dos inhibidors de Pgp (Verapamil o PSC833) en cadascuna de les quatre condicions de processament i emmagatzematge, els limfòcits retenien el doble de Rho123 (mesurada per intensitat de fluorescencia; MFI) que sense inhibidors. No es va trobar diferències significatives en quan a la retenció de Rho123 entre cap de les quatre condicions ni entre els dos inhibidors (p=ns) (taula 1 de l'article). Per citometria de flux, els quatre grups presentaven un perfil de retenció de Rho123 molt similar tant en presència com en absència dels inhibidors (figura 3 de l'article). Per tant, podem dir que l'activitat de Pgp no es veu modificada per les diferents condicions d'emmagatzematge i manipulació.

-Els resultats no mostren diferències significatives entre els individus en la població viable de cèl·lules $CD3^+$ positives en cap de les quatre condicions de manipulació i emmagatzematge (taula 2 de l'article).

-La població de cèl·lules viables $CD3^+$ presentava una major activitat de Pgp que la població de cèl·lules apoptòtiques ($p<0,05$). Es va avaluar la població apoptòtica per separat. Aquesta població no presentava

diferències significatives d'activitat entre les quatre condicions de processament i emmagatzematge (figura 4A de l'article). Quan es va estudiar les diferents condicions sobre un mateix individu en aquesta mateixa població, si que es va observar una variabilitat de l'activitat de Pgp (figura 4B de l'article).

-Així doncs, es va treballar amb la població de cèl·lules viables. El grup de limfòcits acabats d'extreure, frescos no congelats (F/NFr) i el grup de limfòcits mantinguts a temperatura ambient durant 24h i no congelats (NF/NFr) presentaven una viabilitat del 90% mentre que els dos grups restants del 80%. La població de cèl·lules apoptòtiques i necròtiques representen una població molt minoritària (figura 5 de l'article).

2on TREBALL

Do drug transporter (ABCB1) SNPs and P-glycoprotein function influence cyclosporine and macrolides exposure in renal transplant patients? Results of the pharmacogenomic substudy within the Symphony study. Transpl Int. 2013 Feb;26(2):177-86

Objectius

L'objectiu d'aquest estudi, va ser analitzar l'associació de diferents polimorfismes d'ABCB1 (C3435T, C1236T, G2677T i T129C) amb l'activitat de Pgp i l'exposició a diferents fàrmacs immunosupressors en pacients de trasplantament renal de diferents hospitals. Aquest sub-estudi de farmacogenòmica formava part de l'estudi Symphony. L'objectiu del Symphony era avaluar els efectes de diferents dosis de CsA, Tac i SRL sobre la farmacocinètica de l'MPA en pacients tractats amb diferents règims d'immunosupressió (CsA dosis normal, CsA dosis baixa,

Tac dosis baixa i SRL dosis baixa), inducció amb daclizumab, micofenolat mofetil i esteroids. L'estudi es va realizar en 70 pacients, agrupats en: CsA (n=30), tacrolimus (n=13) i rapamicina (n=23). Per l'anàlisis farmacocinètic, es varen mesurar els nivells de tots els immunosupressors i a cada temps (7 dies, 1, 3 i 6 i 12 mesos post-trasplant) es varen obtenir 10 punts: pre-dosis i a temps 20, 40, 75min i 2, 3, 4, 6, 8, 10, i 12h post-dosis pel càlcul de l'AUC. Per dur a terme el sub-estudi de farmacogenòmica, es van genotipar tots els pacients per els diferents SNPs del gen ABCB1 i es va estudiar l'activitat de Pgp en el conjunt de cèl·lules de sang periférica mitjançant l'assaig de Rho123.

Resultats

1.Anàlisis descriptiu

Tant les variables demogràfiques (sexe i edat) com els paràmetres bioquímics (creatinina i albúmina) no presentaven diferències significatives entre els grups de pacients segons cada tractament (p=ns) (taula 2 de l'article).

2.Anàlisis d'haplotips

Es va observar una correlació positiva entre els tres SNPs del gen ABCB1: C3435T-G2677T (r^2=0,91 i P=0,001), C3435T-C1236T (r^2=0,96 i P=0,0001), i G2677T-C1236T (r^2=1,0 i P=0,0001).

3.Influència dels ABCB1 SNPs en l'activitat de Pgp i els fàrmacs immunosupressors

A. <u>ABCB1 SNPs i activitat de Pgp</u>

-L'activitat de Pgp estava influenciada per C3435T, C1236T i G2677T però no per T129C (figura 1 de l'article). Els pacients portadors TT amb els SNPs C3435T, C1236T i G2677T *low pumpers*, presentaven menys

activitat de Pgp que els no portadors (CT/CC) *high pumpers* (p=0,02) (figura 1 de l'article).

B. Fàrmacs immunosupressors i activitat de Pgp

-Els grups tractats amb CsA (n=28) presenten menys activitat de Pgp que el grup de macròlids (n=26) (P=0,04) (figura 2 de l'article). També es va analitzar el grup de macròlids per estudiar possibles diferències en l'activitat de Pgp. Es va analitzar per separat l'efecte de Tac i SRL. Els resultats mostren que els pacients tractats amb CsA tenien menys activitat de Pgp que els pacients tractats amb Tac (39,31±2,72 vs 55,06±8,57 P=0,02; respectivament) i que els pacients tractats amb SRL (ns). A més a més, els pacients tractats amb Tac mostraven una activitat de Pgp major que els pacients amb SRL, però aquesta diferencia no és estadisticament significativa.

-Per altra banda, els pacients tractats amb macròlids, tant Tac com SRL, mostren valors de Pgp semblant als obtinguts en estudis previs amb mostres de voluntaris sans.

C. ABCB1 SNPs, fàrmacs immunosupressors i activitat de Pgp

-Es van classificar els genotips segons si eren *high pumpers* (CT i CC) o *low pumpers* (TT) i es van correlacionar amb l'activitat de Pgp. Com ja era d'esperar, independentment de la teràpia immunosupressora, els pacients *high pumper* tenien més activitat de Pgp que els *low pumper* (figura 3 de l'article).

- Per l'SNP C3435T, els *high pumpers* tractats amb CsA mostraven valors d'activitat més baixos que el grup de macròlids (P<0,05).

- Els pacients *high pumpers* tant del grup tractat amb CsA com del grup macròlids presentaven més activitat de Pgp que els pacients *low pumpers* (P<0,05) (figura 3 de l'article). Aquest mateix perfil d'activitat de Pgp també es va observar pels SNPs G2677T i C1236T.

- Per altra banda, no es va observar el mateix en els pacients amb l'SNP T129. En aquest cas, els pacients *high pumper* eren els heterozigots per TT i mostraven un comportament contrari en quan a l'expressió de Pgp en comparació amb els altres tres SNPs (dades no graficades).

4.Correlació entre paràmetres farmacocinètics de CsA (AUC i Cmin) i activitat de Pgp

-Es va considerar el grup de risc, aquells pacients amb un genotip *high pumper* i baixa dosis de CsA, i es va avaluar aquest grup per separat. Els resultats mostren una correlació negativa entre CsA AUC i Cmin i activitat de Pgp al Mes 1 post-transplant (figura 4 a l'article).
-No es va observar correlació en els pacients *low pumpers* amb dosis alta de CsA, probablement pel poc número de pacients d'aquest grup.
-Els resultats no mostren cap correlació entre els pacients tractats amb macròlids (Tac o Rapa) AUC i Cmin i activitat de Pgp en cap punt de seguiment.

3er TREBALL

Impact of small molecules immunosuppressants on P-glycoprotein activity and T-cell function. J Pharm Pharm Sci. 2012 May;15(3):407-19.

Objectius

A partir dels resultats obtinguts en un estudi *in vivo* realitzat en el nostre grup, on es va descriure un model de nefrotoxicitat en rates, es va estudiar el paper de Pgp en l'associació de Rapa (mTORi) amb dos

inhibidors de calcineurina (CsA i Tac). En aquest estudi, es va analitzar l'activitat de Pgp *in vitro* en diferents subpoblacions de cèl·lula T i els efectes dels mateixos immunosupressors en monoteràpia i associats amb Rapa. Es va mesurar la retenció de Rho123, l'eflux i la cinètica d'extrusió en les poblacions $CD4^+$ i $CD8^+$ en sang perifèrica de voluntaris sans. Es va valorar la resposta antígen-específica de memòria mitjançant l'assaig de proliferació i secreció de citoquines en un cultiu mixt.

Resultats

-La funció de Pgp es va mesurar per citometria de flux mitjançant els següents paràmetres:

1. Acumulació intracel·lular de Rho123

-Es va observar una disminució significativa de la retenció intracel·lular de Rho123 en els grups tractats amb CsA i CsA+Rapa en comparació amb el grup no tractat i els altres immunosupressors (Tac, Rapa i Tac+Rapa) tant en la població de limfòcits $CD4^+$ com en la $CD8^+$ (figura 2 de l'article).

-Per altra banda, la població $CD8^+$ presentava més acumulació intracel·lular de Rho123 que la $CD4^+$.

2. Cinètica d'extrusió

- Per avaluar la cinètica d'extrusió de Rho123 segons cada grup de tractament es van establir diferents temps (0, 30, 60, 90 i 120min). Els grups amb CsA i CsA+Rapa presentaven un eflux més lent de Rho123 comparat amb els altres grups (Tac, Rapa i Tac+Rapa) al minut 60, 90 i 120 ($p<0,001$).

- Per altra banda, els grups tractats amb macròlids presentaven una extrusió similar al grup no tractat (figura 3 de l'article).

-La figura 4 de l'article, mostra els histogrames representatius del perfil cinètic d'extrusió de Rho123. La població $CD4^+$ presentava una extrusió més lenta i menor que la població $CD8^+$. És a dir, després de 2h d'eflux, les $CD4^+$ encara tenien més del 50% de la Rho123 a l'interior de les cèl·lules, mentre que la població $CD8^+$ ja havia extruït entre el 70-80% de Rho123 a l'exterior.

- Aquests resultats es van expressar en valors normalitzats i en forma de percentatge a la taula 1 de l'article on es compara l'extrusió de Rho123 en els diferents temps respecte el minut 0 (100%). Hi havia diferències significatives entre el grup no tractat i els grups de CsA sola i associada amb Rapa, només en la població $CD8^+$.

3. Activitat de Pgp

-En quant a la població $CD8^+$, els valors eren significativament menors en els grups amb CsA respecte als grups de màcrolids en monoteràpia (Tac o Rapa) o associats (Tac+Rapa) comparat amb el grup no tractat. La població de limfòcits $CD4^+$ no presentava diferències significatives entre grups (figura 5 de l'article).

4. t_{50} (taxa mitja d'eflux de fàrmacs)

-Aquest paràmetre definit com la velocitat del transportador o el temps (minuts) que tarda la Pgp en extruïr el 50% de fàrmac fora de la cèl·lula, mostra un patró cinètic similiar a l'observat en l'extrusió de Rho123. La t_{50} de les $CD8^+$ és més ràpida que les $CD4^+$ (figura 6 de l'article).

-Les dues subpoblacions limfocitàries mostraven 50% més de temps en extruïr la CsA (tant en el grup en monoteràpia com associada amb Rapa) respecte als altres grups i al grup no tractat, confirmant el potent efecte inhibitori de la CsA.

5. Assaig de proliferació. Resposta de memòria antígen-específica

-Tant l'inhbidor específic de Pgp (PSC833) com tots els immunosupressors disminuïen la proliferació de cèl·lula T de manera similar.

-La població $CD3^+$ tractada amb els diferents immunosuppressors presentava entre un 5-15% de proliferació mentre que, la proliferació en el grup sense tractament er de l'ordre d'un 30-35% ($p<0,05$) (figura 7 de l'article).

-Els dos grups associats a Rapa (CsA+Rapa i Tac+Rapa) mostren major inhibició de la proliferació $CD3^+$ que els dos fàrmacs (CsA i Tac) en monoteràpia.

-Es va observar una clara inhibició de la proliferació en la població $CD8^+$ respecte a les $CD4^+$ ($p<0,05$).

6. Anàlisis de secreció de citoquines a partir de sobrenedant del cultiu mixt

-La secreció de citoquines que segueixen un perfil pro-inflamatori (Th1) com IFN-Y i TNF-α disminuïen significativament en tots els grups tractats amb immunosupressors així com també el grup amb PSC833 respecte al grup no tractat ($p<0,05$).

-Pel que fa a les citoquines que segueixen un perfil Th2, tant el PSC833 com els immunosupressors (excepte pel grup amb CsA), disminuïen la secreció de IL-6 comparant amb el grup estimulat no tractat. Els grups tractats amb Rapa presentaven una forta inhibició de la IL-10 confirmant el rol que té la Rapa en la modulació de IL-10 (figura 8 de l'article).

DISCUSSIÓ

L'activitat de Pgp es pot mesurar mitjançant l'eflux i/o retenció de Rho123 dins la cèl·lula. En el primer treball, es va estudiar l'efecte de diferents condicions de processament i emmagatzematge de limfòcits T aïllats a partir de mostra de sang de voluntaris, en l'expressió i funció de la Pgp. Amb aquest estudi es volia optimitzar diferents estratègies d'emmagatzematge de limfòcits per tal de millorar la sensibilitat i la reproduïbilitat de l'assaig d'activitat de Pgp. S'ha de tenir en compte que la Pgp és una proteïna molt inestable i que es pot activar i desactivar pels cicles de congelació/descongelació [165]. La majoria d'estudis no especifiquen el processament i emmagatzematge de la mostra abans de l'anàlisis i aquest és un factor important alhora de reproduïr l'assaig d'activitat de Rho123 principalment en estudis multi-cèntrics. Actualment hi ha molt poc descrit a la literatura on es compari l'activitat de Pgp mesurada a partir de limfòcits aïllats directament després de l'extracció o crio-preservats. En aquest treball, no es va observar diferències significatives en quan a l'expressió i l'activitat de Pgp entre limfòcits extrets i aïllats directament (frescos) o congelats.

Se sap que la població de cèl·lules mononucleades expressen proteïnes ABC transportadores. Concretament, la població de limfòcits mostra una forta activitat transportadora, ja sigui mitjançant eflux o influx. Aquest fet es corrobora amb el nostres estudis on s'ha observat activitat de Pgp en les sub-poblacions cel·lulars $CD3^+$, $CD4^+$ i $CD8^+$. A més a més, l'eficiència per la qual la Pgp transporta diferents molècules depèn del tipus de cèl·lula i no implica que hi hagi una correlació entre la funció i l'expressió de Pgp [171, 172]. Les cèl·lules NK tenen un paper important en l'expressió i funció de Pgp, però es tracta d'una població minoritària dins la població de cèl·lules mononucleades. Per aquest motiu, s'ha treballat amb la població de limfòcits $CD3^+$, ja que es troba en un percentatge elevat i juga un paper important en la resposta immune.

Es van utilitzar dos inhibidors de Pgp, un bloquejador de canals de Ca^{+2}, el Verapamil (VP) i el PSC833 o inhibidor específic de Pgp. En l'anàlisis de la retenció de Rho123 en les diferents condicions d'emmagatzematge dels limfòcits no es va observar diferències entre els dos inhibidors. Tot i així, el PSC833 és un inhibidor més potent que el VP, ja que la concentració necesària per inhibir l'eflux de Pgp és la meitat de la que es necessita de VP.

L'activitat d'eflux de Pgp es va estudiar en les tres poblacions de cèl·lules: les viables (70-90%), les apoptòtiques tempranes (4-5%) i les apoptòtiques tardanes (3%) de la població total de $CD3^{+}$. Curiosament els resultats d'activitat de la població de cèl·lules viables no es veien afectats tant si es considerava o no la població de cèl·lules apoptòtiques tempranes. En canvi, les cèl·lules apoptòtiques tardanes i necròtiques si que interferien en els resultats i per això van ser excloses. Tot i així, la població de cèl·lules apoptòtiques la vam analitzar per separat.

Diversos estudis *in vitro* descriuen els immunosupressors com a substractes i/o inhibidors de la Pgp [77, 177-180]. La seva biodisponibilitat ve controlada principalment per les bombes d'eflux ABC transporter i pel sistema enzimàtic del citocrom P450. Els polimorfismes del gen MDR1 poden alterar, de manera diferent en cada individu, tant l'expressió com la funció de Pgp. Aquest fet condueix a l'interès clínic per la farmacocinètica dels fàrmacs immmunosupressors [60]. Aquests polimorfismes podrien afectar l'absorció i concentració en els teixits de diversos substractes de Pgp i per tant jugar un paper important en quan a la disposició del fàrmac i la resposta farmacològica entre diferents individus. No obstant, encara hi ha estudis que mostren controvèrsia alhora de demostrar que el genotip MDR1 podria modificar la farmacocinètica i la farmacodinàmia dels fàrmacs [111, 112, 139, 181-183]. En el següent treball de farmacogenòmica, els resultats confirmen una correlació significativa entre el genotip per MDR1 i l'activitat de Pgp

en *PBMCs* de receptors de transplantament renal. Els pacients homozigots portadors de l'alel T per l'SNP 3435 mostren una activitat menor comparat amb els pacients portadors dels alels CT i CC. Els nostres resultats CC≥CT>TT en relació amb l'activitat de Pgp corroboren dades publicades per altres autors [10, 184-187]. Tot i així, hi ha molta discrepància en quan a la funcionalitat de l'SNP C3435T [189-193]. Diferents estudis experimentals *in vivo* han associat el genotip homozigot per TT de l'SNP 3435 amb una expressió i activitat baixa de Pgp a nivell d'intestí [10, 114, 185, 188]. Aquesta hipòtesis, es recolza amb el fet de que aquests individus tenen un sistema d'eflux dels fàrmacs fora de la cèl·lula, poc actiu. Això es traduïria en un augment en l'absorció dels fàrmacs a través de l'intestí i per tant un augment en la concentració intracel·lular. En els pacients amb els polimorfismes C1236T i G2677T vam observar els mateixos resultats, els homozigots per TT i GG respectivament eren els que presentaven menor activitat de Pgp.

Tenint en compte els resultats obtinguts en el primer treball, on es subratllava la importància dels paràmetres que podien contribuïr en una millora dels estudis d'activitat de Pgp en individus amb diferents temps d'extracció de la mostra, diferent manipulació i inclús diferents àreas geogràfiques, vàrem mesurar l'activitat de Pgp mitjançant l'assaig de Rho123 en PBMCs congelats de mostres de receptors de transplantament renal de diferents hospitals i la seva influència amb els polimorfismes de MDR1.

Els fàrmacs immunosupressors actuals es caracteritzen per tenir un índex terapèutic estret i gran variabilitat intra i inter-individual tant en la farmacocinètica com en la farmacodinàmia. La Pgp és un sistema detoxificador important i per això pot afectar la biodisponibilitat dels fàrmacs que són substracte [136, 190]. La CsA va ser el primer immunosupressor capaç de modular l'activitat de Pgp en assajos clínics. L'activitat de Pgp a nivell d'intestí s'ha vist que pot determinar

l'aclariment oral de CsA. El tacrolimus també és substracte i inhibidor de la Pgp, tot i ser menys actiu que la CsA. El sirolimus és un substracte de Pgp que pot limitar l'absorció intestinal, i la Pgp probablement també està involucrada en la seva excreció [136]. En aquest estudi, els pacients tractats amb CsA mostren una menor activitat de Pgp comparat amb els macròlids, tant Tac com SRL. Amb les concentracions d'immunosupressió que es treballa a la clínica la CsA afecta l'activitat de Pgp, mentre que els macròlids (Tac i SRL) no modifiquen la seva funció.

Diversos estudis recolzen el fet de que la dosi pot estar influenciada per polimorfismes en MDR1. Seria d'esperar que els pacients portadors de polimorfismes tinguin més expressió i/o activitat enzimática de Pgp i CYP3A i per tan major metabolisme dels fàrmacs. Aquests pacients necessitarien més dosis per obtenir la concentració del fàrmac en sang adecuada. Actualment, no hi ha autors que hagin descrit l'associació entre l'activitat de Pgp, polimorfismes d'MDR1 i l'exposició a CsA, Tac i Rapa a diferents temps, 7 dies i 1, 3, 6 i 12 mesos post-transplant. Els nostres resultats es van analitzar en pacients de diferents centres on l'exposició a CsA, Tac i Rapa tenia una AUC ben definida, seguiment durant un any i co-medicació especificada.

Per altra banda, hi havia una correlació negativa entre l'activitat de Pgp en els limfòcits i la AUC i Cmin de CsA en el grup de pacients amb activitat de Pgp alta i dosis baixes de CsA. Pel que fa a la correlació entre l'exposició a CsA i l'activitat de Pgp, un augment en l'exposició a CsA conduïria a una disminució de l'activació de Pgp. Alhora d'interpretar els resultats també cal tenir en compte l'efecte de la Pgp a nivell intestinal. A l'enterocit, s'absorbeix la CsA i es torna a excretar en gran part al lumen intersticial mitjançant les proteïnes transportadores. Per tant, l'intestí prim és el primer que restringeix l'entrada de la CsA a l'organisme. Tanmateix, les ABC transporter també tenen un paper rellevant sobre el metabolisme del fetge abans de la distribució cap als teixits. Tots aquests

processos intervenen en la distribució i posterior eliminació del fàrmac fora de l'organisme. Com a resultat d'aquest complex on intervenen proteines transportadores i enzimàtiques, la concentració de CsA en plasma es podria veure afectada. L'SNP C3435T es correlaciona amb una expressió baixa de Pgp a nivell intestinal, i això afecta directament la biodisponibilitat oral de substractes de Pgp. Per tant, individus amb concentració baixa de Pgp intestinal tindran més absorció en el tracte gastrointestinal i per tant més concentració en plasma.

El rol de la Pgp com a bomba d'extrussió de fàrmacs fora de la cèl·lula és ben conegut però el seu paper en la resposta immune encara no està ben definit. Pendse et al va demostrar l'efecte immunomodulador dels inhibidors de Pgp sugerint les ABC transporter com a noves dianes per a la teràpia immunosupressora, tan en el rebuig agut i crònic de l'empelt com també enfermetats autoimmunes [155].

En aquest últim estudi, es va avaluar la funció de Pgp en la resposta cel·lular T analitzant la influència dels fàrmacs immunosupressors (CsA, Tac i Rapa) en la retenció intracel·lular de Rho123 i el perfil cinètic i activitat de Pgp en diferents subpoblacions limfocitàries. Els primers resultats mostren una disminució significativa de la retenció de Rho123 en els grups tractats amb CsA, sugerint la importància del paper de la Pgp no només en la sortida sinó també en l'entrada de substractes a l'interior de les cèl·lules. Dades sobre la retenció/eflux de Rho123 demostren que la Pgp és funcional tan en la població de limfos $CD4^+$ com en la $CD8^+$, tot i que aquestes dades no aporten una mesura quantitativa de l'expressió de Pgp en aquestes cèl·lules [73]. Els nostres resultats reafirmen l'efecte inhibitori de la CsA en el bloqueig de Pgp, en canvi, l'activitat de Pgp en els grups tractats amb macròlids (Tac i Rapa) tenien un perfil similar al grup no tractat. Per tant, podem dir que les concentracions de Rapa i Tac no modifiquen l'activitat de Pgp. Aquests resultats s'ajusten als obtinguts en un model de nefrotoxicitat en rates on

els autors varen estudiar el rol de Pgp en l'associació de Rapa amb CsA i Tac [207]. És ben conegut que la CsA indueix la sobreexpressió de Pgp *in vitro* i *in vivo* [177] de manera irreversible i dosi-depenent [208].

A la literatura trobem dades que generen controvèrsia sobre la hipòtesis en que l'expressió d'MDR1 representa un mecanisme de resistència en la teràpia en transplant d'òrgans sòlids. Gotzl et al van mesurar l'expressió de Pgp a nivell de mRNA en sang perifèrica de pacients de transplantament renal [209]. Van veure que la falta d'expressió de MDR1 coincidia en un augment en la supervivència de l'injert. També s'ha vist que pacients de transplantament renal, no mostren diferència en l'expressió de Pgp en PBMCs de pacients amb injerts estables enfront pacients amb rebuig crònic [210] o sensibles a CsA o Tac versus pacients resistents a aquests immunosupressors [172]. En canvi, en transplantament de cor [211] o pulmó [212] s'ha vist que un augment de l'expressió de Pgp en PBMCs s'associa amb un rebuig. L'anàlisis de l'eflux de Rho123 en els grups tractats amb CsA (amb i sense Rapa) mostrava un perfil de cinètica més lent comparat amb els altres grups d'immunosupressors (Tac, Rapa i Tac+Rapa). A més a més, l'activitat de Pgp es va calcular com la taxa mitja d'eflux del fàrmac fora de la cèl·lula (T_{50}). Els grups de cèl·lules tractats amb CsA tarden gairebé més del doble de temps en afluïr el 50% de Rho123 comparat amb el grup de macròlids. És a dir, el grup tractat amb CsA+Rapa tardava més en extruïr la Rho123 que el grup de CsA sol confirmant la toxicitat que pot presentar aquesta associació. La nefrotoxicitat induïda per anticalcineurínics amb rapamicina ésta ben descrita en models animals [213-215]. Per altra banda, l'associació de Tac amb Rapa i els dos immunosupressors per separat, tardaven el mateix temps en afluïr el 50% de fàrmac a l'exterior que el grup no tractat. Rapa i Tac afecten l'activitat de Pgp però no modulen la funció de Pgp a dosis pre-establertes.

També es va estudiar l'efecte dels immunosupressors sobre la inhibició de Pgp i el seu perfil cinètic en la població de limfòcits T $CD4^+$ i $CD8^+$. Estudis funcionals demostren un augment significatiu d'activitat de Pgp en la població de cèl·lules $CD8^+$ tot i que l'expressió és més alta a les $CD4^+$ [73]. Encara que es coneix molt bé el rol de la Pgp en el camp de la cèl·lula T, existeix molta discrepància en quan a l'expressió i funció entre les poblacions $CD4^+$ i $CD8^+$. Els nostres resultats mostren més retenció i eflux de Rho123 en la població $CD8^+$ que en la població $CD4^+$, això suggereix que tant l'entrada com la sortida de substractes de Pgp es modula diferent depenent de la subpoblació de cèl·lules T. D'acord amb els resultats publicats anteriorment [150], la cinètica d'extrussió de Rho123 era molt més rápida a les $CD8^+$ que a les $CD4^+$. Aquest fet es correlacionaria amb el rol fisiològic de la Pgp a la població de limfos T citotòxics. Per altra banda, l'extrusió de Rho123 en ambdos grups tractats amb CsA era molt més lenta que en els grups amb Tac, Rapa i Tac+Rapa en les diferents sub-poblacions.

Avui en dia l'interès pel rol que tenen les ABC transporter està creixent. La seva funció d'extrusió i/o inhibició podria convertir-se en un nou target per la farmacodinàmia [164]. En els últims anys, Brennan et al ha disenyat una molècula (LIM-0705) capaç d'activar determinats transportadors en ronyó i fetge, facilitant l'extrusió de Tac fora de les cèl·lules més vulnerables per tal de reduïr al màxim la toxicitat i al mateix temps preservar el seu efecte immunosupressor. *In vitro*, l'augment de la concentració intracel·lular del fàrmac ve associat amb una inhibició més efectiva de la proliferació per CsA. *In vivo*, aquest fet es traduïria en una protecció més efectiva enfront un possible rebuig de l'injert [212]. Davant una segona exposició de l'antigen, les dues sub-poblacions de cèl·lules T de memòria proliferen rapidament i es diferencien en cèl·lules efectores. Es coneix que els anticalcineurínics són els immunosupressors per excel·lència per suprimir la resposta al·loreactiva efectora i/o de memòria de les cèl·lules T [171]. En aquest treball s'ha estudiat, si tant el

PSC833 com els diferents immunosupressors tenen un efecte inhibidor sobre les cèl·lules T de memòria al·loreactives. Es va observar una inhibició més forta de la proliferació sobre les $CD8^+$, comparat amb la sub-pobalció $CD4^+$. Desde un punt de vista clínic, aquests resultats podrien tenir un impacte en el camp del transplantament renal tenint en compte que les cèl·lules T de memòria antigen-específiques tenen menys sensibilitat enfront agents immunosupressors, siguent aquestes cèl·lules les responsables d'episodis de rebuig cel·lular agut.

En els nostres experiments també es va analitzar la secreció de citoquines en el sobrenedant del cultiu mixt limfocitari (MLR) amb els diferents immunosupressors i l'inhibidor specific de Pgp. En quan a la secreció de citoquines pro-inflamatories Th1, es va veure que tant la secreció d'IFN-Y com TNF-α disminuïen en tots els grups de tractament, i aquesta resposta s'accentuava si ens fixem en el grup amb Tac. La secreció de IL-6, una citoquina de tipus Th2, disminuïa en tots els grups de tractament menys en el grup amb CsA i PSC833 en comparació amb el grup estimulat no tractat. Per altra banda, els grups tractats amb Rapa (sola o associat amb CsA) van ser els únics grups que disminuïen significativament la secreció de IL-10, fet que subratlla la importància que té la Rapa en la modulació d'aquesta citoquina.

Globalment, tots aquests resultats evidencien la importància que té la Pgp com a proteïna transportadora de molts immunosupressors en la seva exposició, i el rol que té la Pgp en el camp de la immunitat, en els limfòcits T. Per altra banda, l'estudi dels polimorfismes del gen que codifica per la Pgp, ens permetrà ajustar l'exposició dels immunsupressors, evitant els ajusts erràtics de les dosis i augmentant la seva seguretat i eficacia, minimitzant els nivells sub-terapèutics indesitjables. Un pas endavant cap a la medicina personalitzada.

CONCLUSIONS

1. L'activitat de Pgp mesurada a partir de la retenció de Rho123 per cadascuna de les diferents condicions d'emmagatzematge no es modifica. L'activitat de Pgp en la població de cèl·lules necròtiques interfereix en els resultats i per tant s'ha descartat aquesta població, tenint només en compte la població de cèl·lules viables.

2. L'ús dels diferents paràmetres de mesura d'activitat de Pgp són una eina fonamental per la normalització d'estudis d'activitat de Pgp en individus amb diferent temps d'extracció i processament de la mostra o diferents zones geogràfiques, d'aquesta manera s'optimitza la sensibilitat i reproduïbilitat de l'assaig de Rho123.

3. Els polimorfismes d'MDR1 (C3435T, C1236T i G2677T) estan influenciats per la funció de Pgp en la població de limfòcits T $CD3^+$ en pacients de transplantament renal. Els pacients amb l'SNP C3435T portadors de l'al·lel C presenten una activitat major, mentre que els pacients amb genotip TT mostren menor activitat. Els pacients amb els SNPs C1236T i G2677T, presenten el mateix perfil d'activitat. L'activitat de Pgp no es veu influenciada per l'SNP T129C.

4. Variacions en l'activitat de Pgp depenen de la teràpia immunosupressora. Els pacients tractats amb CsA mostren una menor activitat de Pgp en comparació amb els grups tractats amb macròlids (Tac i Rapa). Els pacients tractats amb baixa dosis de CsA i genotip *high pumper* tenen menys concentració intracel·lular de CsA ja que la capacitat d'extrusió que tenen és més alta. Aquest grup de pacients tenen la resposta terapèutica modificada ja que mostren una menor exposició al fàrmac.

5. Hi ha una correlació negativa entre els individus *high pumper* tractats amb baixa dosis de CsA (AUC i Cmin) i activitat de Pgp. No hi ha correlació entre els individus *low pumper* amb dosis altes de CsA. A més a més, no hi ha correlació entre els grups de macròlids (Tac i SRL) AUC i Cmin i activitat de Pgp als 7dies, 1, 3 i 12 mesos post-transplant.

6. L'activitat de Pgp mesurada com la taxa mitja d'eflux de fàrmacs mostra un perfil cinètic similar a l'activitat de Pgp mesurada mitjançant l'extrusió de Rho123. La cinètica d'extrusió de Rho123 és més ràpida en la població de cèl·lules $CD8^+$ respecte a la població $CD4^+$.

7. La inhibició de l'activitat de Pgp per part de la CsA té un efecte més potent en la sub-població de cèl·lules $CD8^+$ respecte a la sub-població de cèl·lules $CD4^+$.

8. Els immunosupressors disminueixen significativament la proliferació de les sub-poblacions $CD4^+$ i $CD8^+$. La inhibició de Pgp seria una nova diana terapèutica a tenir en compte per tal d'evitar el rebuig de l'empelt en transplantaments d'òrgans sòlids.

ANNEX 2

Paper I

Dendritic cells phenotype fitting under hypoxia or lipopolysaccharide; adenosine 5_-triphosphate-binding cassette transporters far beyond an efflux pump.

Lloberas N, Rama I, Llaudó I, Torras J, Cerezo G, Cassis L, Franquesa M, Merino A, Benitez-Ribas D, Cruzado JM, Herrero-Fresneda I, Bestard O and Grinyó JM.

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Dendritic cells phenotype fitting under hypoxia or lipopolysaccharide; adenosine 5′-triphosphate-binding cassette transporters far beyond an efflux pump

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[1]Both authors are considered co-directors of this study.

Summary

This study examines adenosine 5′-triphosphate-binding cassette (ABC) transporters as a potential therapeutic target in dendritic cell (DC) modulation under hypoxia and lipopolysaccharide (LPS). Functional capacity of dendritic cells (DCs) (mixed lymphocyte reaction: MLR) and maturation of iDCs were evaluated in the presence or absence of specific ABC-transporter inhibitors. Monocyte-derived DCs were cultured in the presence of interleukin (IL)-4/granulocyte–macrophage colony-stimulating factor (GM-CSF). Their maturation under hypoxia or LPS conditions was evaluated by assessing the expression of maturation phenotypes using flow cytometry. The effect of ABC transporters on DC maturation was determined using specific inhibitors for multi-drug resistance (MDR1) and multi-drug resistance proteins (MRPs). Depending on their maturation status to elicit T cell alloresponses, the functional capacity of DCs was studied by MLR. Mature DCs showed higher P-glycoprotein (Pgp) expression with confocal microscopy. Up-regulation of maturation markers was observed in hypoxia and LPS-DC, defining two different DC subpopulation profiles, plasmacytoid *versus* conventional-like, respectively, and different cytokine release T helper type 2 (Th2) *versus* Th1, depending on the stimuli. Furthermore, hypoxia-DCs induced more B lymphocyte proliferation than control-iDC (56% *versus* 9%), while LPS-DCs induced more CD8-lymphocyte proliferation (67% *versus* 16%). ABC transporter-inhibitors strongly abrogated DC maturation [half maximal inhibitory concentration (IC_{50}): P-glycoprotein inhibition using valspodar (PSC833) 5 μM, CAS 115104-28-4 (MK571) 50 μM and probenecid 2·5 μM], induced significantly less lymphocyte proliferation and reduced cytokine release compared with stimulated-DCs without inhibitors. We conclude that diverse stimuli, hypoxia or LPS induce different profiles in the maturation and functionality of DC. Pgp appears to play a role in these DC events. Thus, ABC-transporters emerge as potential targets in immunosuppressive therapies interfering with DCs maturation, thereby abrogating innate immune response when it is activated after ischaemia.

Keywords: ABC transporter blockers, dendritic cells maturation, immunosuppression, P-glycoprotein

Introduction

Dendritic cells (DCs) are professional antigen-presenting cells whose differentiation, migration and activities are linked intrinsically to the microenvironment. The capacity of DCs to activate and regulate T cell responses is acquired during a complex differentiation and maturation programme [1,2]. DCs originate in bone marrow, and at an immature stage (iDC) they migrate through diseased peripheral tissue before reaching their final destination in the lymph node [1,3,4]. In view of their physiological function, DCs play an important role in adverse immune reactions such as autoimmune diseases, chronic inflammatory processes and allograft rejection [2,5]. Thus, modulation of

DC function is a promising strategy in the treatment and prevention of such diseases [6,7]. Furthermore, their ability to change phenotype and function, depending on their stage of maturation, is an interesting target in immune system modulation towards tolerance in solid organ transplantation.

One of the most obvious scenarios in which hypoxia may play a role in immune-mediated renal damage is the transplantation setting. It is clear that ischaemia– reperfusion injury during transplantation contributes to the adaptive and innate immune response. In recent years, DCs have been studied regarding their important role in immune response as a bridge between innate and acquired immune responses [1,4,5]. In a previous report we investigated the functional changes shown by immature DCs (iDCs) after hypoxia-induced differentiation [8]. In that study we confirmed that hypoxia, similar to allogeneic stimulus, induced maturation of DCs, which was associated with an increase in hypoxia-inducible factor (HIF)-1α protein levels and was attenuated by mammalian target of rapamycin inhibition. We presented hypoxia as a novel maturation signal not only for monocyte-derived DCs, but also for renal resident iDCs exposed to ischaemia [8]. This new mechanism for renal DC maturation invites speculation about the role of these cells in the immune-mediated response to renal ischaemia. Thus, we might hypothesize that ischaemia-induced maturation of renal DCs drive their migration to regional lymph nodes, as well as bringing about T cell activation and additional immune-mediated damage to the kidney.

Proteins of the adenosine 5′-triphosphate-binding cassette (ABC) transporter superfamily are involved in the active transport of a broad range of substrates, ranging from xenobiotics, peptides and proteins to sugars, metal ions and lipids [9,10]. The primary role of these molecules in various physiological processes is as an efflux pump, conferring resistance by driving out cytotoxic xenobiotics, toxic molecules and various cellular products [11,12]. ABC proteins identified for their role in cancer multi-drug resistance (MDR) chemotherapy are the *MDR1* gene-encoded P-glycoprotein (Pgp; ABCB1) [13] and multi-drug resistance protein 1 (MRP1; ABCC1) [14–16]. In fact, ABC transporters are described fully in nephrotoxicity models in kidney allografts, and play a key role in the pharmacokinetics of many immunosuppressors. Pgp and MRP1 have been found to be expressed in skin DC and monocyte-derived DC (interstitial DC), and functionally, both transporters have been described as being required for efficient DC maturation and T cell migration [12]. In this field, Pgp is implicated in interleukin (IL)-12 secretion, resulting in the activation of nuclear factor kappaB (NF-κB) in DCs, which is a key event in the initiation of DC maturation [12].

As DCs are the most potent antigen-presenting cells of the immune system, it is important to know which molecules are essential in their function. ABC transporters, Pgp and MRP1, have already been shown to be required for DC differentiation and maturation after tumour necrosis factor (TNF)-α stimuli [17]. During hypoxia, extracellular adenosine 5′-triphosphate (ATP) levels often increase and these extracellular ATP act as a *find me* signal for many phagocytic cells, including DCs. Thus, it is important to understand the effects of hypoxic environment on local or lymph node DCs and other immune cells. As the putative contribution of ABC transporters as well as other mechanisms defined previously in studies of drug resistance to DC functioning is still relatively unknown, we were tempted to explore this issue under hypoxic conditions.

Notably, immune responsiveness might benefit from such mechanisms. Thus, we aimed to study whether ABC transporters were also essential in maturation of DCs in a hypoxic microenvironment, a well-known stimulus in pathological events such as ischaemia–reperfusion injury. Modulation of DC hypoxia-related maturation through ABC transporters could be an interesting target to reduce immunoinflammatory responses in organ transplantation.

Materials and methods

Antibodies and reagents

The following monoclonal antibodies were obtained from Becton Dickinson Pharmingen (San Diego, CA, USA): anti-human CD3-allophycocyanin (APC), CD20-phycoerythrin (PE), CD14-APC, CD11c-PE-cyanin 5 (Cy5), CD40-fluorescein isothiocyanate (FITC), CD80-APC, CD83-APC, CD86-FITC, CD54-APC and human leucocyte antigen D-related (HLA-DR)-FITC. Mouse anti-human JSB1 (Pgp) (Calbiochem, Darmstadt, Germany), rat anti-human 4124 (MRP) (Chemicon International, Temecula, CA, USA), anti-human DC-lysosomal-associated membrane protein (LAMP) (T-20; Santa Cruz, Madrid, Spain) and secondary antibodies were purchased from Invitrogen (Molecular Probes, Eugene, OR, USA) and 4′,6-diamidino-2-phenylindole (DAPI) mounting medium from Santa Cruz (Madrid). The MDR1 Pgp antagonist PSC833 was provided by Novartis AG (Basel, Switzerland). Purified recombinant human IL-4 and granulocyte–macrophage colony-stimulating factor (GM-CSF) were purchased from R&D Systems (Minneapolis, MN, USA). Lipopolysaccharide (LPS) (*Escherichia coli* serotype 011:B4) and phytohaemagglutinin (PHA) were purchased from Sigma-Aldrich (Madrid, Spain) and MK571 was obtained from Alexis Biochemicals (Grupo Taper SA, Madrid, Spain). Medium and supplements were purchased from PAA (Linz, Austria) and Lonza (Verviers, Belgium). Annexin-V and 7-aminoactinomycin D (7-AAD) were purchased from Sigma-Aldrich (Madrid). Anti-human HIF-1α-fluorescein monoclonal antibody and mouse immunoglobulin (Ig)G1 isotype control-CFS was obtained from R&D Systems. Cytometric bead array (CBA) and carboxyfluorescein

diacetate succinimidyl ester (CFSE) were from Molecular Probes (Madrid, Spain).

Ethics statement

Human cells were obtained in accordance with protocols approved by the Ethics Committee of the Hospital Bellvitge of Barcelona (Barcelona, Spain) and in accordance with the principles of the Declaration of Helsinki.

Dendritic cell culture and drug treatment

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient centrifugation of blood obtained from buffy coats from healthy donors. PBMCs (200×10^6 cells/ml) were incubated for 2 h at 37°C in 5% CO_2 in 25 cm^2 flask plates. After washing, the adherent monocytes were cultured in the presence of 500 U/ml of IL-4 and 1000 U/ml of GM-CSF in RPMI-1640 medium with 10% human serum at 37°C in a humidified atmosphere of 5% CO_2, obtaining 90% DC purity at day 7. ABC inhibitors were added once after 48 h of monocyte isolation: MDR1 inhibitor (PSC833, 5 μM), MRP1 and MRP2 inhibitors (MK571, 50 μM) and probenecid (PBN), 2·5 μM. Cells were kept at 37°C in a humidified atmosphere with 5% CO_2. Medium with supplements and inhibitors was changed every second day and prior to experiments. The gating of DC populations was validated in our previous study [8]. Lymphocytes were obtained by Ficoll-Percoll gradient and purified by non-adherence.

Dendritic cell differentiation under LPS and hypoxia stimuli

Immature DCs (2×10^6 cells/ml RPMI 10% human serum) were exposed at day 5 to hypoxia conditions for 48 h [8]. Hypoxic (0·5% oxygen) conditions were generated at day 5, exposing iDCs to hypoxia (0·5% O_2, 5% CO_2) in a hypoxia atmosphere-controlled incubator (Binder), keeping cells unmanipulated for 48 h, thereby avoiding O_2 pressure changes. To compare with a standard stimulus for DCs maturation, LPS (2 μg/ml) was added for 24 h at day 6 after PBMC isolation.

Cell phenotype analysis by flow cytometry

Flow cytometry (fluorescence-activated cell sorting: FACS) analysis was performed using a FACS Canto and DIVA software (Becton Dickinson). The study subpopulation was defined using different cell markers: CD3 for lymphocytes, CD14 for monocytes, CD20 for B cells and CD56 to stain natural killer (NK) cells. Thereafter, FACS was performed at day 7 of DCs to assess mean fluorescence and expression of mature cell phenotype. CD14, CD11c and CD123 were used to identify the DC nature and different markers were used to define the mature population of DCs (mDCs) (CD40/CD80/CD83/CD86/CD54/HLA-DR). To assess the DC phenotype, we used the markers according to standard methods in the literature for DCs [18–20]. Incubation was carried out at 4°C for 30 min. Apoptosis was measured by annexin-V using flow cytometry.

HIF-1α protein expression on hypoxia DCs ± ABC transporter inhibitors

Intracellular HIF-1α was assessed by flow cytometry (FACS Canto; Becton Dickinson). DCs were identified with two membrane markers as HLA-DR^+ and $CD11c^+$. After phenotyping, cells were permeabilized with saponine buffer (Sigma, Madrid) and labelled with HIF-1α or isotype control (R&D Systems). Intracellular HIF-1α was analysed in the double-positive region for HLA-DR^+ and $CD11c^+$.

Analysis of Pgp and MRP1 expression (iDC and mDC) by immunofluorescence

To assess Pgp and MRP1 expression in iDCs and mDCs, double-surface immunostaining and dual-colour flow cytometry of freshly isolated PBMCs were carried out following incubation overnight at 37°C in human serum. Cells were washed twice in phosphate-buffered saline (PBS), fixed with cold methanol for 3 min and washed twice with PBS. DCs were blocked with fetal bovine serum (FBS) 20% for 2 h. For indirect staining of Pgp in mDCs, $0{\cdot}5 \times 10^6$ DCs were incubated overnight at 4°C with the primary anti-Pgp monoclonal antibody (mAb) JSB1 (1/50 with FBS 10%), anti-MRP1 mAb (4124) and DC LAMP antibody (1/50 with FBS 10%). Before incubation, cells were permeabilized to anti-Pgp mAb JSB1 incubation. After incubation, cells remained for 30 min at room temperature. The DCs were then incubated with the secondary antibodies Alexa 647 and Alexa 488 (1/100 with FBS 1%) for 45 min and washed. Finally, DCs were mounted in DAPI. Analysis of cell surface marker expression was performed using the dual-colour inmunofluorescence technique (Leica TCS-SL confocal espectral microscope, Mannheim, Germany) equipped with image analysis software (Leica confocal software).

Cell proliferation CFSE assay by flow cytometry

Distinguishing DCs from monocytes was also defined functionally by the ability to stimulate an allogeneic mixed leucocyte reaction (MLR) [20,21]. Thus, we tested not only phenotypical changes, but also functionally tested CD3 proliferation. We performed a CFSE study to analyse the effector function of these DCs; the results supported the phenotypical changes and also emphasized the distinction from macrophages. Lymphocytes were stained with CFSE and exposed to mDCs (under hypoxia or LPS stimuli) with

or without ABC transporter inhibitors. After 24 h, medium was removed and co-culture was performed with fresh medium. Allogeneic CFSE-labelled PBMCs (2×10^5) were cultured alone (negative control) or in the presence of DCs collected at the end of the 7-day culture after stimuli exposure (DC : T cell ratio 1:10; final volume 200 µl RPMI 10% FBS). As positive control responder, PBMCs were stimulated with 1 µg/ml (PHA). After 5 days of culture (37°C, 5% CO_2) the proliferation of responder cells was determined by flow cytometry after labelling with CD20, CD4 and CD8 antibody to exclude DCs and to define different B and T lymphocyte subpopulations. No ABC transporter inhibitors were used in T and DC co-cultures.

In addition, MLR with purified T and B cells was performed with the RosetteSep human T cell enrichment cocktail and the RosetteSep human B cell enrichment cocktail, respectively (Stemcell Technology, Grenoble, France) After cell isolation the MLR technique was carried out as described.

Cell cytokine analysis by flow cytometry

Flow cytometry analysis was performed using FACS Canto and DIVA software (Becton Dickinson). Interleukin-2, -4, -6, -10, -17a, TNF-α and interferon (IFN)-γ secretion protein levels from cell supernatant were measured quantitatively following cell stimulation by CBA (BD Biosciences). Cytokine quantification was performed on stimulated and non-stimulated, and treated and non-treated (with ABC inhibitors) DCs, and on lymphocytes after MLR.

Statistical analysis

Each experiment was performed at least three times and representative data are shown. Significant differences between samples were determined by analysis of variance (ANOVA) with the SPSS statistical package. Data in bar graphs are given as the mean ± standard deviation (s.d.). A value of $P < 0{\cdot}05$ was considered significant.

Results

Analysis of monocyte, iDC and mDC populations after hypoxia and LPS

Monocytes were isolated and cultured with GM-CSF and IL-4; the resulting iDCs were exposed to hypoxia on day 5 for 48 h or to LPS for 24 h to induce cell maturation. Figure 1a shows the analysis of different cellular subpopulations during the differentiation and maturation of DCs. At day 0 we had a high percentage of monocytes ($CD14^+$) and the presence of several lymphocyte subtypes ($CD3^+$, $CD20^+$ and $CD56^+$). During differentiation, the $CD14^+$ population expressed DCs markers ($HLA\text{-}DR^+$ and $CD11c^+$) and the lymphocyte percentage diminished after removing the medium and replacing it with fresh culture medium. At the end of the differentiation (at day 7) the purity of DCs was greater than 90% (Fig. 1b). DC population was gathered in two subpopulations, depending on the degree of maturation according to the forward-/side-scatter profile and specific phenotypic markers established in our previous study [8]. We also performed a follow-up of DC differentiation at different time-points. We observed that after hypoxia or LPS stimulus, cells changed their morphology, acquiring a stellate form characteristic of the mDCs shifting to the upper window. LPS stimulus induced a more homogeneous and stronger maturation response, while hypoxia stimulus showed a different magnitude of response (Fig. 1b). To evaluate further the changing phenotype after stimuli of the DC population, FACS analysis was performed at days 1, 5 and 7. CD40 mean fluorescence revealed that mDCs appeared at day 5 of decreasing monocytes and iDCs populations. After LPS and hypoxia stimuli at day 7, DCs were well differentiated from non-stimulated cells.

Analysis of Pgp and MRP1 expression in iDC and LPS/hypoxia-DCs by immunofluorescence

To characterize mDCs we used DC-LAMP, a type I transmembrane glycoprotein restricted to mDCs and expressed in the endosomal/lysosomal compartment. DCs exposed to LPS or hypoxia showed a clear DC LAMP-positive up-regulation, confirming the mature phenotype. Dual staining with the Pgp (JSB1) or MRP1 (4124) antibodies also showed an over-expression of Pgp and MRP1 in those DC-LAMP-positive DCs, differing from non-stimulated cells ($P < 0{\cdot}05$) (Fig. 2a,b, respectively). This may indicate that in DC maturation there is an increase in Pgp and MRP1 in the cell membrane. Furthermore, this effect was more evident after LPS stimuli than after hypoxia.

Effect of ABC transporter blockade in hypoxia and LPS DC maturation

To evaluate the ABC transporters involvement in DC maturation, PSC833, MK571 or PBN were added to inhibit MDR1, MRP1 and MRP2, respectively.

After hypoxia stimulation the percentage of mature DCs was evaluated by the forward-/side-scatter profile. Hypoxia resulted in an induction of 67·8% of mDCs *versus* 32·2% of iDCs (Fig. 3), lower compared to LPS, which induced 80·8% of mDCs and 19·2% of iDCs ($P < 0{\cdot}05$). The addition of ABC transporter inhibitors shifted the ratio of mature and immature DCs achieved after both stimuli (Fig. 3) ($P < 0{\cdot}05$). MDR1 and MRP inhibitors induced a marked decrease in mDCs [half maximal inhibitory concentration (IC_{50}): P-glycoprotein inhibition using valspodar (PSC833 5 µM, CAS 115104-28-4 (MK571) 50 µM and probenecid 2·5 µM] and an increase in iDCs. Thus, after

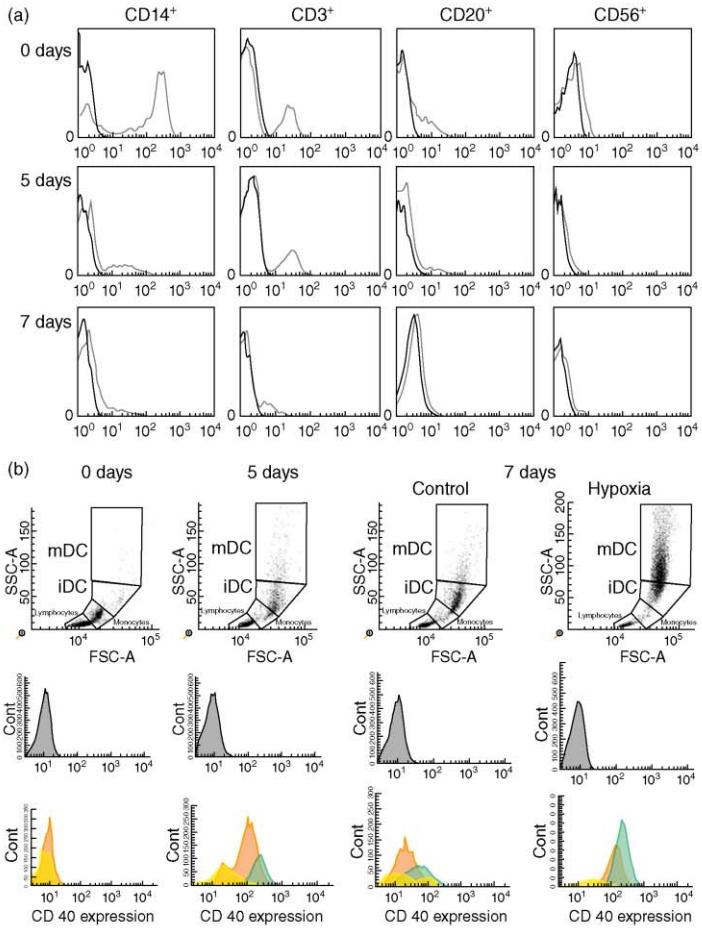


Fig. 1. (a) Analysis of different cellular subpopulations during the differentiation and maturation of dendritic cells (DCs). Cells were stained at different times (days 0, 5 and 7) from isolation by antibodies specific for monocytes, T, B and natural killer (NK) cells in control condition ($CD14^+$, $CD3^+$, $CD20^+$ and $CD56^+$, respectively). (b) Follow-up of increasing dendritic cell maturation under hypoxia by flow cytometry. At day 0, monocytes were cultured with granulocyte–macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4. At day 5, immature dendritic cells (iDCs) were stimulated for a further 48 h with hypoxia, acquiring mature phenotype with high expression of $CD40^+$ (mDCs) with respect to non-stimulated cells (control). The percentage (%) of monocytes is shown in yellow, iDC in orange and mDC in green.

hypoxia, PSC inhibited mDCS to 31·4% ($P < 0·05$), MK571 to 40% ($P < 0·05$) and PBN to 45·6% ($P < 0·05$). The effect of ABC blockers on DC maturation after LPS showed similar results: PSC833 reduced mDCS to 48·8% ($P < 0·05$), MK571 to 51·6% ($P < 0·05$) and PBN to 50·6% ($P < 0·05$). All mean values were analysed for 10 experiments.

To rule out a toxic effect of inhibitors on DC viability, cell apoptosis was analysed by annexin V/7-ADD assay. A comparable percentage of viable cells was observed after hypoxia exposure with or without ABC inhibitors exposure (H: 86·1%, H + PSC: 84·25%, H + MK: 85·29% and H + PBN: 83·7%). We found no statistical changes between hypoxia DC and non-stimulus.

Phenotype switch on DC maturation: effect of ABC transporter blockade

Hypoxic conditions induced a twofold DC maturation compared to control non-stimulated DCs ($P < 0·05$), analysed as intensity of different maturation markers (CD40, CD83, HLADR and CD54). This confirmed the results validated in a previous study [8]. ABC inhibitors showed a clear decrease in both plamacytoid-like and conventional DC phenotype maturation, depending on the stimuli (Table 1).

When iDCs were stimulated by LPS the mean fluorescence intensity (MFI) of CD80, CD86, HLA-DR and CD54 maturation markers increased MFI threefold with respect to control, and there was a twofold increase of MFI with respect to hypoxia stimulus (Table 1). Interestingly, CD83 and CD40 were similarly up-regulated under both stimuli, and CD86 was down-regulated under hypoxia-achieving control values, suggesting a plasmocytoid-like phenotype pattern with respect to LPS-DC. Despite these differences in the maturation response of DCs after the two stimuli, the up-regulation of maturation markers was abrogated strongly when ABC inhibitors were added at a similar intensity (Table 1). All results are representative of six experiments.

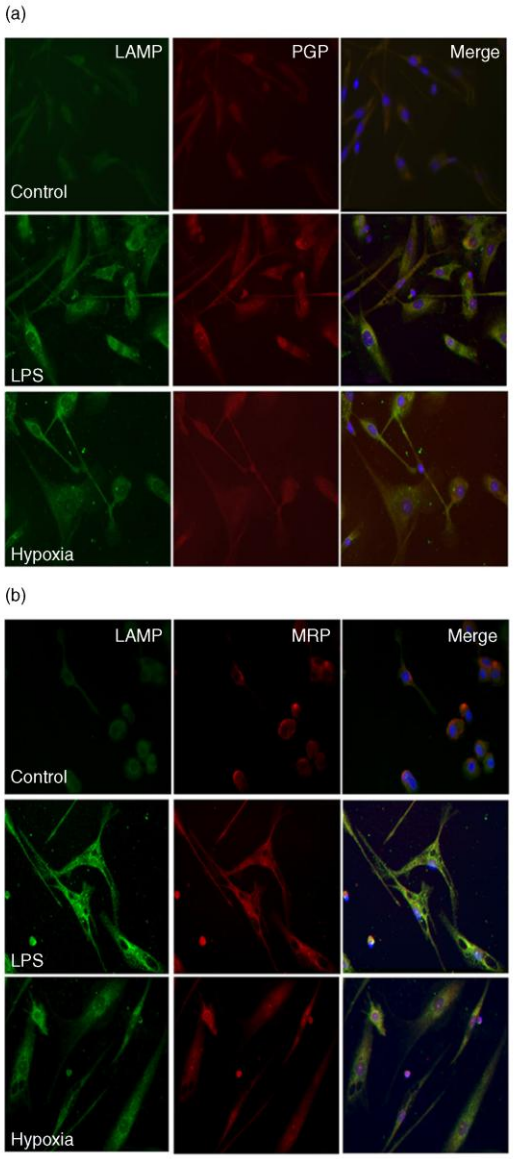
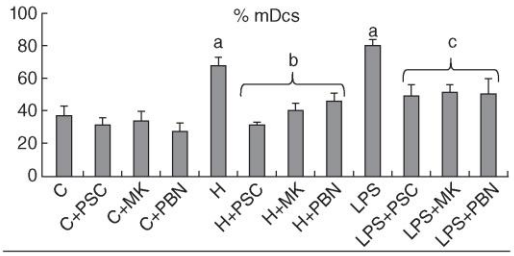


Fig. 2. Over-expression of P-glycoprotein (Pgp) and multi-drug resistance protein 1 (MRP1) by immunofluorescence in dendritic cells (DCs) after hypoxia and lipopolysaccharide (LPS). Two-colour immunofluorescence images were obtained from DC lysosomal-associated membrane protein (LAMP) antibody [first panel of each column (1), green] and P glycoprotein antibody (JSB1) Pgp (a) or MRP1 (b) antibody [second panel of each column (2), red]. The two images were merged and double-positive cells are shown in the third panel of each row (3). iDCs appear in the first row, LPS-DC in the second row and hypoxia-DCs in the third row.



a = $P < 0{\cdot}05$ *versus* control
b = $P < 0{\cdot}05$ *versus* hypoxia
c = $P < 0{\cdot}05$ *versus* LPS

Fig. 3. Effect of P-glycoprotein (Pgp) blockade in dendritic cells (DCs) after hypoxia and lipopolysaccharide (LPS) maturation. After DC maturation stimuli (hypoxia or LPS), the percentage of mDCs increased with respect to non-stimulated cells. Additionally, the Pgp inhibitors blockaded the maturation of the DCs. Each solid bar represents the average percentage of 10 different experiments [$P < 0{\cdot}05$ *versus* non-stimulated cells and adenosine 5′-triphosphate-binding cassette (ABC) transporter inhibitors].

Figure 4 is a representative histogram of the most relevant changes in DC maturation markers after hypoxia or LPS.

HIF-1α protein expression on hypoxia DCs ± ABC transporter inhibitors

HIF-1α expression in control cells was 37·5 ± 5·2%, when DCs stimulated by hypoxia were increased significantly with respect to control (67·6 ± 3·7). Interestingly, when ABC inhibitors were added to hypoxic-DC, HIF-1α results were similar to hypoxia-DCs (H + PSC833 57·5 ± 4·4 and H + MK571 62·3 ± 5·1) (Fig. 5).

Impact of ABC transporter inhibition on lymphocyte proliferation

To address the functional impact of ABC transporter inhibition on DCs, we next assessed the effects of these cells on lymphocyte proliferation in the MLR, evaluated by CFSE staining. Hypoxia- and LPS-matured DCs were capable of inducing a significantly ($P < 0{\cdot}05$) higher lymphocyte proliferation than non-stimulated iDCs. Functional studies showed a higher T cell proliferation after LPS than after hypoxia stimulus (53·9% with LPS *versus* 28·5% with hypoxia). ABC transporter inhibitors reduced alloimmune lymphocyte proliferation after exposure to hypoxia and LPS mDCs by approximately 20–30% with respect to non-treated cells ($P < 0{\cdot}05$) (data not shown).

To explore further the impact of different DC subtypes on lymphocyte proliferation, lymphocyte subpopulations were assessed. Interestingly, the LPS stimulus induced higher lymphocyte proliferation in the CD8 lymphocyte

Table 1. Representative fluorescence activated cell sorter (FACS) mature phenotype markers for dendritic cells (DCs).

	Control		LPS								Hypoxia							
	Control		LPS		LPS+PSC		LPS+MK		LPS+PBN		H		H + PSC		H + MK		H + PBN	
	%	MFI	%	MFI	%	MFI	%	MFI	%	MFI	%	MFI	%	MFI	%	MFI	%	MFI
Presentation molecules																		
MHC II	96·5	549·9	98·6	2260·9[a]	96·9	1106·6[b]	94·2	700·7[b]	91·8	484·2[b]	99·2[b]	1085·2	93·1	660·7	75·9	160·9	82	367·9
Co-stimulatory/signalling molecules																		
CD40	64·8	35·8	90·9[a]	95·1[a]	80·2	47·2[b]	73·8[b]	43·1[b]	59·8[b]	36·2[b]	91·3[a]	78·2[a]	67·9[c]	36·6[c]	48·5[c]	25·4[c]	40·3[c]	23·5[c]
CD80	5·6	10·5	69·2[a]	48·8[a]	24·2	19[b]	36·3[b]	25·4[b]	23·7[b]	19·4[b]	28[a,b]	22·1[a,b]	3	8·7[c]	3·2	10·1	35·3	10·5
CD86	90·7	317·7	97·5	855·6[a]	97	592·8	90·5	326·2[b]	86·7	404·3[b]	96·3	389·3[b]	88·4	391·1	71·5	143·5	81·5	321·7
CD54	98·8	780·2	99·3	5343[a]	98·5	2629·5[b]	98·1	1416·6[b]	97·7	1197[b]	96·8	2504·4[a,b]	96·2	867·4[c]	97·7	673[c]	99·3	1197[c]
Maturation antigen																		
CD83	12·4	16·4	49·7[a]	47·3[a]	17·4[b]	18·8[b]	16·6[b]	19·1[b]	34·9[b]	26·5[b]	54·1[a]	43·8[a]	5·2[c]	12·1[c]	5·4[c]	13·3[c]	18[c]	19·1[c]

[a]*Versus* C. $P < 0{\cdot}05$, [b]*versus* lipopolysaccharide (LPS) $P < 0{\cdot}05$, [c]*versus* H $P < 0{\cdot}05$. Hypoxia and LPS conditions cause phenotypic and morphological changes in immature dendritic cells (iDCs). Stimulated DCs were analysed by flow cytometry for CD40, CD80, CD83, CD86, CD54 and human leucocyte antigen D-related (HLA-DR) surface marker expression. Mean fluorescence intensity (MFI) for hypoxia-DC and LPS-DC was higher than for non-stimulated cells, although conventional markers showed lower MFI intensity under hypoxia compared to LPS. MFI and expression of each marker are presented as mean ± standard deviation of six experiments. MHC: major histocompatibility complex; PSC: P-glycoprotein inhibition using valspodar; PBN: probenecid; MK: CAS 115104-28-4.

subtype. Further, plasmocytoid-like hypoxia-DC induced a higher B lymphocyte proliferation than LPS-DC (Fig. 6). MLR performed with purified T and B cells showed similar results to those with unfractionated PBMCs (data not shown).

Interestingly, when lymphocyte subpopulations were analysed, ABC transporter inhibitors showed a different profile depending on the stimuli for DC maturation; that is, under hypoxia, ABC inhibitors presented a clear inhibition of B and T CD4 lymphocyte proliferation ($P < 0{\cdot}05$) (Fig. 6).

Cytokine release from PBMCs after MLR

Cytokine release in the mixed culture with mDCs and lymphocytes showed a different pattern depending on the maturation stimuli. Lymphocytes stimulated by LPS-mDCs presented over-production of IL-2, IL-6, IFN-γ and TNF-α, related mainly to a T helper type 1 (Th1) response, compared with control ($P < 0{\cdot}05$). IL-2 and IL-6 were higher in lymphocyte-LPS-mDCs than lymphocyte-hypoxia-mDCs ($P < 0{\cdot}05$) (Fig. 7). In contrast, IL-4 was over-expressed in PBMCs exposed to hypoxia-mDCs, suggesting a switch to a Th2 response. IL-17 was up-regulated similarly in PBMCs exposed to the two conditions (Fig. 7).

All cytokine release was abrogated by the addition of ABC transporter inhibitors. However, only IL-4 and IL-17 release from PBMCs exposed to hypoxia-mDCs and IL-2, IL-6, IFN-γ, TNF-α and IL-17 release from PBMCs exposed to LPS-mDCs were statistically significantly different compared to samples of DCs not exposed to ABC blockers ($P < 0{\cdot}05$) (Fig. 7).

Discussion

Since we first described the impact of hypoxia on DC maturation, there have been further DC studies in the literature confirming a cross-talk between the hypoxic environment and DC maturation [22,23]. In the transplant setting, immune-mediated injury is not only caused by alloimmune response, but also points to the 'injury hypothesis' as a result of other factors that may play an important role (for example, ischaemia–reperfusion injury). In fact, there is increasing evidence that ischaemia modulates immune and inflammatory responses, but the precise role of hypoxic signalling in renal immune-mediated injury is largely unexplored and unclear [24]. Our group proposed hypoxia as a key regulator of DC maturation in the kidney [8], suggesting a novel mechanism by which the lack of oxygen regulates immune responses. This work targets new investigation into the role of molecular oxygen-sensing in dendritic cell maturation and function, which may have implications in acute and chronic renal injuries in both the transplantation and non-transplantation settings.

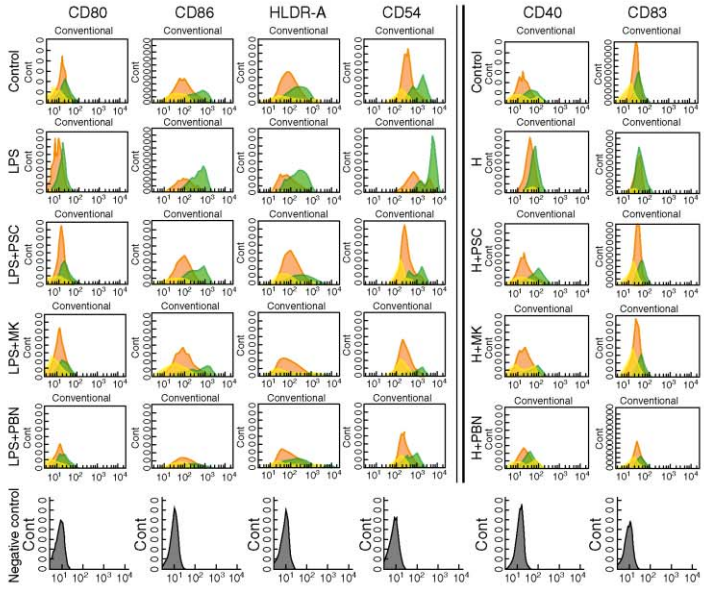


Fig. 4. Conventional/plasmacytoid-like phenotype switch on dendritic cell (DC) maturation. Effect of adenosine 5′-triphosphate-binding cassette (ABC) transporter blockade. Human monocyte derived-DCs were used after a 5-day culture with granulocyte–macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4. To inhibit multi-drug resistance (MDR1) protein, DC culture was performed in separate flasks with 5 μM P-glycoprotein inhibition using valspodar (PSC833), and to inhibit multi-drug resistance protein (MRP) with 50 μM CAS 115104-28-4 (MK571) or 2·5 mM probenecid (PBN). At day 5 DCs were stimulated for a further 48 h with hypoxia (hypoxia-mDCs) or for 24 h with lipopolysaccharide (LPS) (1 μg/ml, LPS-mDCs), or left non-stimulated. Cells were then collected and analysed with fluorescence activated cell sorter (FACS) for different mature DC subsets.

LPS stimulus has been used classically in several studies to induce DC migration and maturation. Here we show that the LPS stimulus induced a stronger homogeneous maturation effect, while the hypoxia stimulus showed a diverse degree of response. It is well known that in activating innate immunity, LPS induces DC maturation by ligand-driven Toll-like receptor (TLR) activation [25]. Our current results show that LPS and hypoxia induced mean fluorescence of mature phenotype DC markers differently from non-stimulated iDCs, but examining these markers individually to compare the two stimuli we found a down-regulation of CD86 for only hypoxia DC. Also, only CD40 and CD83 were expressed to the same degree for both hypoxia and LPS stimulation, whereas for the other surface markers (CD80, CD86, CD54 and HLA-DR) LPS induced a significant up-regulation at least two times greater than did hypoxia. Recently, Jantsch *et al.* [26] described similar results with an increase in CD80, CD86 and major histocompatibility complex (MHC)-II expression in DCs treated with LPS together with hypoxia, compared to cells treated only with LPS. In contrast, CD80 and CD86 expression decreased slightly under hypoxia alone, whereas MHC-II expression remained unchanged. Sekar *et al.* [27] generated plasmacytoid-like DC, attenuated IFN-γ production and

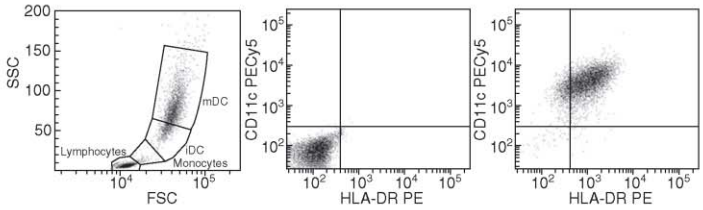


Fig. 5. Hypoxia-inducible factor 1 (HIF-1α) over-expression in hypoxia-dendritic cells (DCs). Effect of adenosine 5′-triphosphate-binding cassette (ABC) transporter inhibitors. Representative HIF-1α flow cytometry histogram from a double positive region (human leucocyte antigen D-related$^+$ and CD11c$^+$). Results are presented as mean ± standard error of three experiments. (H, H + PSC and H + MK *versus* control: $P < 0{\cdot}05$).

	HIF 1α%	MFI
Control	37·5±5·2	564±42
Hypoxia	67·6±3·7	713±53
Hypoxia+PSC833	57·5±4·4	680±44
Hypoxia+MK571	62·3±5·1	705±51

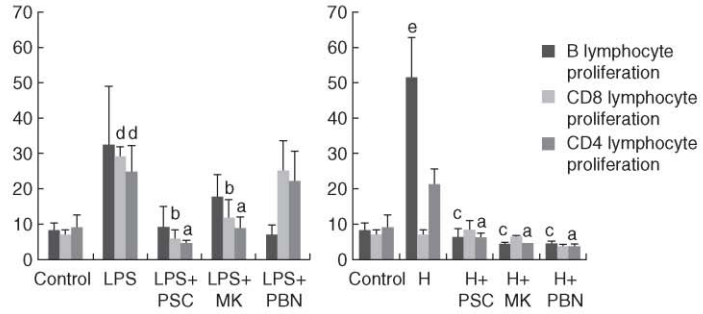


Fig. 6. T cell proliferation in the mixed lymphocyte reaction (MLR). Lymphocytes were stained with carboxyfluorescein diacetate succinimidyl ester (CFSE) and exposed to mature dendritic cells (DCs) [under hypoxia or lipopolysaccharide (LPS) stimuli] with or without adenosine 5′-triphosphate-binding cassette (ABC) transporter inhibitors. Cell proliferation was determined by flow cytometry after labelling with CD20, CD4 and CD8 antibodies. The results are representative of six independent experiments and expressed as the mean ± standard error.

a: **CD4**: $P < 0{\cdot}05$ H, LPS *versus* ABC transporter inhibitors (PSC, MK or PBN)
b: **CD8**: $P < 0{\cdot}05$ H, LPS *versus* ABC transporter inhibitors (PSC, MK or PBN)
c: **B lymphocyte**: $P < 0{\cdot}05$ H, LPS *versus* ABC transporter inhibitors (PSC, MK or PBN)
d: $P < 0{\cdot}05$ H, LPS *versus* control
e: $P < 0{\cdot}05$ H, *versus* control

decreased CD86 as well as MHC-I surface exposure under hypoxia. These findings suggest that LPS probably promotes a more conventional DC profile, while hypoxia appears to create an imbalance in plasmacytoid-like DC phenotypes [28,29].

ABC transporters are described fully in nephrotoxicity models in kidney transplantation, modulating the pharmacokinetics of many immunosuppressors. It is also known that *P*-glycoprotein is involved in DC maturation. Pendse *et al.* [12] defined a novel role for Pgp in DC maturation, identifying this transporter as a potential novel therapeutic target in allotransplantation. Schroeijers *et al.* [30] showed that human monocyte-derived DCs express Pgp at all maturation stages, and that they are up-regulated during DC maturation. Randolph *et al.* [31] found that Langerhans cells express Pgp and observed that their blockade inhibited migration of these cells. Although there is some consistent literature in this field, the precise role of Pgp and MRP1 in DC migration and maturation is, as yet, not known precisely, especially under hypoxia [32]. Concerning our results, the immunofluorescence staining that revealed higher expression of Pgp and MRP1 in DC LAMP-positive mDCs *versus* iDCs suggested initially that Pgp plays a role in the maturation of iDCs under hypoxia.

To explore further the mechanisms involved in DC maturation under hypoxia, and taking into account the potential role of ABC transporters in this process, we were tempted to analyse the role of the ABC transporters. The addition of three specific inhibitors shifted the ratio of mature and immature DCs achieved after hypoxia or LPS stimuli. Both MDR1 and MRP inhibitors showed a marked decrease in the mean fluorescence of all DC maturation markers except for CD86, which did not reach statistical significance. These results suggest that both MDR1 and MRPs are involved in DC maturation under LPS and hypoxia. In fact, our results under hypoxia point to a possible downstream mechanistic pathway via hypoxia-induced expression of HIF-1α. Interestingly, HIF-1α achieved similar values in hypoxia-DCs under both ABC transporter (MDR1 and MRPs) inhibitors to those under hypoxia alone. These findings are in agreement with recent studies in cancer therapy which argue for the contribution of HIF-1α in drug resistance, as HIF-1α is

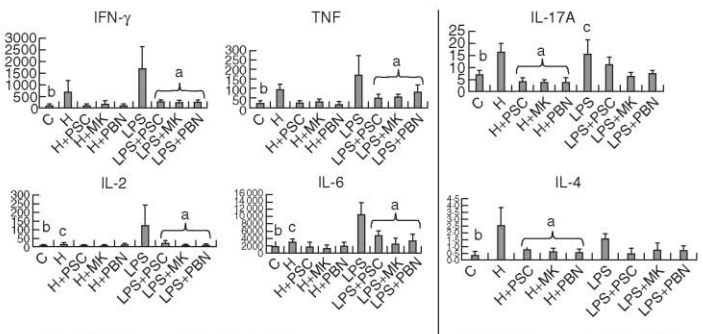


Fig. 7. Different pattern on cytokine release observed after hypoxia-dendritic cells (DCs) or lipopolysaccharide (LPS)-DCs exposition. Interleukin-2, -4, -6, -10, -17a, tumour necrosis factor (TNF)-α and interferon (IFN)-γ secretion protein levels from cell supernatant were measured following cell stimulation by fluorescence activated cell sorter (FACS). Cytokine quantification was performed on stimulated and non-stimulated, treated and non-treated [with adenosine 5′-triphosphate-binding cassette (ABC) inhibitors] DCs, and on lymphocytes after mixed lymphocyte reaction (MLR). Cytokine secretion results are expressed as pg/ml (*y*-axis)

a: $P < 0{\cdot}05$ LPS *versus* LPS+ABC inhibitors
b: $P < 0{\cdot}05$ LPS *versus* control
c: $P < 0{\cdot}05$ LPS *versus* H

a: $P < 0{\cdot}05$ H *versus* H+ABC inhibitors
b: $P < 0{\cdot}05$ LPS and H *versus* control
c: $P < 0{\cdot}05$ LPS *versus* LPS+MK

able to activate MDR1 [33]. Currently, it is well known that DCs are a bridge between innate and adaptative immunological responses and that LPS and hypoxia are involved in DC stimulation, but the role of ABC transporters in this context has been not explored [34]. Also, this link between hypoxia and LPS-DCs and ABC transporters may be inhibited by some of the most potent immunosuppressive drugs such as cyclosporin, tacrolimus and sirolimus, and this suggests an excellent target for preventing ischaemia-derived inflammation mediated by innate immunity.

As described previously, hypoxia is able to increase the release of proinflammatory cytokines and the expression of co-stimulatory molecules by murine and human DCs, thus enhancing their potential to induce allogeneic lymphocyte proliferation [8,26]. Hypoxia- and LPS-matured DCs induced significantly higher T cell proliferation than immature untreated DCs, achieving different degrees of T cell proliferation depending on the stimuli. Interestingly, when different subpopulations were assessed, CD8 lymphocyte proliferation was up-regulated remarkably in DCs treated with LPS, while the proliferation of B lymphocytes was higher under hypoxia. Recently it has been reported that plamacytoid DCs are able to induce B lymphocyte proliferation, which lends support to our findings [35]. DCs differentiated in the presence of MDR1 and MRP inhibitors reduced alloimmune T cell proliferation twofold. Furthermore, ABC transporter inhibitors showed different profiles of lymphocyte proliferation inhibition depending on DC maturation stimuli. Thus, inhibiting ABC transporters could be an effective approach to reducing the stimulatory capacity of DC, thereby decreasing lymphocyte proliferation.

DCs are usually exposed to diverse pathological and physiological conditions. In fact, LPS and hypoxia are some of the possible *in-vitro* stimuli that can simulate the different environments that arise in wide-ranging types of cytokines that may trigger assorted inflammatory processes. However, the effects of these stimuli on phenotype differentiation patterns of DC and of the cytokine prompt cascade remain unclear [36,37]. In our study, we showed that lymphocytes exposed to LPS-DCs generated higher levels of proinflammatory cytokines (IL-2, IL-6, IL-10, IFN-γ and TNF-α), balanced mainly to the Th1 response. Higher levels of immunoregulatory cytokine IL-4 were found in lymphocytes stimulated by hypoxia-DCs, suggesting a switch to the Th2 response. In fact, plasmacytoid DCs have just been found to secrete substantial amounts of IL-4-producing Th2 cells [27,38]. Cytokine secretion was abrogated by the addition of MDR1 and MRP1 inhibitors. The inhibition of DC maturation through ABC transporter blockers probably has a downstream impact on cytokine release.

These findings allow us to suggest that the modulation of different DC phenotype profiles depends upon the initial stimulus and defines subsequent diverse cytokine activators, markers and functions. This is the first time that the role of ABC blockers as inhibitors of DCs maturation after hypoxia and LPS stimuli has been described. The impact of this immune activation, depending on DC maturation stimulus leading to different lymphocyte subtype proliferation, confirms the plasticity of the immunological response in the face of pathological stimuli. In addition, both ABC transporter MDR1 and MRP1 blockers interfere in DC differentiation and maturation, modifying mature DC phenotype and lymphocyte activation. ABC transporters could be a potential target in DC-based immunosuppressive therapies designed to abrogate innate immune response when it is activated after ischaemia or endotoxin stimulus. The cellular and molecular mechanisms underlying the innate adaptive immune response to ischaemia–reperfusion are an active area of research with much more to tell us. These findings add more information about the specific functional role of ABC transporters as a potential therapeutic target in alloimmunity modulation.

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Disclosure

None.

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Paper II

Pretransplant Immediately Early-1-Specific T Cell Responses Provide Protection For CMV Infection After Kidney Transplantation.

Bestard O, Lucia M, Crespo E, Van Liempt B, Palacio D, Melilli E, Torras J, Llaudó I, Cerezo G, Taco O, Gil-Vernet S, Grinyó JM and Cruzado JM.

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Pretransplant Immediately Early-1-Specific T Cell Responses Provide Protection For CMV Infection After Kidney Transplantation

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Cytomegalovirus (CMV) infection is still a major complication after kidney transplantation. Although cytotoxic CMV-specific T cells play a crucial role controlling CMV survival and replication, current pretransplant risk assessment for CMV infection is only based on donor/recipient (IgG)-serostatus. Here, we evaluated the usefulness of monitoring pre- and 6-month CMV-specific T cell responses against two dominant CMV antigens (IE-1 and pp65) and a CMV lysate, using an IFN-γ Elispot, for predicting the advent of CMV infection in two cohorts of 137 kidney transplant recipients either receiving routine prophylaxis ($n = 39$) or preemptive treatment ($n = 98$). Incidence of CMV antigenemia/disease within the prophylaxis and preemptive group was 28%/20% and 22%/12%, respectively. Patients developing CMV infection showed significantly lower anti-IE-1-specific T cell responses than those that did not in both groups ($p < 0.05$). In a ROC curve analysis, low pretransplant anti-IE-1-specific T cell responses predicted the risk of both primary and late-onset CMV infection with high sensitivity and specificity (AUC > 0.70). Furthermore, when using most sensitive and specific Elispot cut-off values, a higher than 80% and 90% sensitivity and negative predictive value was obtained, respectively. Monitoring IE-1-specific T cell responses before transplantation may be useful for predicting posttransplant risk of CMV infection, thus potentially guiding decision-making regarding CMV preventive treatment.

Key words: Cytomegalovirus infection, Elispot, kidney transplantation, T cell response

Abbreviations: BPAR, biopsy-proven acute rejection; CMV, cytomegalovirus; CNIs, calcineurin-inhibitors; Elispot, enzyme-linked immunosorbent spot assay; IE-1, immediately early-1; mTor-i, mTor-inhibitors; PBMCs, peripheral blood mononuclear cells; PP65, phosphoprotein 65; rATG, rabbit antithymocyte globulin; ROC, receiver operating characteristic

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Introduction

Human cytomegalovirus (CMV) infection is still a major complication after kidney transplantation. Because of T cell immunosuppression, transplant recipients are at increased risk to develop CMV infection a short time after transplantation, critically challenging both graft and also patient survival (1,2). On the one hand, CMV infection may *directly* lead to persistent viremia and tissue-invasive injury such as pneumonitis, enteritis or retinitis, and on the other, *indirect*-related CMV effects have also been associated to acute and chronic allograft rejection, diabetes and accelerated atherosclerosis (3,4).

Noteworthy, the advent of preventive strategies using either universal prophylaxis or preemptive treatment initiated on the basis of viral detection in blood has significantly helped to reduce morbidity and mortality. Indeed, while recent reports have shown that routine prophylaxis with valganciclovir may reduce the incidence of posttransplant CMV infection and improve long-term kidney graft survival (5–8), other groups have also shown that preemptive therapy is also able to decrease the incidence of CMV disease, avoiding development of antiviral resistance, drug toxicity (7,9–10) and the advent of late-onset CMV infection (11,12). Furthermore, some CMV seronegative patients receiving a kidney allograft from a CMV seropositive donor never develop CMV infection despite not receiving any prophylaxis treatment (13–15). Altogether, it suggests that current immune assessment of the CMV risk before kidney transplantation exclusively

evaluating detectable circulating CMV IgG titers is not accurate and informative enough to predict the risk of CMV infection in all transplant recipients.

Cytotoxic CMV-specific T cells play a crucial role controlling CMV survival and replication (16,17). While host CD8+ T cells may target a wide range of CMV immunogenic proteins, particular dominant T cell responses against immediately early-1 (IE-1) antigens and to phosphoprotein 65 (pp65) seem to be essential for CMV control (18–20). Recent relevant reports using different T cell immune-monitoring tools have shown the importance of such CMV-specific T cell responses for controlling CMV infection after transplantation. However, most of them have mainly focused on the posttransplant period, gathering different solid organ transplants and assessing rather low numbers of kidney transplant recipients (21–25).

Since all kidney transplant patients display an intrinsic baseline functionality of CMV-specific T cell responses, thus predisposing to CMV replication after transplantation, we aimed to evaluate the clinical usefulness of monitoring prior to transplantation CMV-specific T cell responses against dominant CMV antigens (IE-1 and pp65) and a CMV lysate, using an IFN-γ Elispot assay, for predicting the advent of posttransplant CMV infection in two cohorts of kidney transplant recipients either receiving routine prophylaxis ($n = 39$) or preemptive treatment ($n = 98$). Furthermore, changes in 6-month posttransplant CMV-specific T cell responses were also analyzed in both groups of patients.

Methods

Patients and study groups

This is a single-center retrospective study performed at our Renal Transplant Unit at Bellvitge University Hospital in Barcelona, Spain. Between June 2009 and June 2011, consecutive kidney adult renal transplant recipients were enrolled to the study if pretransplant peripheral blood mononuclear cells (PBMCs) were available. The study was approved by the Ethics Committee of our center.

Patients were divided in two groups, depending on the CMV preventive strategy performed; either prophylaxis or preemptive therapy was done following the clinical protocol established in our Transplant Unit during the study time period. Until June 2010, prophylaxis treatment posttransplantation was restricted to CMV seronegative transplant recipients receiving a seropositive donor (R−/D+), and preemptive therapy was carried out in all CMV positive recipients either receiving a positive or a negative donor allograft (R+/D+ and R+/D−, respectively), including those receiving T cell depleting antibodies. Subsequently, from July 2010 on, a prophylaxis policy was also extended to all CMV positive transplant recipients (R+) receiving T cell depleting antibodies. In addition, six R− patients because of either hypersensitivity history to acyclovir or showing posttransplant absolute leukocyte count <2000 cells/μL, platelet count <100 000 cells/μL or hemoglobin levels lower than 8.0 g/dL, preemptive therapy was assigned.

CMV preventive strategies

In the *prophylaxis* group, including those transplants recipients receiving T cell depleting agents such as rATG, patients received 900 mg (2 × 450 mg) per day oral valgancyclovir tablets starting within 14 days after transplantation until Day 100 posttransplantation, and in the *preemptive* group, quantitative CMV monitoring by means of antigenemia was performed once weekly at weeks 1–4; every 2 weeks at weeks 6–12; every 4 weeks at months 4–6; and every 3 months at months 9 and 12, or additionally as clinically indicated.

Patients in either group who tested positive (detectable CMV antigenemia higher than 20 positive cell/2 × 10^5 PBMC) at any time after transplantation received 1800 mg (2 × 900 mg) per day oral valganciclovir for at least 14 days, until CMV antigenemia became negative on two consecutive assessments within 1 week. Thereafter, secondary prophylaxis was given using 900 mg (2 × 450 mg) per day oral valganciclovir for 1 month. In case of CMV disease or if the patient was unable to take oral medication, intravenous ganciclovir at 2 × 5 mg/kg body weight per day was permitted. In all cases, doses of all antiviral regimens were adjusted by kidney allograft function.

Clinical data and definitions

CMV *antigenemia* was defined as a positive antigenemia for CMV with no symptoms. CMV *disease* included both viral syndrome and tissue invasive disease. Identification of the viral syndrome caused by CMV required the following: (1) positive antigenemia for CMV; (2) temperature of >38°C with no other source to account for it and (3) one of the following findings: leukocyte count of <4000/mm^3, atypical lymphocytes of >3%, elevation of transaminases and platelet count of <100 000/mm. Tissue invasive disease required histopathological evidence of CMV, with or without virus culture of the tissue. This included identification of inclusion bodies or viral antigens in biopsy material or in bronchoalveolar lavage specimen cells by immunocytochemistry (3,22–25).

Microbiological studies

Surveillance by means of CMV antigenemia was routinely performed (approximately every 1–2 weeks) during the first 3 months after transplantation in both preemptive and prophylaxis strategies. CMV antigenemia was determined in polymorphonuclear Leukocytes, obtained by dextran sedimentation, formaldehyde fixed, stained and read under a fluorescence microscope (rapid antigenemia anti-human CMV ppUL83, Argene, Varilhes, France; Ref 14-002). The maximum sensitivity of the method in our laboratory was 1 positive cell/2 × 10^5 PBMCs.

ELISA for CMV-IgG

CMV serostatus was determined using a commercial CMV IgG ELISA Kit (BioCheck, Inc., Burlingame, CA) according to the manufacturer's instructions.

CMV peptides

Pools of peptides derived from a peptide scan (15 mers with 11aa overlap), covering the whole antigen length through the immediate-early protein 1 (IE-1) and through the 65 kDa phosphoprotein (pp65; Jerini Peptide Technologies, Swiss-Prot ID: P13202 and Swiss-Prot ID: P06725, respectively) of Human CMV (HHV-5), as well as a CMV lysate (Autoimmune Diagnostik®, Strasberg, Germany), were used as stimuli for the IFN-γ Elispot assay, allowing us to avoid HLA restrictions.

Anti-CMV T cell immune response assessment

IFN-γ Elispot assay: A multiscreen, 96-well filtration plate (AID®, Strasberg, Germany) coated with antihuman IFN-γ antibody (AID®, Autoimmune Diagnostika) was used. Cryopreserved PBMCs from either pretransplantation and/or 6 months after transplantation were thawed and

incubated for at least 3 h at 37°C before peptide stimulation. Thereafter, 3×10^5 of lymphocytes (in a 100 μL volume) were added to each well together with each different peptide, medium alone as a negative control and with PHA (Sigma–Aldrich®, Madrid, Spain) as a positive control. All Elispot assays were carried out in triplicate. After 18 h incubation at 37°C/5% CO_2, cells were removed by washing the plates four times with PBS containing 5% Tween 20 and twice with PBS. Fifty microliters of biotinylated anti-IFN-γ antibody was added (1:1000 dilution, 7-B6-1-biotin; Mabtech) and incubated for 3 h at room temperature. The Elispot plate was washed a further six times with PBS/Tween 20 and incubated for 2 h with streptavidin-ALP substrate (AID®) followed by the addition of an alkaline phosphatase conjugate substrate (50 IL; AID®, Autoimmune Diagnostika). The resulting spots were counted semi-automatically with an Elispot reader (AID® Elispot Reader HR, 4th generation). Results were expressed as percentage of cells secreting IFN-γ after subtracting the number of spots due to spontaneous IFN-γ release (measured in the control wells) from the number of spots obtained in the wells incubated with each peptide.

IFN-γ flow cytometry: Following incubation with respective peptides, PBMC were tested for intracellular IFN-γ production by the cytokine flow cytometry assay in five transplant recipients showing relevant anti-viral T cell responses against all three evaluated stimuli in the Elispot assay. PBMC were washed and stained for 30 min in ice with APC-conjugated mAb anti-CD3 (clone HIT3a; BD®, Madrid, Spain), PE-conjugated mAb anti-CD4 (clone RPA-T4; BD®) and PERP-CY.5-conjugated mAb anti-CD8 (clone RPA-T8; eBioscience®, Barcelona, Spain) in PBS + 5% FBS, containing 5% human immunoglobulin and 0.01% sodiumazide. Cells were then washed with PBS + 5% FBS, fixed and permeabilized using the FIX and PERMJ kit (BD®), according to the manufacturer's instructions, and stained for 45 min with FITC-conjugated mAb anti-IFN-γ (clone 4s.B3; BD®).

Statistical analysis

All data are presented as mean ± SD. Groups were compared using the χ^2 test for categorical variables, the one-way ANOVA or t-test for normally distributed data and the nonparametric Kruskal–Wallis or Mann–Whitney U test for non normally distributed variables. Both CMV antigenemia and disease were considered the outcome variables of the study. Bivariate correlation analyses were done using Pearson or Spearman test for non-parametric variables. A sensitivity/specificity ROC curve test was done to investigate the value of the Elispot test for predicting the advent of posttransplant CMV infection. The statistical significance level was defined as 2-tailed $p < 0.05$.

Results

Patient demographics

As shown in Figure 1, 137 consecutive kidney transplant recipients were assessed for their anti-CMV T cell response before transplantation. Of these, 39 patients received posttransplant CMV prophylaxis and 98 followed a pre-emptive protocol. Six-month CMV-specific T cell responses could be evaluated in 58 patients, 21 receiving prophylaxis and 37 preemptive therapy. Mean follow-up of the study was 25 months (range 37–15 months).

Main demographic and baseline characteristics of all patients are depicted in Table 1. Incidence of posttransplant antigenemia or CMV disease was not different between patients receiving prophylaxis or preemptive therapy. No CMV infection events were observed beyond 6 months after transplantation. Among prophylactic-treated patients, the advent of CMV antigenemia appeared in all but one patient after completing valganciclovir treatment with a median of 45 days after stopping treatment. Within preemptive-followed patients, most CMV infection episodes occurred during the first 3 months after transplantation with a median of 38 days after transplantation. Incidence of CMV recurrence after treatment was equally distributed between both groups (three within preemptive and two among prophylaxis). Among patients receiving preemptive therapy, the advent of CMV antigenemia was significantly more common in older recipients (54.8 ± 9 vs. 48.3 ± 13, $p < 0.005$) and in those experiencing delayed graft function (DGF; 45.5% vs. 14.4%, $p < 0.005$). To note, T cell depletion induction treatment was associated to a significantly increased risk of both posttransplant antigenemia and CMV disease (63.6% vs. 38% and 80% vs. 40% for antigenemia and disease, respectively, $p < 0.005$). Conversely, type of maintenance immunosuppression was not associated with CMV infection. At 6 months, allograft function was significantly worse among those patients experiencing either CMV antigenemia or disease as compared to those that did not.

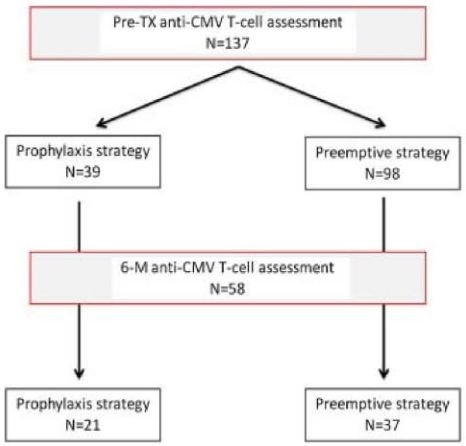

Figure 1: Illustration of the study course.

Main clinical data of patients with and without CMV antigenemia/disease within preemptive and prophylactic-treated patients are displayed in Tables 2 and 3, respectively. Most CMV infections in both cohorts of patients were asymptomatic CMV-detected antigenemia (28% and 22% in prophylactic and preemptive, respectively) and clinical disease was observed in 20% and 12% of prophylactic and preemptive groups, respectively. To note, the majority of clinical diseases were diagnosed as viral syndromes (11/18) whereas tissue invasive diseases were observed in seven patients, located in the gastrointestinal tract and two in the pulmonary tract.

Table 1: Main demographic and baseline characteristics of the entire study group

	All patients (N = 137)	Prophylaxis (N = 39)	Preemptive (N = 98)
Gender (male/female)	91/46	26/13	65/33
Age (years, mean ± SD)	48.9 ± 13.2	46.4 ± 14.7	49.8 ± 12.7
Type of kidney TX (living/deceased)	63/74	20/19	43/55
Pre-TX CMV donor (D)/recipient (R) serostatus			
R−/D+ (%)	28 (20.4)	22 (56.4)*	6 (6)
R+/D+ (%)	83 (60.6)	12 (30.8)	71 (72.5)
R+/D− (%)	26 (19)	5 (1.2)	21 (21.5)
Maintenance immunosuppression			
CNI-based (CsA/TAC; %)	8 (6)/112 (82)	3 (8)/3 (8)	5 (5)/81 (83)
CNI-free (mTor-i; %)	17 (12)	5 (13)	12 (12)
Mycophenolate mofetil (%)	137 (100)	39 (100)	98 (100)
No Induction therapy (%)			
Induction immunosuppression	10 (7.3)	1 (2.5)	9 (9.2)
rATG (%)	68 (49.7)	25 (6)	43 (43.8)
Anti-CD25 monoclonal Ab (%)	59 (43)	13 (33.4)	46 (47)
DGF (yes/no)	30/107	9/30	21/77
BPAR (%)	18 (13)	6 (15)	12 (12)
Allograft function (eGFR; mL/min)			
Month 6	40.6 ± 25	45.8 ± 26	38.4 ± 24
Month 12	52.6 ± 15	53.4 ± 17	52.2 ± 14
Month 18	52.5 ± 16	53.8 ± 14	52 ± 16
Pre-TX anti-CMV IgG titers (UA/mL)	166.7 ± 99	99.9 ± 116*	190.7 ± 81
Pre-TX anti-CMV T cell response (spots/3 × 10^5 PBMC)			
CMV lysate	128.9 ± 183	61.9 ± 112*	155.5 ± 198
Pp65 antigen	101.7 ± 168	39.4 ± 65*	126.5 ± 189
IE-1 antigen	39.8 ± 86.1	21.5 ± 29*	47 ± 99
CMV infection (antigenemia/disease)	33 (24)/18 (13)	11 (28)/8 (20)	22 (22)/10 (10)
Exitus (%)	8 (5.8)	2 (5)	6 (6)

*$p < 0.05$.

Pp65- and IE-1-specific T cell responses are predominantly provided by the CD8+ T cell compartment

While T cell responses against both pp65 and IE-1 CMV peptides were predominantly CD8+, CD4+ T cell responses could also be detected against the CMV lysate (Figure 2).

Low pretransplant IE-1-specific T cell responses is associated with posttransplant CMV infection

All anti-CMV T cell responses within prophylactic patients were significantly lower as compared to patients with preemptive therapy (Table 1). Pretransplant pp65 and CMV lysate but not anti-IE-1-specific T cell responses positively correlated with pretransplant CMV IgG titers ($r = 0.298$, $p = 0.001$ and $r = 0.325$, $p < 0.001$, respectively). Although pretransplant CMV-specific T cell responses could be detected among some seronegative transplants recipients (12/28), they were significantly lower than within seropositive recipients (Figure 3).

Patients receiving either preemptive or prophylaxis therapy developing CMV infection showed significantly lower anti-IE-1 T cell responses as compared to patients that did not. No association was observed between pretransplant anti-pp65 and CMV lysate T cell responses and incidence of CMV infection (Figure 4). Similar findings were observed among those patients receiving rATG (Figure 5). Furthermore, prophylaxis-treated transplant recipients developing CMV disease, did also show lower pretransplant pp65-specific T cell responses as compared to those that did not. When all patients of the study were assessed together, those with posttransplant CMV infection showed significantly lower pretransplant anti-IE-1 T cell responses than patients not experiencing CMV infection (data not shown). Patients under mTor-i did not show a different CMV-specific T cell immunity as compared to those receiving CNI-based regimens.

Frequencies of pretransplant anti-IE-1 T cell responses independently predict the risk of posttransplant CMV infection

Receiver operating characteristic curve (ROC) analysis for predicting either posttransplant antigenemia or disease in patients receiving prophylaxis and preemptive therapy is depicted in Figure 6. As shown, considerably high AUC, ranging from 0.635 up to 0.760, were obtained for pretransplant anti-IE-1 T cell responses for prediction of both CMV antigenemia and disease in the different treatment groups, respectively. Sensitivity and specificity of anti-IE-1 T cell IFN-γ Elispot is summarized in Table 4. No additive effect for predicting posttransplant CMV infection

Table 2: Main demographic and baseline characteristics of preemptive-treated patients

	CMV antigenemia		CMV disease	
Preemptive strategy (N = 98)	Yes (N = 22)	No (N = 76)	Yes (N = 10)	No (N = 88)
Gender (male/female)	17/5	48/28	7/3	58/30
Age (years, mean ± SD)	54.8 ± 9*	48.3 ± 13	53.3 ± 10	49.5 ± 13
Type of kidney TX (deceased/living)	6/16	37/39	1/9	42/46
DGF (yes/no)	10/12*	11/65	3/7	18/70
BPAR (%)	3 (14)	9 (12)	2 (20)	10 (11)
Pre-TX CMV donor (D)/recipient (R) serostatus				
R−/D+ (%)	1 (4.5)	5 (6.5)	1 (10)	5 (5.5)
R+/D+ (%)	17 (77)	60 (79)	7 (70)	72 (82)
R+/D− (%)	4 (18)	11 (14.5)	2 (20)	11 (12.5)
Pre-TX anti-CMV IgG titers	216.6 ± 59	183.4 ± 85	177.9 ± 85	192 ± 81
Maintenance immunosuppression				
CNI-based (CsA/TAC; %)	0/19	5/62	0/9	5/72
CNI-free (mTor-i; %)	3	9	1	11
Mycophenolate mofetil (%)	22	76	10	88
Mycophenolate acid tough levels (mean ± SD)				
Month 1	2.9 ± 1.9	2.9 ± 1.9	2.9 ± 2.1	2.9 ± 1.9
Month 3	2.9 ± 2	3.3 ± 2	2.7 ± 1.8	3.2 ± 1
Month 6	3.5 ± 2	2.7 ± 1.7	3.2 ± 1	2.8 ± 1.7
No induction therapy (%)	1 (4.4)	8 (10.5)	0 (0)	9 (10)
Induction immunosuppression				
rATG (%)	14 (63.6)*	29 (38)	8 (80)*	35 (40)
Anti-CD25 monoclonal Ab (%)	7 (32)	39 (51.5)	2 (20)	44 (50)
Allograft function (eGFR; mL/min)				
Month 6	28.3 ± 23*	41.2 ± 23	12.3 ± 14*	41.2 ± 22
Month 12	47.1 ± 17	53.8 ± 12	48.1 ± 19	53 ± 13
Month 18	50.1 ± 19	52.7 ± 15	53 ± 22	52 ± 15
Exitus (%)	3 (13)	3 (4)	1 (10)	5 (5.6)

*$p < 0.05$.

was observed when using pp65 and IE-1 T cell responses together (data not shown).

When risk of CMV infection was categorized as a binary variable, taking into account most sensitive and specific cut-off values of pretransplant IE-1-specific T cell IFN-γ Elispots for each group of transplant recipients (7 and 8 IFN-γ spots per 3×10^5 stimulated PBMCs, for preemptive and prophylaxis-treated patients, respectively) a higher than 80% and 90% sensitivity and negative predictive value were obtained, respectively (Table 4).

Anti-CMV T cell responses at 6 months after transplantation between CMV infected and noninfected transplant recipients

As all CMV infection events appeared before the first 6 months after transplantation, changes in 6-month CMV-specific T cell responses were evaluated. In general, anti-CMV T cell responses significantly increased after transplantation despite that patients were receiving immunosuppression (128.9 ± 183 vs. 278 ± 433, $p = 0.012$; 101.7 ± 168 vs. 127 ± 183, $p = 0.006$ and 39.8 ± 86.1 vs. 126 ± 454, $p < 0.001$, for CMV-lysate, pp65 and IE-1 for 3×10^5 stimulated PBMCs, respectively). There were no differences between 6-month anti-CMV T cell responses among patients having received prophylaxis or preemptive therapy, CNI or non-CNI-based immunosuppression or different type of induction therapy (data not shown). However, when patients with or without posttransplant CMV infection were compared regarding their change in the CMV-specific T cell response at 6-month, patients having experienced CMV infection showed a significantly increase in pp65 and IE-1-specific T cell responses as compared to those that did not (Figure 7).

Discussion

While current clinical immune assessment of the CMV risk of infection before transplantation exclusively relies on donor and recipient CMV IgG-serostatus, our study shows that CMV-specific T cell response, particularly against the IE-1 dominant CMV antigen, may improve the identification of those kidney allograft recipients at high-risk for CMV infection. Importantly, our approach is capable to discriminate such patients already before transplantation, with high sensitivity and specificity, regardless the type of preventive strategy used. Furthermore, the high negative

Table 3: Main demographic and baseline characteristics of prophylactic-treated patients

	CMV antigenemia		CMV disease	
Prophylaxis treatment (N = 39)	Yes (N = 11)	No (N = 28)	Yes (N = 8)	No (N = 31)
Gender (male/female)	7/4	19/9	6/2	20/11
Age (years, mean ± SD)	44.7 ± 15	47.1 ± 14	38.8 ± 13	48.5 ± 13
Type of kidney TX (deceased/living)	3/8	17/11	3/5	17/14
DGF (yes/no)	5/6	4/24	2/6	7/24
BPAR (cellular/humoral)	3/0	2/1	1/0	4/1
Pre-TX CMV donor (D)/recipient (R) serostatus				
R−/D+ (%)	5 (45.5)	1 (3.5)	4 (50)	18 (58)
R+/D+ (%)	4 (36.4)	8 (28.5)	3 (37.5)	9 (29)
R+/D− (%)	2 (18.2)	3 (11)	1 (12.5)	4 (13)
Pre-TX anti-CMV IgG titers	90 ± 117	104 ± 118	50.1 ± 92	115 ± 120
Maintenance immunosuppression				
-CNI-based (CsA/TAC)	0/10	3/21	0/7	3/24
-CNI-free (mTor-i)	1	4	1	4
-Mycophenolate mofetil	11	28	8	31
Mycophenolate acid tough levels (mean ± SD)				
Month 1	3.1 ± 1.6	3.2 ± 1.8	3.1 ± 1.4	3.2 ± 1.8
Month 3	3.7 ± 2	3.6 ± 2	3.4 ± 2	3.7 ± 2
Month 6	3.1 ± 2	3.6 ± 2.4	3.2 ±	3.1 ± 2
No induction therapy (%)	0 (0)	1 (3.5)	0 (0)	1 (3.2)
Induction immunosuppression				
-rATG (%)	8 (73)	17 (61)	7 (87.5)	18 (58)
-Anti-CD25 monoclonal Ab (%)	3 (27)	10 (35.5)	1 (12.5)	12 (38.8)
Allograft function (eGFR; mL/min)				
Month 6	41.7 ± 23	47.5 ± 28	48.2 ± 21	45.1 ± 28
Month 12	50.1 ± 11	55.3 ± 20	53.4 ± 8	53.4 ± 20
Month 18	48.6 ± 12	57 ± 16	53.8 ± 13	54.6 ± 15
Exitus (%)	1 (9)	1 (3.5)	0 (0)	2 (6)

*$p < 0.05$.

predictive value of the test highlights the usefulness of such approach.

Noteworthy, our study shows that monitoring IE-1 CMV-specific T cell frequencies before transplantation would help transplant physicians on the one hand to better discriminate those patients with no need of CMV prophylactic treatment from those in whom prophylaxis should preferentially be indicated and on the other to better predict those patients in whom prophylaxis treatment could safely be discontinued. Interestingly, intrinsic impairment of the IE-1-specific T cell response was not only associated with the advent of posttransplant CMV infection but also with the development of CMV disease, thus reinforcing the importance of such functionally active CMV-specific T cell precursors for achieving CMV control under immunosuppression.

The observation that patients receiving T cell depleting antibodies experiencing CMV infection were those with significantly lower pretransplant IE-1-specific T cell frequencies, suggests that the increased susceptibility for CMV infection after T cell depletion is particularly facilitated by the impairment of IE-1-specific T cell precursors already before transplantation rather than to a generalized T cell subset depletion after rATG therapy. Differently from what has been shown among normal individuals (26), within our seronegative group of chronic kidney disease patients, CMV-specific T cell responses were also detectable in a group of them, though at significantly lower frequencies than among seropositive patients. Nevertheless, only patients with adequate pretransplant anti-IE-1-speciifc T cell frequencies were at significant low-risk for CMV infection. This finding supports the notion that although CMV triggers both humoral and cellular responses, only the latter and particularly that directed to IE-1 CMV antigens seem to be crucial for posttransplant viral replication control, therefore being the former of limited utility in the clinical practice (27,28). Nonetheless, whether the detection of CMV-specific peptide T cell responses among CMV-seronegative patients could result from cross-reactive recognition of CMV epitopes by memory T cells originated from distinct (e.g. non-HCMV) antigenic exposures or if a more accurate assessment of CMV-specific memory B-cell IgG frequencies would increase the sensitivity to detect patients already sensitized to CMV antigens deserves further evaluation.

To date, studies in transplant recipients evaluating the impact of CMV-specific cellular responses have mainly

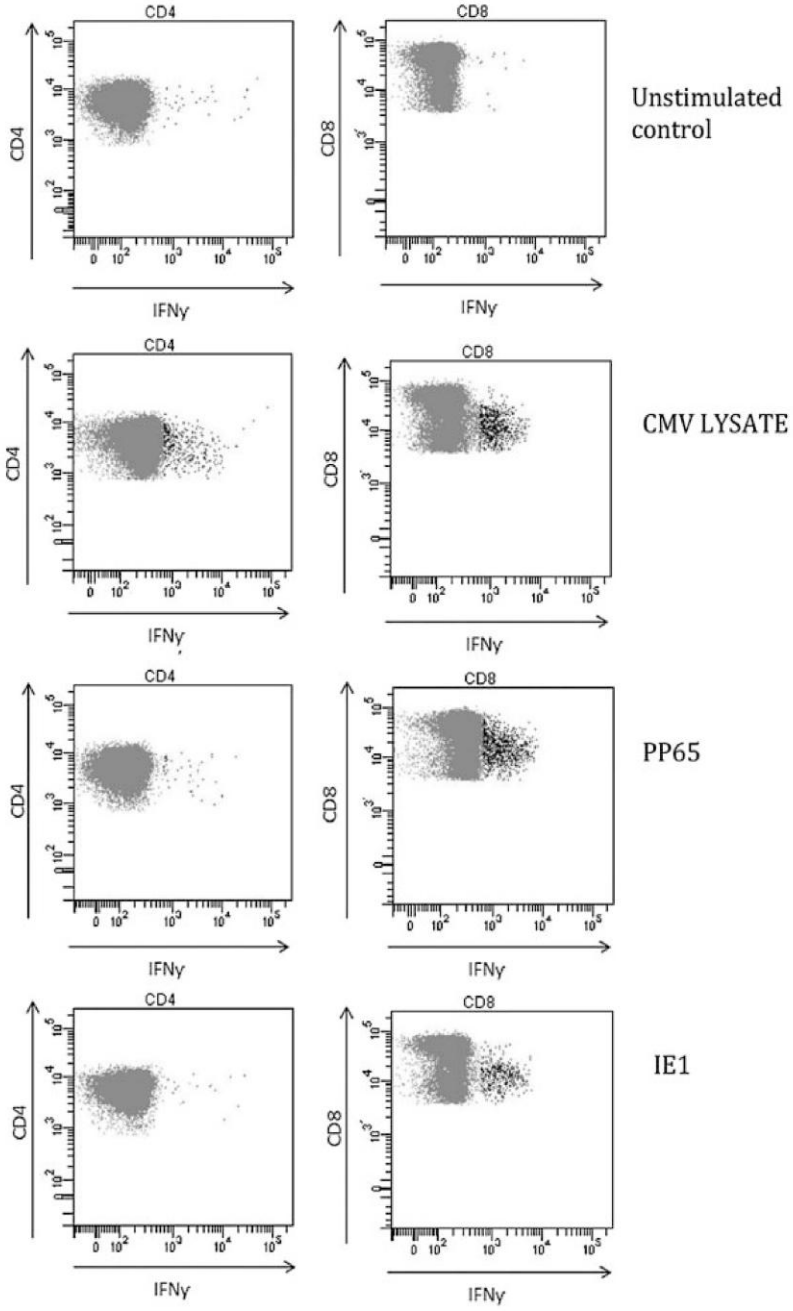

Figure 2: Intracellular IFN-γ FACS analysis on CD4+ and CD8+ T cells stimulated with three different CMV stimuli in a representative kidney transplant patient with high frequencies of IFN-γ producing T cells assessed by the Elispot assay before transplantation. CD8+ T cell subsets accounted for the most predominant anti-IE-1 and pp-65 T cell responses. CD4+ T cell responses were also detected against the CMV lysate stimuli.

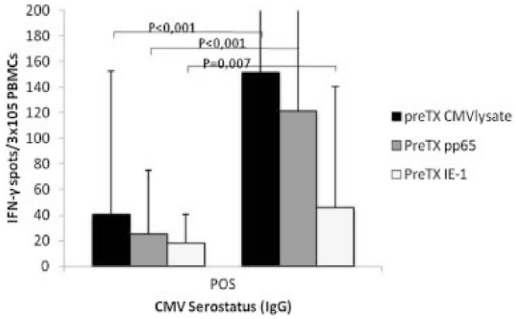

Figure 3: CMV-specific T cell responses between CMV-seropositive and seronegative transplant recipients. Pretransplant CMV-specific T cell responses were significantly lower among seronegative patients than within seropositive transplant recipients (40.3 ± 112 vs. 151.6 ± 191, $p < 0.001$; 25.2 ± 50 vs. 121.3 ± 181, $p < 0.001$ and 17.6 ± 23 vs. 45.5 ± 95.1, $p = 0.007$ for CMV-lysate, pp65 and IE-1 for 3×10^5 stimulated PBMCs, respectively).

focused at the posttransplant period and used different cellular immune assays with distinct CMV stimuli (21–26,29–32). Our study is in consonance with these previous reports, but also shows that the increased risk to develop posttransplant CMV infection (even after a course of prophylactic treatment) seems to rely in an individual immune susceptibility already manifested prior to transplantation. Likewise, but in lung and heart transplant patients, Bunde et al. (21) showed that frequencies of IE-1 but not pp65-specific CD8+ T cells already at Day 0, discriminated patients who did not develop CMV disease from patients at risk. Although focusing on the association between allogeneic and CMV-specific effector T cell responses, Nickel et al. (33) reported similar findings in a group of 36 kidney transplant patients.

Although different studies have suggested a preponderant role of CD8+ T cells for CMV control (21,24,26,29,30), others have also shown the concomitant key function of CD4+ T cells, which seem to even have a preferential role conferring long-lasting protection (29,32). In our study, we

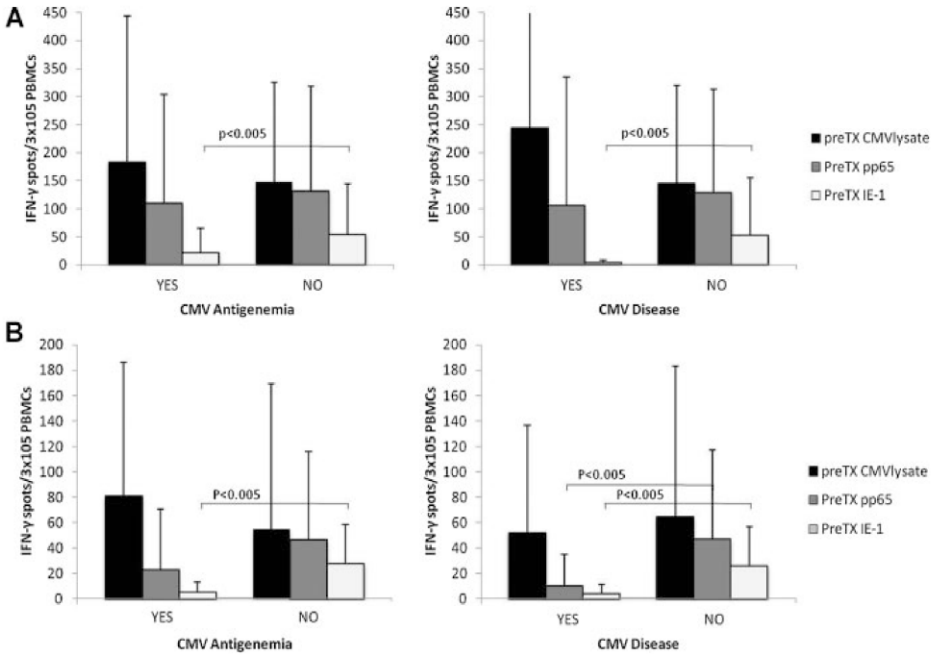

Figure 4: Pretransplant anti-CMV T cell responses and risk of CMV infection. (A) CMV-specific T cell responses between patients developing CMV viremia and those that did not, in patients receiving preemptive treatment (182.7 ± 262 vs. 147.6 ± 178, 109.4 ± 194 vs. 131.4 ± 188, 21.5 ± 44 vs. 54.4 ± 90 IFN-γ spots for CMV-lysate, pp65 and IE-1 for 3×10^5 stimulated PBMCs, respectively. $p < 0.05$ only for IE-1 responses), and between patients developing CMV disease and those that did not (244.3 ± 342 vs. 145.4 ± 175, 105.9 ± 229 vs. 128.8 ± 185, 4 ± 4.8 vs. 52 ± 103 IFN-γ spots for CMV-lysate, pp65 and IE-1 for 3×10^5 stimulated PBMCs, respectively. $p < 0.05$ only for IE-1 responses). (B) CMV-specific T cell responses between patients developing CMV viremia and those that did not, in patients receiving prophylaxis treatment (80.5 ± 106 vs. 54.6 ± 115, 22.7 ± 48 vs. 46 ± 70, 5.4 ± 8 vs. 27.8 ± 31 IFN-γ spots for CMV-lysate, pp65 and IE-1 for 3×10^5 stimulated PBMCs, respectively. $p < 0.05$ only for IE-1 responses), and between patients developing CMV disease and those that did not (51.8 ± 85 vs. 64.5 ± 119, 10.2 ± 25 vs. 47 ± 70, 4.2 ± 7 vs. 26 ± 31 IFN-γ spots for CMV-lysate, pp65 and IE-1 for 3×10^5 stimulated PBMCs, respectively. $p < 0.05$ for both pp65 and IE-1 responses).

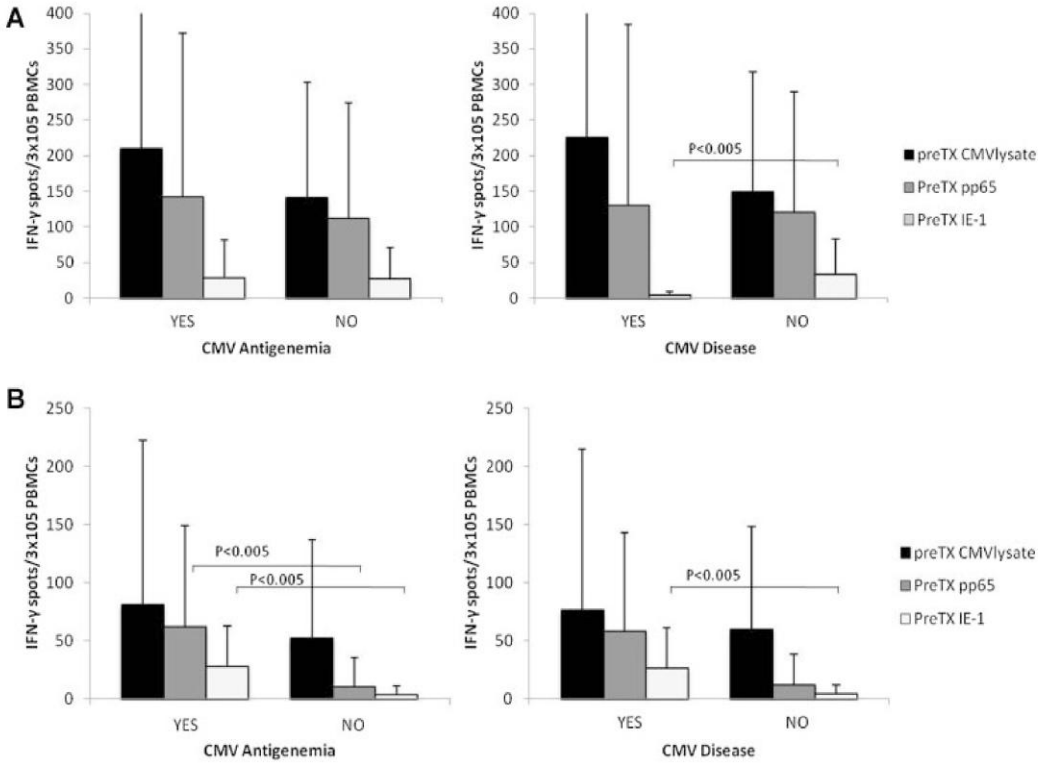

Figure 5: CMV-specific T cell responses and development of CMV viremia and disease within rATG-treated patients receiving preemptive and prophylaxis therapy. (A) CMV-specific T cell responses between patients developing CMV viremia and those that did not, in patients receiving rATG and preemptive treatment (209.4 ± 292 vs. 141.5 ± 162, 142.6 ± 230 vs. 112 ± 162, 28.3 ± 53 vs. 27.4 ± 44 IFN-γ spots for CMV-lysate, pp65 and IE-1 for 3×10^5 stimulated PBMCs, respectively. P = NS for any sCMV stimuli), and between patients developing CMV disease and those that did not (226.2 ± 3456 vs. 149.3 ± 168, 129.8 ± 254 vs. 120.2 ± 170, 4.8 ± 4 vs. 33 ± 50 IFN-γ spots for CMV-lysate, pp65 and IE-1 for 3×10^5 stimulated PBMCs, respectively. $p < 0.05$ only for IE-1 responses). (B) CMV-specific T cell responses between patients not developing CMV viremia and those experiencing CMV viremia, in patients receiving rATG and prophylaxis treatment (80.5 ± 142 vs. 52 ± 85, 61.6 ± 87 vs. 10.2 ± 25, 27.7 ± 35 vs. 3.5 ± 7.6 IFN-γ spots for CMV-lysate, pp65 and IE-1 for 3×10^5 stimulated PBMCs, respectively. $p < 0.05$ for both pp65 and IE-1 responses), and between patients developing CMV disease and those that did not (76 ± 139 vs. 59.2 ± 89, 58.2 ± 85 vs. 11.7 ± 27, 26.2 ± 35 vs. 4 ± 8 IFN-γ spots for CMV-lysate, pp65 and IE-1 for 3×10^5 stimulated PBMCs, respectively. $p < 0.05$ for IE-1 responses).

found that pp65 and IE-1-specific T cell responses are predominantly but not exclusively restricted to CD8+ thus, CD4+ T cells responses could similarly be required to confer long-term protection against CMV infection.

Even though T cell responses may target multiple CMV-specific proteins (18,34,35), it appears that protective cellular immunity is mainly directed against the tegument protein ppUL83 and to the immediately early protein ones (19,21,33,36). To note, IE-1 is the first protein expressed upon CMV reactivation (37), thus IE-1-specific T cells would be the first to be activated and directed to sites of replication (38,39). Hence, this mechanism could explain why high levels of IE-1 but not other CMV-specific T cells would be associated with protection from CMV disease. Some other groups have shown lack of correlation with exclusive IE-1-specific T cell responses and risk of CMV disease (40,41). To note, most of them focused at the posttransplant setting and evaluated a rather low number of transplant recipients. In our study, at 6 months while there was a general increase of all CMV-specific T cell responses (both against IE-1 and also pp65), this feature was specifically observed within those having recovered from CMV infection, suggesting that broader CMV-antigen

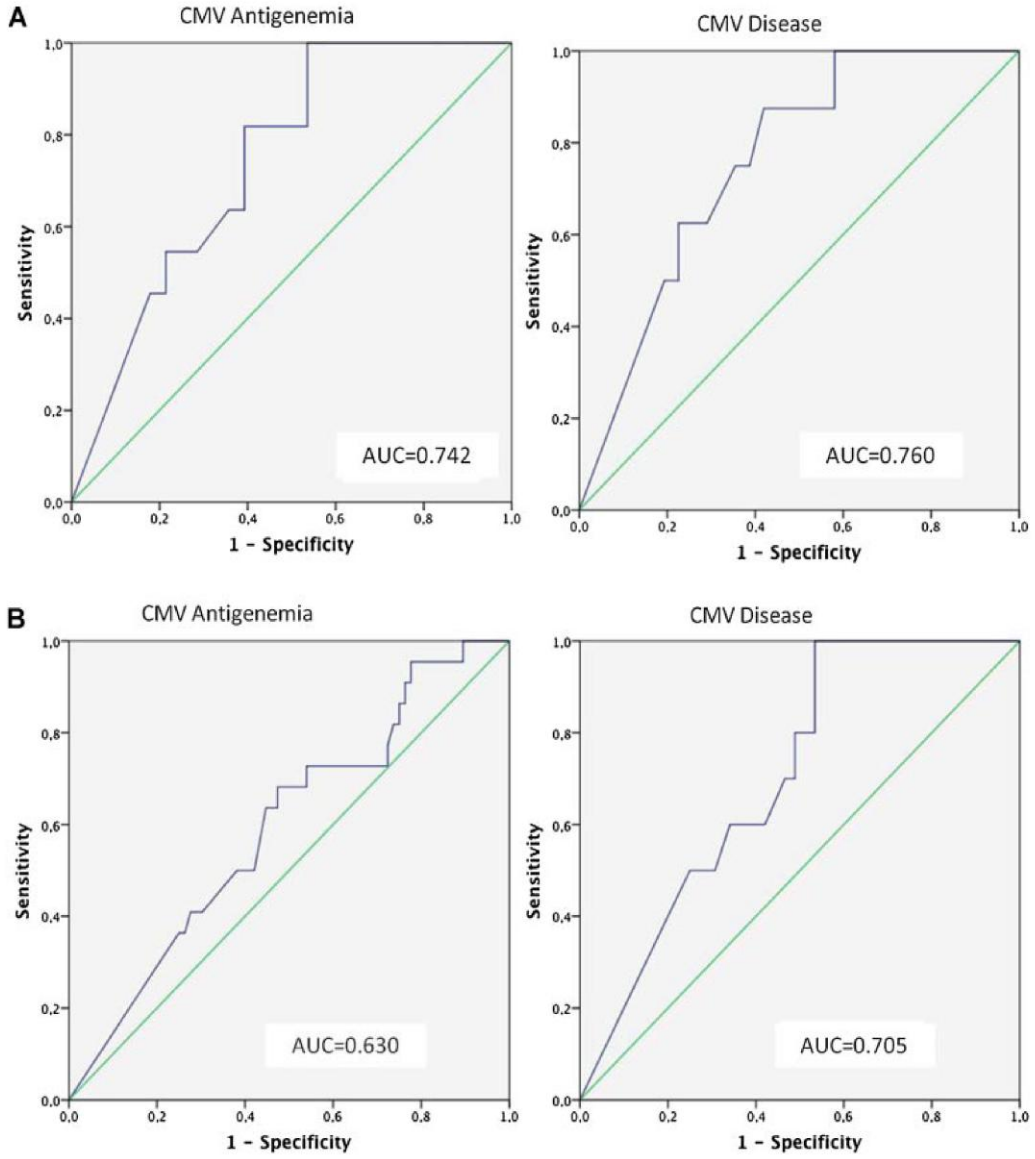

Figure 6: ROC curve analysis estimates sensitivity and specificity of pretransplant anti-IE-1 T cell frequencies for predicting the risk of both CMV viremia and disease in prophylactic and preemptive-treated patients.

specific T cell responses might be also necessary for controlling CMV replication. This data reinforces the potential value of preventive strategies using recombinant CMV proteins as vaccines, preferentially containing immunogenic IE-1 antigens already before transplantation.

There are some limitations in this study. First, although we used a non-standardized immune assay, the IFN-γ Elispot has already been shown to be highly reproducible for measuring antigen-specific cellular responses in other relevant fields of medicine (42–44), allowing a comprehensive quantitative-dynamic idea of the antigen-specific cellular strength at a single cell level. Another limitation is the lack of PCR-CMV viremia monitoring in our study that could have induced misleading diagnosis. Nonetheless, although PCR-CMV viremia has shown higher sensitivity as

Table 4: Predictive value of anti-IE-1 T cell response for posttransplant CMV infection

			Predictive value		
Treatment group	Variables	Cut-off values (IFN-γ spots)	Specificity (%)	Sensitivity (%)	NPV (%)
Prophylaxis	Pre-TX anti-IE-1 (CMV infection)	8 spots 3×10^5 PBMCs	65	82.5	89.5
Preemptive	Pre-TX anti-IE-1 (CMV infection)	7 spots 3×10^5 PBMCs	55	80	95.7

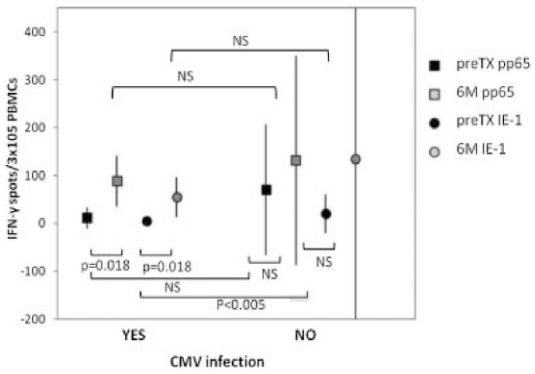

Figure 7: Anti-CMV T cell response changes between infected and non-infected transplant recipients. Patients having experienced CMV infection showed a significantly increase in their anti-pp65 and anti-IE-1 T cell responses as compared to those that did not (4.8 ± 2 vs. 54.4 ± 42, $p = 0.018$; 11.7 ± 22 vs. 88.8 ± 53 $p = 0.018$ for IE-1 and pp65, respectively among patients having experienced CMV infection, and 20 ± 41 vs. 135 ± 532, p = NS and 70.2 ± 137 vs. 131.3 ± 219 p = NS for IE-1 and pp65 for 1.5×10^5 stimulated PBMCs, respectively among non-infected transplant recipients).

compared to CMV antigenemia (45), the incidence of CMV antigenemia or disease among our two cohorts of kidney transplant recipients fitted with that reported in the literature using PCR-based assays (46,47).

In conclusion, we have shown that monitoring frequencies of IE-1-specific T cell responses before transplantation may be useful for predicting posttransplant risk of CMV infection, thus being potentially valuable for guiding decision-making regarding CMV preventive treatment. To further support this result and validate its potential clinical utility, large-scale prospective randomized trials are highly warranted and should be preferentially performed in the context of multicenter cooperative networks.

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Disclosure

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

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