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Role of NFATc activation in innate immune cells in acute transplant rejection

COORDINATOR: Prof. Andrea Biondi

TUTOR: Prof.ssa Francesca Granucci

CO-TUTOR: Dott. Ivan Zanoni

Dr.ssa Clara Cigni

Matr. No. 701619

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Abbreviation list

DCs: Dendritic cells

MHC: Major histocompatibility complex

APCs: Antigen presenting cells

PRR: Pattern recognition receptors

PAMPs: Pathogen-associated molecular patterns

TLRs: Toll-like receptors

LPS: Lipopolysaccharide

DAMPs: Damage-associated molecular patterns

LNs: lymph nodes

Tregs: T regulatory cells

OVA: ovalbumin

NFAT: Nuclear factor of activated T cells

IL-2: interleukin-2

miH: minor histocompatibility antigens

NPs: nanoparticles

Chapter 1: Introduction

1.1 Inflammation

Inflammation is an adaptive response that is triggered by noxious stimuli and conditions, such as infections and tissue injury [1]. The acute inflammatory response results in events that lead to the delivery of blood components normally restricted to the blood vessels (plasma proteins and leukocytes) to the site of infection or injury.

The process of acute inflammation involves a rapid activation (over a period of hours) of the immune system which underlies the four cardinal signs of inflammation in the interested tissues. These comprise an increase in local blood flow, accounting for the redness (rubor) and warmth (calor), and a localized leakage of plasma-protein fluid (the exudate) accompanied by the recruitment of circulating leukocytes into the tissues, accounting for the swelling (tumor). Pain (dolor) is caused by leukocyte-released mediators that activate C-type sensory nerve fibers [2].

A typical inflammatory response consists of four components; the inflammatory inducers, the sensors that detect them, the inflammatory mediators induced by the sensors, and the target tissues that are affected by the inflammatory mediators. Depending on the nature of the inflammatory trigger, the sensors, mediators and target

tissues vary such that the appropriate type of inflammatory response is induced. Inducers, of exogenous or endogenous origin, are the signals that initiate the inflammatory response and activate specialized sensors. The sensors in turn elicit the production of a specific set of mediators, which alter the functional states of tissues and organs. A successful acute inflammatory response results in the elimination of the triggering insult, followed by a resolution and repair phase of the damaged tissues (Figure 1).

If the inflammatory trigger is not eliminated by the acute inflammatory response, the resolution phase may not be appropriate and the inflammatory process persists and acquires new characteristics, leading to a chronic inflammatory state [3]. An excessive inflammatory response is detrimental due to its negative effects on tissue function, resulting in tissue damage and, then, in a dysregulated tissue repair response, accompanied by tissue remodeling, fibrosis, and metaplasia. Moreover, the chronic inflammatory state may involve the formation of granulomas and tertiary lymphoid tissue [4]. Finally, chronic inflammation can accompany many pathological states without infections or injury and is associated with many chronic diseases, including diabetes, atherosclerosis, arthritis, cancer and autoimmune disease.

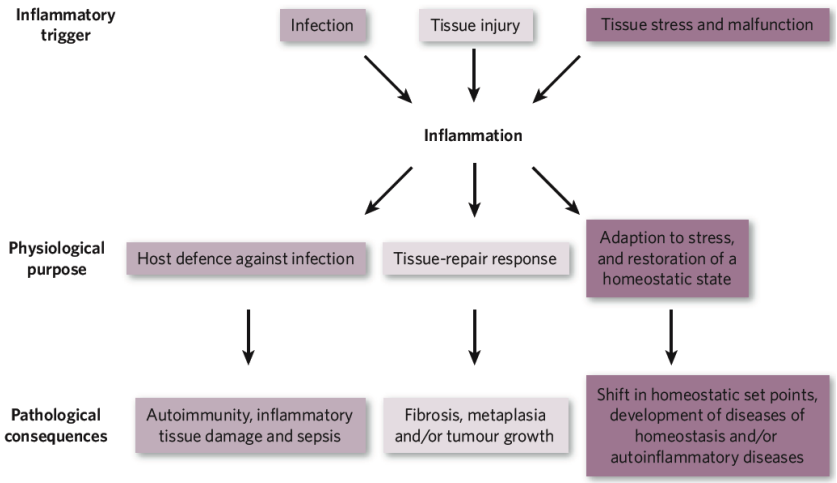

Figure 1. The inflammatory "pathway". Depending on the trigger, the inflammatory response has different purpose, including elimination of the triggering insult, a tissue- repair response and the restoration of a homeostatic state. However, if the inflammatory response fail to eliminate the insult, a chronic inflammatory status could arise, resulting in a shift in the homeostatic set points and development of diseases [5].

1.1.1 Innate Immunity

The inflammatory response is activated when tissue-autonomous defences are insufficient. The acute inflammatory response is based on specialized cell types and mediators which are recruited from the circulation and that are component of the immune system [6].

The immune system exploits numerous strategies evolved to respond to infection and tissue injury. In higher organisms, like mammals, the immune system consists of two interrelated arms, the innate and adaptive ones, each with different functions and roles.

During evolution, the innate immune system appeared before the adaptive immune system and comprised soluble factors (antimicrobial peptides and complement pathway) and myeloid cells, including granulocytes, mast cells, macrophages and dendritic cells (DC). Innate immunity could be consider as the first line of defense against infectious organisms as it rapidly controls the replication of the infecting pathogens and it is highly efficient to combat infections. In particular, the innate immune system is based on non-clonally, germ-line encoded receptors that recognize molecular structures unique to classes of infectious microbes [7].

On the other hand, the adaptive immune system appeared only recently during evolution, in particular at the time of the differentiation of vertebrates [8], and is mainly based

on two classes of specialized cells, T and B lymphocytes. Adaptive immunity is characterized by specificity and uses randomly generated, clonally expressed receptors produced via somatic recombination, which allows to generate a high specific and vast repertoire of lymphocytes that leads to the generation of immunological memory. However, primary immune responses of T and B cells can be induced only in organized secondary lymphoid organs and is involved in elimination of pathogens in the late phases of infection, since it takes three to five days to produce sufficient numbers of clones and to differentiate into effector cells [9].

The combination of innate and adaptive immunity makes the mammalian immune system highly efficacious to recognize and eliminate invading pathogens with maximal efficacy and minimal damage to self. The innate immune system appears to be fundamental in the induction and control of adaptive immunity; for instance, naïve T cells undergo clonal expansion only when they recognize small peptides presented on the cell surface by major histocompatibility complex (MHC) antigens expressed on antigen-presenting cells (APC), such as DC [10]. In this regard, the innate and adaptive immune systems are ideal partners since they show different strategies to recognize infections. The innate immunity recognizes microorganisms via a limited number of germline-encoded pattern recognition receptors (PRRs) able to bind highly

conserved structures present on the surface of the pathogens, while adaptive immunity receptors are produced by somatic recombination, generating a virtually unlimited repertoire able to recognize different antigens. However, by using randomly generated receptors, adaptive immune system cannot distinguish between self from non-self and it must be instructed on the origin of the antigens by innate immunity. As proposed by Charles Janeway Jr almost thirty years ago [11], the innate immune system is able to determine, with high fidelity, whether an antigen is derived from self, non-self or innocuous, relaying its conclusions to the adaptive immune system. Janeway postulated that the innate immune system would sense the presence of infection via recognition of conserved microbial pathogen-associated molecular patterns (PAMPs), by PRRs. PAMPs are structures unique and common to a broad class of microbes and they are essential for their life. PAMPs can vary in their molecular structure and nature from lipids, lipoproteins, proteins which are component of the bacterial cell wall to nucleic acids and are redundantly or non-redundantly detected by PRRs [12]. Importantly, the recognition by PRRs on cells of the innate immunity lead to the induction of signals involved in activation of the adaptive immune system [13]. Different PRRs react with specific PAMPs, show distinct expression patterns, activate specific signaling pathways and lead to distinct anti-pathogen responses. Indeed,

PRRs belong to several families of proteins and they can be divided into different classes [14]. Once PRRs recognize PAMPs, they activate specific signal transduction pathways, inducing the expression of many immune-related genes and the production of inflammatory cytokines. Among signaling receptors, Toll-like receptors (TLRs) are the best-characterized family, since TLR4 were first discovered in human by R. Medzhitov and C.A. Janeway [15]. In particular, it has been demonstrated in mouse that TLR4 is the receptor for the lipopolysaccharide (LPS), a cell wall component of Gram-negative bacteria, together with other proteins, such as the co-receptor CD14 and the glycoprotein MD-2 [16]. This complex was shown to induce the activation of NF-κB signaling pathway.

Finally, the activation of TLRs on the surface of APCs, such as DCs, is able to induce the expression of co-stimulatory molecules CD80/CD86 and the production of pro-inflammatory cytokines, leading to the activation of naïve T cells (figure 2).

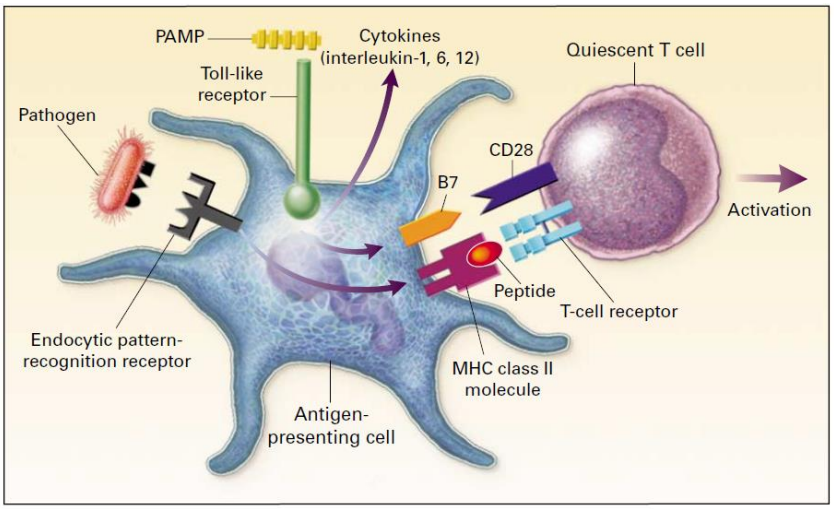

Figure 2: Interplay between innate and adaptive immune systems
Recognition of PAMPs by PRRs generates signals that activate the adaptive immune system. Endocytic PRRs mediate the uptake and phagocytosis of pathogens by APCs; proteins derived from microbes are then processed and presented by MHC class II molecules, which are recognized by T cell receptors (TCR). Instead signaling receptors (TLRs) lead to the activation of pathways that induce the expression of pro-inflammatory cytokines and co-stimulatory molecules [17].

Recent studies have also demonstrated that PRRs recognize non-infectious material that can cause tissue damage and endogenous molecules that are released during cellular injury, without the presence of microorganisms. Inflammation can also arise as a result of trauma, ischaemia-reperfusion injury or chemically induced injury even in the absence of any pathogen and has been therefore termed “sterile inflammation” [18]. In this context, Polly Matzinger hypothesized that as the inflammation induced in response to sterile injury is similar

to that observed during microbial infection, host receptors that mediate the immune response to microorganisms may be involved in the activation of sterile inflammation [19]. The endogenous molecules released during sterile inflammation have been termed damage-associated molecular patterns (DAMPs), as these host-derived non-microbial stimuli are released following tissue injury or cell death and have similar functions as PAMPs in terms of their ability to activate pro-inflammatory pathways. A common feature of DAMPs is that they are endogenous factors that are normally sequestered intracellularly and are therefore hidden from recognition by the immune system under normal physiological conditions. Instead, under conditions of cellular stress or injury, these molecules can be released into the extracellular environment by dying cells and trigger inflammation under sterile conditions [20]. For instance, an important consequence of necrotic cell death is the loss of plasma membrane integrity which allows to the escape of intracellular material from the cell. Prototypical DAMPs derived from necrotic cell include the High mobility group box (HGMB1), heat shock proteins (HSPs) and purine metabolites (ATP and uric acid) [21-22]. In addition to DAMPs from an intracellular source, there are also extracellularly located DAMPs, such as hyaluronan, heparin sulphate and biglycan. These are tipically released by extracellular matrix degradation during tissue

injury [23]. Despite the growing list of sterile immune stimuli, the mechanisms by which these stimuli trigger an inflammatory response are still not fully understood. However, even when endogenous DAMPs are structurally heterogenous, the outcome of inflammatory responses to these is generally uniform. Although further studies are required, it has been demonstrated that DAMPs activate similar inflammatory signaling pathways (mediated by TLRs, inflammasomes and IL1R) compared to PAMPs. The activation of these pathways induce the production of pro-inflammatory cytokines and chemokines from innate immune cells, which are released to recruit and activate additional inflammatory cells crucial for tissue and wound repair.

1.1.2 Inflammatory mediators and prostaglandins

Inducers of inflammation trigger the production of numerous inflammatory mediators, which in turn alter the functionality of many tissues and organs. These mediators can be derived from plasma proteins or secreted by cells; the cellular mediators can be produced by specialized leukocytes or by cells present in local tissues, while other mediators are preformed and circulate as inactive precursors in the plasma. These inflammatory mediators induce tissue modification in order to remove or sequester the source of the disturbance, to allow the host to adapt to the abnormal conditions and, ultimately, to restore functionality and homeostasis to the tissue.

Inflammatory mediators can be classified into seven groups according to their biochemical properties: vasoactive amines, vasoactive peptides, fragments of complement components, lipid mediators, cytokines, chemokines and proteolytic enzymes [24].

1. - Vasoactive amines (histamine and serotonin) are produced by mast cells and platelets. They have complex effects on the vasculature, causing increased vascular permeability and vasodilation, or vasoconstriction, depending on the context.

2. - Vasoactive peptides can be stored in an active form in secretory vesicles or generated by proteolytic processing of inactive precursors in the extracellular fluid. Other vasoactive peptides are generated through proteolysis by the Hageman factor, thrombin or plasmin and cause vasodilation and pain.
3. - Complement fragments C3a, C4a and C5a are produced by several pathways of complement activation. C5a promotes granulocyte and monocyte recruitment and induce mast-cell degranulation, thereby affecting the vasculature.
4. - Lipid mediators (eicosanoids and platelet-activating factors) are derived from phospholipids, such as phosphatidylcholine, that are present in the inner leaflet of cellular membranes. The eicosanoid, such as prostaglandins, cause vasodilation. Platelet-activating factors are generated by the acetylation of lysophosphatidic acid and activate several processes that occur during the inflammatory response, including recruitment of leukocytes, vasodilation and vasoconstriction, increased vascular permeability and platelet activation.
5. - Inflammatory cytokines are produced by many cell types and they have several roles in the inflammatory response, including activation of the endothelium and leukocytes and induction of the acute-phase response.

6. - Chemokines are produced by many cell types in response to inducers of inflammation. They control leukocyte extravasation and chemotaxis towards the affected tissues
7. - Proteolytic enzymes (including elastin, cathepsins and matrix metalloproteinases) have diverse roles in inflammation, in part through degrading ECM and basement-membrane proteins. These proteases have important roles in many processes, including host defence, tissue remodelling and leukocyte migration.

Many inflammatory mediators have effects in common on the vasculature and on the recruitment of leukocytes. Indeed, vascular endothelial cells have a major role in the inflammatory process, changing their phenotypes in response to the different mediators in order to favor local swelling and edema formation, which finally terminates with leukocytes extravasation [25].

In this context lipid mediators, in particular prostanoids, are particularly effective in inducing vascular permeability and edema formation. Prostanoids are arachidonic acid metabolites and are generally accepted to play pivotal functions in inflammation, platelet aggregation, and vasoconstriction/relaxation.

All prostanoids exhibit roughly the same structure as all are oxygenated fatty acids composed of 20 carbon atoms and containing a cyclic ring, a C-13→C-14 *trans*-double

bond, and a hydroxyl group at C-15. Prostanoids can be classified into prostaglandins (PG), which contain a cyclopentane ring, and thromboxanes (Txs), which contain a cyclohexane ring [26].

Prostanoids are rapidly synthesized in a variety of cells in response to various stimuli, such as inflammation, and act in an autocrine and paracrine fashion.

Prostaglandin E_2 (PGE_2), also known as dinoprostone, is the most abundant prostanoid in mammals and it is involved in regulating many different fundamental biological functions including normal physiology and pathophysiology [27].

The synthesis of PGs is initiated by the liberation of arachidonic acid, in response to various physiological and pathological stimuli, from the cell membrane by phospholipase A_2 (PLA_2). Arachidonic acid is converted to the prostanoid precursor PGG_2, which is subsequently peroxidized to PGH_2. Both enzymatic reactions are catalyzed by the protein cyclooxygenase (COX), which consists of two forms: the constitutively expressed COX-1 is responsible for basal, and upon stimulation, for immediate PG synthesis, which also occurs at high AA concentrations. COX-2 is induced by cytokines and growth factors and primarily involved in the regulation of inflammatory responses. Following COX activity, prostanoid synthesis is completed by cell-specific synthases. In particular, PGE_2 is synthesized from PGH_2

by cytosolic (cPGES) or by membrane-associated/microsomal (mPGES-1 or mPGES-2) prostaglandin E synthase [28]. Of these enzymes, cPGES and mPGES-2 are constitutively expressed and preferentially couple with COX-1, whereas mPGES-1 is mainly induced by pro-inflammatory stimuli, with a concomitant increased expression of COX-2 (Figure 3).

In a flogistic context, PGE_2 plays a key role as an inflammatory mediator because it is involved in all processes leading to the classic signs of inflammation: redness, swelling and pain [29-30]. Redness and edema result from increased blood flow into the inflamed tissue through PGE_2-mediated augmentation of arterial dilatation and increased microvascular permeability [31]. In fact, PGE_2 binds on smooth muscle cells and endothelial cells (components of blood vessels), inducing a local vasodilation that results in edema formation [32]. This process is a very important event in order to orchestrate early inflammatory immune responses.

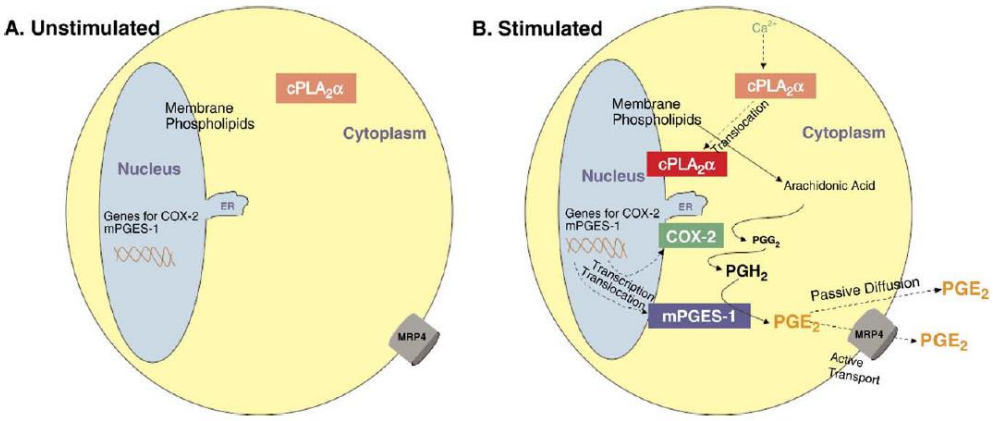

Figure 3: Coordinate production of PGE_2 by $cPLA_2\alpha$, COX-2 and mPGES-1.

(A) Prior to activation by inflammatory stimuli, $cPLA_2\alpha$ is present in the cytoplasm of unstimulated cells, while COX-2 and mPGES-1 are not expressed. (B) Inflammatory stimulation results in calcium influx which leads to the translocation of $cPLA_2\alpha$ from cytosol to nuclear membrane where it hydrolyzes phospholipids to release arachidonic acid. Inflammatory stimuli also induce the expression of both COX-2 and mPGES-1 at the nuclear membrane and endoplasmic reticulum, where they finally generate PGE_2 [33].

1.2 Dendritic cells

Dendritic cells (DCs) are a sparsely distributed, migratory group of bone-marrow derived leukocytes that are specialized for the uptake, transport, processing and presentation of antigens to T cells [34]. DCs are APCs which constitute an extremely complex cell population composed of numerous cell types that differentiate from distinct precursors, colonize distinct tissues (both peripheral and lymphoid organs) and have different functions [35].

At an “immature” stage of development, one of the major role of DCs is to patrol the body periphery, continuously sampling the antigenic environment. Any encounter with microbial products or tissue damage initiates the migration of the DCs to lymph nodes (LNs). To this purpose, DCs are equipped with different microbial sensors and other probes (PRRs) that are able to initiate a signaling cascade which drive phenotypic and functional maturation after encounter with the antigen [36]. The antigenic sample is processed and fixed on the DCs surface as peptides that are presented by MHC molecules. APCs, such as DCs, usually present exogenous antigens on MHC class II (MHC II) molecules, whereas they usually present endogenous antigens (from self-component or a viral infection) on MHC class I (MHC I) molecules [37-38].

Once encounter the antigens, DCs also upregulate the co-stimulatory molecules that are required for effective interaction with T cells. In the LNs, the now “mature” DCs efficiently trigger an immune response by any T cells with a receptor that is specific for the peptide-MHC complexes on the DC surface, promoting T cell proliferation. Although triggering of T cells into cell-cycle progression is a central function of DCs, it is now clear that DCs can also influence the subsequent development of these dividing T cells (figure 4).

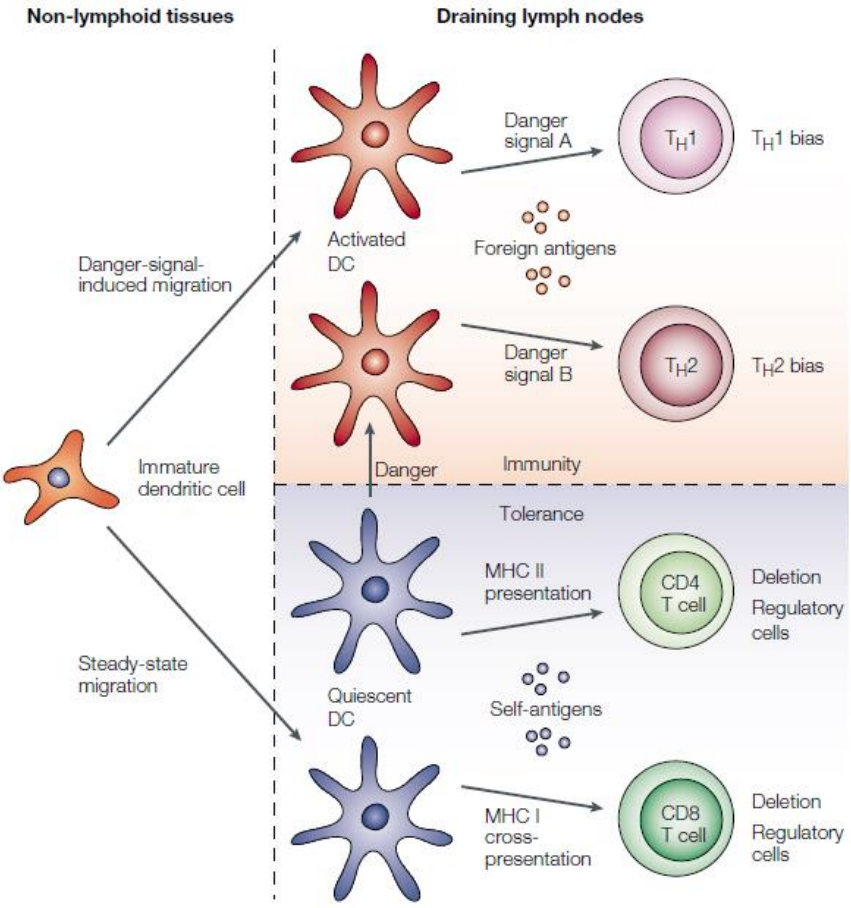

Figure 4: Dendritic cells and immunoregulation.

In the absence of microbial infections and "danger" signals, there is a low-level, steady-state entry of DCs into lymphoid tissues, in which quiescent DCs help to maintain peripheral T cell tolerance to self-antigens. Inflammation activates DCs and increases their rate of migration into LNs, where they signal to T cells [39]. T_H1, T helper 1 cell; T_H2, T helper 2 cell

1.2.1 DCs heterogeneity and functions

DCs are present throughout the body, including both lymphoid and non-lymphoid tissues where they exert different functions, such as T cell activation, interaction with other cell types (B cells and NK cells) and cytokine productions. In order to perform these different functions, DCs comprise a heterogeneous group of cells that have originally been divided into different subsets on the basis of the expression of some surface markers (Table 1). Subsequently, DCs have further been divided into many different subsets on the basis of their ontogeny, localization and immunological functions. They are principally divided into four main categories; the plasmacytoid DCs (pDCs), the conventional or classical DCs (cDCs), the Langerhans cells (LCs) and the monocyte-derived DCs. However, the first two categories (pDCs and cDCs) shares their ontogeny, arising from the same adult hematopoietic stem-cell (HSC)-derived precursor distinct from the precursor of monocyte and macrophages, which is known as common-derived progenitor (CDP). CDPs then give rise to distinct DCs subsets; cellular markers have been identified to help delineate distinct CDPs populations that are biased towards the generation of cDCs or pDCs. In particular, the intermediate stage between CDPs and cDCs is the precursor for cDCs (pre-cDCs), which develop in the bone

marrow and then migrate to peripheral organs where it develop locally into cDCs. On the other hand, pDCs terminally differentiate in the bone marrow via a pre-pDCs intermediate stage [40].

Table 1: Phenotype of mouse lymphoid and non-lymphoid tissue DCs [41]

ND, not determined

Phenotypical marker	pDC	Lymphoid tissue cDC		Nonlymphoid tissue cDC			Langerhans cell
		$CD8^+$ cDC	$CD11b^+$ cDC	$CD103^+$ $CD11b^-$ cDC	$CD103^+$ $CD11b^+$ intestinal cDC	$CD103^-$ $CD11b^+$ cDC	
CD45	+	+	+	+	+	+	+
CD11c	+	+++	+++	++	++	++	++
MHC class II	+	++	++	++	++	++	++
CD8	subset	+	–	–	–	–	–
CD4	+	–	+/–	–	–	–	ND
CD11b	–	–	+	–	+	+	+
CD103	–	subset	–	++	++	–	–
Langerin	–	subset	–	+	–	–	++
EpCAM	–	–	–	–	–	–	++
B220	+	–	–	–	–	–	–
CD24	ND	++	+	++	++	+/–	++
Btla	+	++	+	++	+	+/–	ND
c-kit	–	+	+	+	+	+/–	ND
CD26	+	+	+	+	+	+/–	ND
Xcr1	–	+	–	+	–	–	–
CD36	–	+	–	+	ND	–	–
Cystatin C	+	++	+	ND	ND	ND	ND
Clec9a (DNGR1)	+	++	–	++	–	–	–
Cadm1 (Necl2)	–	+	–	ND	ND	ND	ND
CD205	–	++	+	++	ND	ND	++
CX_3CR1	–	subset	–	–	–	++	+
CD209 (dc-sign)	++	–	+	–	+	+/–	–
F4/80	–	–	+	–	–	+	+
CD172a (Sirpα)	+	–	++	–	–	++	+
CD64 (Fcγr1)	–	–	–	–	–	++	ND
Ly6C	++	–	–	–	–	+/–	–

Plasmacytoid DCs:

Plasmacytoid DCs respresent a small subset of DCs that share a similar origin with, but a different life cycle than, cDCs. pDCs are rare cells (0,3-0,5% of the human peripheral blood or of murine lymphoid organs) that accumulate mainly in the blood and lymphoid tissues at the steady state and enter the LNs through blood circulation [42].

pDCs express low levels of MHC II and co-stimulatory molecules and low levels of the integrin CD11c in the steady state, but they are positive for the B cell marker B220/CD45RA. They present antigen inefficiently in the steady state but are fully capable of antigen presentation after pathogen-induced activation [43]. At this end, they particularly express a narrow range of PRRs that include TLR7 and TLR9 that make them capable to specifically recognize foreign nucleic acids. Upon recognition, pDCs are able to produce massive amounts of type I interferon (interferon α/β, IFN-I) and other cytokines [44].

Classical DCs:

Classical DCs form a small subset of tissue hematopoietic cells that populate most lymphoid and non-lymphoid tissues. These cells are specialized to sense tissue injuries, capture environmental and cell-associated

antigens, and process and present phagocytosed antigens to T lymphocytes. cDCs can be grouped into two main classes (lymphoid organ-resident and non-lymphoid resident cDCs) based on their localization in tissues and their migratory pathways as they circulate in the body.

Lymphoid organ-resident cDCs:

Lymphoid tissue-resident cDCs differentiate in, and spend their entire lives within, lymphoid tissues. LNs include both lymphoid organ-resident and non-lymphoid migratory cDCs, whereas lymphoid-tissue resident cDCs make up the entirety of the splenic cDCs compartment [45]. Lymphoid organ-resident cDCs consists of mainly two subsets, $CD8^+$ and $CD11b^+$ cDCs.

The $CD8^+$ cDCs subset represent 20-40% of spleen and LNs cDCs; in the spleen, they are located in the marginal zone, whereas in the LNs they are present in the subcapsular sinus. From these strategic locations to filter blood antigens, $CD8^+$ cDCs migrate to the T cell zone to present blood or tissue antigens to T lymphocytes.

$CD8^+$ cDCs subset express the CD8α protein, but not CD8αβ, commonly express by $CD8^+$ T cells. $CD8^+$ cDCs express no or low levels of the integrin CD11b and other macrophage markers, but they express high levels of the cytokine FMS-like tyrosine kinase 3 (Flt3) and proliferate in response to Flt3L [46]. They differentially expressed

lectin receptors, such as CD205, Clec9A and langerin (CD207), and TLRs compared to $CD11b^+$ cDCs. Moreover, they are closely related to non-lymphoid tissue migratory $CD103^+$ cDCs. However, in contrast to tissue-migratory cDCs that arrive in the LNs in a mature state, $CD8^+$ cDCs are phenotypically immature at the steady state and activation to a mature state occurs only upon stimulation with microbial products [47].

On the other hand, the $CD11b^+$ cDCs subset lack the marker CD8 and most often predominates the lymphoid-resident cDCs population in all organs except the thymus. Similarly to $CD8^+$ cDCs, $CD11b^+$ cDCs proliferate in response to Flt3L. In the spleen, $CD11b^+$ cDCs are heterogeneous and consist of two populations that differentially express the endothelial cell-specific adhesion molecules (ESAM). $ESAM^{hi}CD11b^+$ cDCs express higher levels of CD4, CD11c and Flt3 and derive from DC-restricted progenitors, whereas $ESAM^{lo}CD11b^+$ express lower levels of these markers and are thought to derive from circulating monocytes [48].

Non-lymphoid organ-resident cDCs:

Non-lymphoid tissue resident cDCs represent 1-5% of tissue cells depending on the organ and consist of two major subsets: $CD103^+CD11b^-$ and $CD11b^+$ cDCs.

The $CD103^+CD11b^-$ cDCs subset populates most connective tissues at the interface with the environment and efficiently migrate to the T cell zone of the draining LN. The proportion of these cells among total cDCs rarely exceed 20-30%. $CD103^+$ cDCs lack the macrophage markers CD11b, CD115, CD172a and F4/80, but they commonly express higher Flt3 levels and the C-type lectin receptor langerin compared to $CD11b^+$ cDCs [49].

The $CD103^+CD11b^-$ cDCs subset shares its origin and function with lymphoid-organ resident $CD8^+$ cDCs; indeed, these two subsets express similar TLR, C-type lectin receptor and chemokine receptor profile. In particular, $CD8^+$ and $CD103^+$ cDCs are the only subsets that express the double-stranded viral RNA sensor,TLR3 and TLR11 [50].

Ex vivo studies have also revelead the superior ability of $CD8^+$ and $CD103^+$ cDCs to present microbial or cell-associated antigens to $CD8^+$ T cells. These subsets indeed express more genes related to MHC I presentation compared to $CD11b^+$ cDCs and are the main source of IL-12 and IL-15 [51-52]. On the other hand, the role of these subsets in the activation of $CD4^+$ T cells is not as clear as their role in the activation of $CD8^+$ T cells. However, it has been demonstrated that dermal $CD103^+$ cDCs control the induction of pathogen-specific $CD4^+IFN\text{-}\gamma^+$ T cells upon cutaneous infection with *C.albicans* [53].

The $CD11b^+$ cDCs subset consists of a mixture of tissue cDCs and macrophages, which express the integrin CD11b and most often lack the integrin CD103. $CD11b^+$ cDCs can sense pathogens and migrate from non-lymphoid tissues to regional LNs charged with self and foreign antigens. These cells seem to have a predominant role in MHC II presentation and are the predominant subset to drive the accumulation of antigenic-specific $CD4^+$ T effector cells and T regulatory (Tregs) cells [54]. In this context, it has also been demonstrated that a specific subset of migratory lamina propria $CD103^+CD11b^+$ cDCs have a superior ability to induce peripheral Treg differentiation in vivo, due to their expression of aldehyde dehydrogenase (ALDH), an enzyme that metabolizes dietary vitamin A into retinoic acid. [55].

Langerhans cells:

Langerhans cells are radio-resistant cells that reside in both the mouse and human epidermis and exhibit specific differentiation and homeostatic features distinguish them from other cDC and pDC populations.

LCs originate from yolk sac-derived myeloid precursors that are recruited to the epidermis prior to birth, around embryonic day 18 [56], and from fetal liver-derived monocytes [57]. The epidermal LC precursors acquire a DC-like morphology immediately after birth and then

undergo extensive proliferation between postnatal day 2 and 7. Following this early proliferative burst, the differentiated LCs show a low rate of in situ proliferation in steady state conditions, which allows LCs to self-renew in adulthood independently of the bone marrow [58]. On the other hand, in inflammatory conditions that lead to severe LCs depletion and damage of the epidermal-dermal basal membrane, LCs are repopulated by blood-borne monocytes [59].

LCs are identified in the epidermis based on the expression of the hematopoietic markers CD45, MHC II, CD11c, epithelial cell adhesion molecule (EpCAM) and the lectin langerin (CD207).

LCs differs for many aspects from cDCs and monocyte-derived DCs present in the dermis; this aspect has led to epidermal LCs to be characterized as a lineage related to non-lymphoid tissue macrophages. However, in contrast to such tissue macrophages, after encounter with an antigen LCs present a migratory ability similar to that of non-lymphoid tissue resident cDCs [60]. To this purpose, LCs interact closely with the surrounding keratinocytes in the epidermis through interactions that involve E-cadherin. Upon recognition of a pathogen or tissue-damage, LCs downregulate E-cadherin expression, resulting in a detachment from keratinocytes and in a switch from a sessile state to a mobile state, which enables them to

reach the dermal compartment and then the draining LNs [61].

Several studies have demonstrated that LCs could have a large number of sometimes opposing functions. Although LCs are able to pick up antigens present in the epidermis and convey them to the LNs to display them in an immunogenic manner to naïve T cells, recent studies have shown that LCs can have immunosuppressive functions and can dampen T cell responses elicited by dermal DCs [62]. However, another study showed that the direct presentation of antigens by LCs is essential for the development of T helper 17 (Th17) cells in a mouse model of *C. albicans* [53]. Moreover, by projecting dendrites through keratinocytes tight junctions, LCs can also elicit humoral immunity to antigens that have not yet penetrated the epidermal barrier [63].

Monocyte-derived DCs:

Over the last decade, the potential of monocytes to differentiate into DCs has been demonstrated. Although the ability of monocytes to behave as DCs precursors was firstly shown by an in vitro system [64], experimental models have now been proved the differentiation of DCs from monocytes in vivo during inflammatory reactions [65]. The phenotype of monocyte-derived or inflammatory DCs is influenced by the nature of the stimuli and by the tissues

in which they arise. However, most monocyte-derived DCs are characterized by the expression of Ly6C, CD11b, MHC II and intermediate levels of CD11c.

Circulating monocytes consist of two main subsets, $Ly6C^{hi}$ and $Ly6C^{low}$ monocytes. $Ly6C^{hi}$ monocytes, which correspond to conventional monocytes, are CX_3CR1^{low}, $CCR2^{+}$, $CD62L^{+}$ and $CCR5^{-}$, whereas $Ly6C^{low}$ monocytes are CX_3CR1^{hi}, $CDr2^{-}$, $CD62L^{-}$ and $CCR5^{+}$. $Ly6C^{hi}$ monocytes are commonly considered to be the direct precursors of inflammatory DCs. It has been demonstrated that $Ly6C^{hi}$ monocytes recruited to the inflamed dermis differentiated locally into macrophages, as well as DCs that subsequently migrated to the draining LNs [66]. Moreover, several studies have also established that in a condition of inflammation, the chemokine receptor CCR2 controls monocyte exit from the bone marrow and recruitment to the site of inflammation; according to these studies, monocyte-derived DCs are greatly reduced in $CCR2^{-/-}$ mice [67].

1.2.2 DCs populations in the skin

The skin is composed of the epidermis, attached to a basement membrane, and the dermis, underlained by a subcutaneous fatty region. Each layer is highly complex and contain different structures, such as hair follicles, sebaceous glands, nerves, blood vessels and lymphatics. Moreover, the epidermis and dermis are populated by a variety of cell types that together form an extremely resistant barrier to pathogens [68].

The epidermis is a tightly packed stratified epithelium largely composed by keratinocytes, which form an outer enucleated, cornified layer referred as to the stratum corneum; this is followed by the stratum granulosum, then the stratum spinosum and finally the basal layer attached to a complex basement membrane. In the epidermis, LC are found in the interfollicular epidermis and the epithelium of hair follicles.

The dermis is not as densely packed with cells as the epidermis, and is instead composed of elastin and collagen fibers and other extracellular matrix components largely produced by fibroblasts. Blood vessels and capillary beds are spread throughout the dermis, while lymphatics begin in this structure and then traverse the deeper layers of the skin to eventually access the lymph nodes. Moreover, many of the immunologically relevant cell types are present into the dermis, such as mast cells,

macrophages, innate lymphoid cells and DCs subsets [69].

The DC populations in the skin comprise five different subsets (Figure 5) that are able to migrate to draining LNs in order to transport skin-sequestered antigens. A small proportion of cDCs present in healthy skin are able to undergo a maturation process, referred as homeostatic maturation, which involves morphological and phenotypical changes that lead to migration to the draining LNs. Following homeostatic maturation, cDCs upregulate MHC II molecules and can present cutaneous self antigens to the T cell in the LNs. Here, if they encountered T cells that have escaped central tolerance, cDCs trigger an abortive program for these autoreactive T cells. On the other hand, in the presence of inflammatory stimuli, cDCs that reside in the skin undergo a terminal differentiation program that additionally results in the up-regulation of co-stimulatory molecules expression and then in the promotion of clonal expansion of antigen-specific T cells in the draining LNs [70].

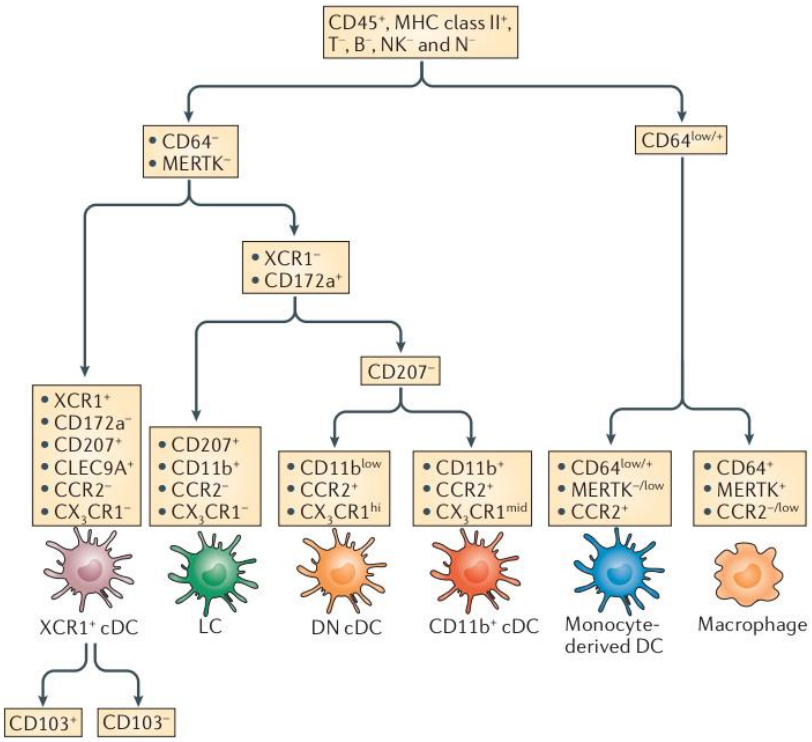

Figure 5: Subsets of skin dendritic cells and macrophages [71]
DC and macrophage populations in the skin can be identified among $CD45^+MHC\ II^+$ cells through a small number of cell surface markers, such as CD64, CCR2 and MERTK. The DC population can be further characterized by the expression of other cell surface markers, such as XCR1, CD207, CD11b and CX_3CR1.

The DCs populations that are found in the dermis have a short lifespan and are replaced by bone marrow-derived blood-borne precursors that continually extravasate from blood vessels. By contrast, macrophages have a longer resistence in healthy skin and they comprise a pool that is established prenatally and a pool that develops after birth

from blood monocytes [72]. cDCs can be distinguished from monocyte-derived DCs and macrophages by their expression of CD64 and of the tyrosine protein kinase MER (MERTK), a receptor that recognizes apoptotic cells. In this regard, cDCs do not present expression of these markers and they can be distinguished from monocyte-derived DCs ($CD64^{low/+}MERKT^{-/low}$) and macrophages ($CD64^{+}MERKT^{+}$) [73].

The cDC population can be further divided into three different subsets; dermal $XCR1^{+}$ cDCs, dermal $CD11b^{+}$ cDCs and the double negative dermal cDCs.

All the $CD11b^{-}$ and $CD8^{+}$ cDCs that are found in lymphoid and non-lymphoid tissues express the XC-chemokine receptor 1 (XCR1) [74]; among the $XCR1^{+}$ cDCs, dermal $XCR1^{+}$ cDCs are the only subset that expresses high levels of CD207. This subset comprises both $CD103^{-}$ and $CD103^{+}$ cells. It has been demonstrated that dermal $XCR1^{+}CD103^{+}$ cDCs present a superior ability in cross-presentation of keratinocyte-derived self antigens. For instance, a transgenic mouse known as K5-mOVA has been used to determine the ability of different cDCs subsets to cross-present a self antigen; in a K5-mOVA mouse, a membrane-bound form of the ovalbumin (OVA) peptide is expressed by skin keratinocytes. Two recent studies based on this model have demonstrated that $CD207^{+}CD103^{+}$ dermal cDCs are the only subset able to

cross-present OVA in both in vitro and in vivo experiments [75-76].

On the contrary, $CD11b^+$ dermal cDCs are the most abundant type of DC in the healthy mouse and they can be distinguished from the other subsets thanks to their $XCR1^-CD172a^+$ phenotype. Many studies that attempt to analyze the function of this subset are difficult to interpret since the $CD11b^+$ were contaminated with monocyte-derived DCs and macrophages. However, it has been showed that a proportion of these cells express ALDH and they are able to produce retinoic acid and to induce Treg cells upon migration to the draining lymph nodes [77]. Granucci and co-workers have also found that peripheral lymphoid Treg cells are induced exclusively by migratory DCs through retinoic acid production, confirming that only migratory cDCs present the peculiar ability to induce Treg cells. Indeed, when autoantigen presentation is extended to all classical DCs, Treg cells develop only in the LNs and not in the spleen [78].

Finally, the mouse dermis contains a quantitatively minor population of cDCs known as double-negative (DN) cDCs; these cells are $XCR1^-CD207^-$ and also express very low levels of CD11b. In contrast to $XCR1^+$ cDCs, DN and $CD11b^+$ cDCs express intermediate or high levels of CCR2, but their development is only mild affected by the lack of CCR2 compared to monocyte-derived DCs and macrophages. Moreover, DN and $CD11b^+$ dermal cDCs

also express CX_3CR1 at high and intermediate levels [60]. However, although a migratory counterpart of DN cDCs is found in the skin-draining LNs, its specific functions remains to be established and no human equivalent of this subset has been identified as yet.

1.2.3 DCs mediated T_H cell polarization

DCs are central in the orchestration of the different forms of immunity and tolerance. This role mainly relies on their ability to sense pathogens or tissue injury through specific receptors, such as PRRs, that modulate their maturation process resulting in the development of effector DCs that selectively promote the immune responses. The various classes of specific immune responses are driven by the biased development of specific effector CD4+ T cell subsets, principally divided into T helper 1 (T_H1) and T helper 2 (T_H2) cells, that activate different component of cellular and humoral immunity. However, T_H1/T_H2 cell-mediated immunity is controlled by the action of T regulatory cells (Treg); Treg cells are defined as a diverse class of natural and adaptive regulatory T cells, that prevent autoimmunity and potentially lethal tissue destruction by chronic innate or adaptive immune responses [79].

The fate of naïve T cells is determined by three signals that are provided by pathogen-primed mature DCs (Figure 6). The stimulatory signal 1 results from the ligation of T cell receptors (TCRs) by antigen peptides presented by MHC molecules on the cell surface of DCs. The stimulatory signal 1 determines the antigen-specificity of the response, but the initiation of protective immunity also requires T cell co-stimulation, which represents the co-stimulatory signal 2. This kind of signal is mainly mediated by triggering of CD28 on T cells by CD80 and CD86 that are expressed by DCs after ligation of PRRs from both pathogens or inflammatory tissue factors [80]. In the absence of this co-stimulatory signal 2, naïve T cells become anergic, which might lead to tolerance. Instead, TCR stimulation and co-stimulation allow naïve T cells to develop into protective effector cells, normally accompanied by high level expression of selective set of cytokines. The balance of the cytokines and the resulting class of immune response strongly depend on the conditions under which DCs are primed for the expression of the T cell-polarizing signal 3 [81].

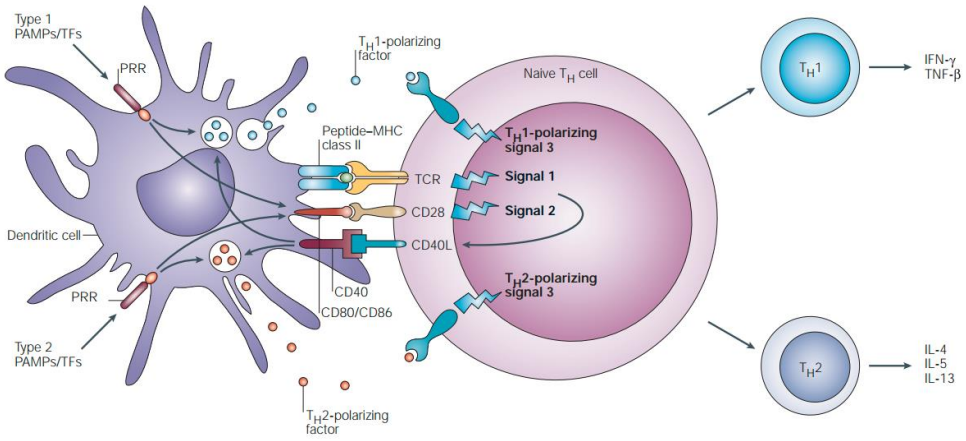

Figure 6: T cell stimulation and T_H1/T_H2 polarization require three DCs-derived signals [82].

Signal 1 is the antigen-specific signal that is mediated by binding between TCRs and MHC-associated peptides. Signal 2 is the co-stimulatory signal, mediated by triggering of CD28 by CD80 and CD86 expressed by DCs. Signal 3 is the polarizing signal that is mediated by various soluble or membrane-bound factors that promote the differentiation of T_H1 or T_H2 cells, respectively.

The way in which DCs bias the development of T_H-cell subsets and, consequently, the expression profile of T_H-cell polarizing factors both depend on the way in which DCs are activated. Therefore, the conditions of activation and degree of maturation of the immature DCs are crucial for the way in which the three signals are delivered to the T cells.

Although both mouse and human DC subsets might vary in their abilities to recognize the various pathogens and tissue injuries, and although they might differ in their T cell

polarizing abilities in the absence of pathogens, they are flexible and can adopt mature T_H1/T_H2 or regulatory T cell-inducing abilities, instructed by the priming signals from microbial and tissue-derived factors.

Over the years there are about three networks of inflammatory messengers discovered to be associated with the development of effector T_H subsets. A first network is associated with the development of T_H1 cells and can be activated by viruses or intracellular bacteria. The responses to bacteria and viruses are dominated by TLRs, such as TLR3, TLR4, TLR7 or TLR9 [83-84]. This signals mediate the production of DC-derived T_H1-polarizing factors such as the interleukin-12 (IL-12) family members, IL-23 and IL-27, type I interferons (IFNs) and cell-surface expressed intercellular adhesion molecule 1 (ICAM 1) [85-86]. This network promotes the development and function of T_H1 cells and cytotoxic T lymphocytes (CTLs).

A second network of factors is associated with infection with extracellular worms and parasitic helminths, and the development of T_H2 cells. T_H2 cell-polarizing factors are the monocytic chemotactic protein 1 (MCP1, also known as CCL2) or OX40 ligand (OX40L). These signals stimulate the T_H2 effector cells-mediated production of high levels of IL-4, IL-13 and IL-5, cytokines that are important in the defense against helminths [87].

Finally, a third network results in the downregulation of immunity and the induction of tolerance that is associated with the production of interleukin-10 (IL-10) and TGF-β and the development of Treg cells [88-89].

1.3 Nuclear Factor of Activated T cells (NFAT)

Nuclear factor of activated T cells (NFAT) was identified almost thirty years ago in nuclear extracts of activated T cells as an inducible nuclear factor that could bind the interleukin-2 (IL-2) promoter [90]. Since the discoveries of the first NFAT protein, the NFAT family has grown to include five members. The NFAT family comprises the NFATc members, which are regulated by calcium signaling and includes NFAT1, NFAT2, NFAT3 and NFAT4 (also known as NFATc2, NFATc1, NFATc4, NFATc3, respectively), and the NFAT5 member, which is the most ancient form and is instead regulated by osmotic stress (Table 2) [91-92]. When all the NFAT family has been isolated and characterized, it became clear that their expression and function is not limited to T cells; instead, it has been discovered that at least one NFAT family member is expressed by almost every cell type that has been examined, including other cells of the immune system and non immune cells [93]. Many studies have uncovered crucial regulatory roles in numerous developmental programs in vertebrates, including those of the heart and blood vessels, skeletal muscle, bone, neurons, kidney, pancreas and skin [94-95-96].

However, the NFAT proteins function and regulation are best understood in the immune system and it is now clear that NFAT regulates not only T cell activation and differentiation, but also the function of other immune cells, such as DCs, B cells and macrophages [97-98].

Finally, it has recently demonstrated that a dysregulation of NFAT signaling can be associated with malignant transformation and development of cancer [99].

Table 2: NFAT family of transcription factors [100]

TonEBP; tonicity-responsive enhancer-binding protein

Table 1 | **NFAT family of transcription factors**

NFAT family member	Alternative names	Regulation	Expression in the immune system
NFAT1	NFATc2 and NFATp	Calcium–calcineurin	Yes
NFAT2	NFATc1 and NFATc	Calcium–calcineurin	Yes
NFAT3	NFATc4	Calcium–calcineurin	No
NFAT4	NFATc3 and NFATx	Calcium–calcineurin	Yes
NFAT5	TonEBP and OREBP	Osmotic stress	Yes

1.3.1 NFAT structure and regulation

The NFAT family is evolutionarily related to the REL-nuclear factor-κB (REL-NF-κB) family of transcription factors [101]. Four members of the NFATc family, NFAT1-4, are regulated by the Ca^{2+} / calcineurin signaling pathway. Each protein has two or more alternatively spliced forms, which results in variation at the amino (N) and carboxyl (C) termini with the core region being conserved [102].

The NFAT proteins contain an amino-terminal transactivation domain (TAD), a regulatory domain known as the NFAT homology region (NHR), a highly conserved DNA-binding domain known as the Rel-homology domain (RHD), and a carboxyl-terminal domain (Figure 7a). The regulatory domain is moderately conserved among NFAT proteins and contains multiple serine-rich regions (SRRs) that are phosphorylated by different kinases. Of the fourteen phosphorylation sites that have been mapped in NFAT proteins, all but one are dephosphorylated by calcineurin and are located in three motifs; the serine-rich region 1 (SRR1) motif and the SPXX (where X indicates any amino acids) repeat motifs, that are SP2 and SP3. The regulatory domain also contains the docking sites for calcineurin and for the kinases, which control NFAT activation by regulating the phosphorylation status of the

SRR1 region [103]. The RHD shares structural homology with REL proteins and it is the unifying characteristic of NFAT proteins. RHD contains the DNA-binding loop that confers base-specific recognition, while the C-terminal domain makes contact only with the phosphate backbone of DNA [104].

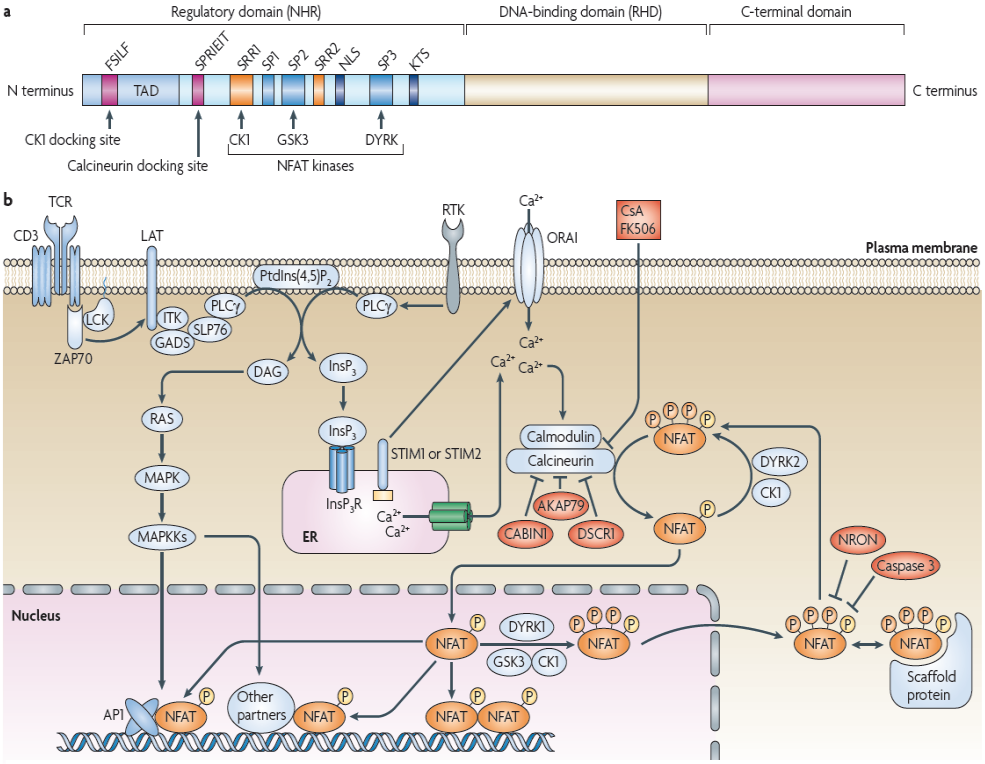

Figure 7: The Ca^{2+}-NFAT signaling pathway [105].

(A) General structure of NFAT transcription factors. NFAT proteins consist of an amino-terminal regulatory domain (NHR), a DNA-binding domain (RHD) and a carboxyl-terminal domain. (B) NFAT activation and regulation. Immunoreceptors bind to their ligands and activate phospholipase Cγ (PLCγ), which in turn leads to the production of inositol-1,4,5-triphosphate ($InsP_3$). $InsP_3$ induces the efflux of calcium

from the endoplasmic reticulum. The emptiness of intracellular stores induces in turn the activation of calcium-release-activated channels (CRAC) on the plasma membrane, leading to the intracellular Ca^{2+} increase. Ca^{2+} binds to calmodulin, which in turn activates calcineurin. Calcineurin dephosphorylates NFAT, allowing its nuclear translocation. Once into the nucleus NFAT collaborates with other partner proteins in order to regulate gene expression. Finally, NFAT proteins are rephopshorylated and inactivated by multiple kinases.

The activation of the NFAT proteins is induced by the engagement of immunoreceptors that are coupled to the calcium-signaling pathway, such as the antigen receptors that are expressed by T and B cells (Figure 7b). Receptor ligation leads to the activation of phospholipase Cγ (PLCγ), which hydrolyses the phosphatidylinositol-4,5-bisphopshate to produce inositol-1,4,5-triphosphate ($InsP_3$) and diacylglycerol. $InsP_3$ induces then the release of calcium from intracellular stores. This event triggers the opening of calcium-release-activated calcium (CRAC) channels on the plasma membrane, leading to a substained increase of intracellular calcium [106]. Calcium binds to calmodulin, which in turn activates the calmodulin-dependent phosphatase calcineurin. The activity of calcineurin is also controlled by several calcineurin inhibitors, which include the calcineurin-binding protein 1 (CABIN1), the A-kinase anchor protein (AKAP79) and calcipressin [107-108]. Interactions between NFAT and calcineurin occur at a specific motif in the N terminus of NFAT, which has the consensus

sequence PXIXIT (X indicates any amino acid) [109]. The NFAT proteins are dephosphorylated at the SRRs present in the regulatory domain by activated calcineurin. The dephosphorylation of these motifs leads to exposure of the NFAT nuclear-localization signal and to nuclear import. Once into the nucleus, NFAT proteins collaborate with other transcriptional partners, including AP1, forkhead box P-family protein (such as FOXP3) and proteins of the GATA family, to initiate and maintain specific transcriptional programs that vary with cell type and the pattern of stimulation [110].

The activity of NFAT is also controlled by several kinases, such as glycogen-synthase kinase 3 (GSK3), casein-kinase 1 (CK1), p38 and JUN N-terminal kinase (JNK), that have been reported to phosphorylate the SRRs and to control NFAT nuclear shuttling. The several kinases are divided between maintenance kinases, which act in the cytosol to keep NFAT in a fully phosphorylated state and prevent its translocation into the nucleus in resting cells, and export kinases, which re-phosphorylate NFAT in the nucleus and promote its nuclear export. For instance, CK1 acts both as maintenance and an export kinase for SRR1, whereas GSK3 functions as an export kinase for the SP2 motif of NFATc2 and for both the SP2 and SP3 motifs of NFATc1 [111-112].

Finally, the member NFATc1 (NFAT2) is uniquely regulated by a positive autoregulatory loop at the

transcriptional level among the five NFAT family members. NFATc1 can exist in three different isoforms, NFATc1A, NFATc1B and NFATc1C, and the mechanism of this loop is isoform specific. Although the B and C isoforms are constitutively expressed in the cells, the shorter isoform A is under the control of an NFAT-dependent inducible promoter. Only when NFATc1 expression levels are sufficiently high, the self-sustaining positive autoregulatory loop function to maintain high levels of expression [113].

1.3.2 NFAT functions in the immune system

The high degree of similarity among the Rel-homology domain of the NFAT family confers common DNA-binding specificities and interaction partners among the members, which might explain the redundancy in some NFAT-regulated functions. Gene-knockout mice that lack individual NFAT proteins show only mild alterations in immune functions, and it is only when more than one family member is eliminated that severe changes in many cells and functions of the immune system become apparent. Cell-specific differences in expression levels and distinct mechanisms of regulation might explain why certain functions are specifically regulated by different NFAT family members, as indicated by the phenotypes of NFAT-deficient mice [114-115-116].

The NFAT functions are firstly recognize in adaptive immunity, in particular T cells. However, recent studies have now highlight new roles for NFAT members in B cells and also in innate immune cells, such as DCs, macrophages and mast cells.

NFAT proteins functions in T cell:

The NFAT proteins have many roles and account for different aspects of T cells biology, such as the regulation of T cell development, T cell activation, T_H cell differentiation, and T cell tolerance.

T cell development:

In the thymus, immature precursors that are generated in the bone marrow differentiate into mature T cells. This process involves double-negative (DN) T cell precursors that do not express neither CD4 or CD8; DN precursors then rearrange the TCR and become double-positive (DP) cells that express both CD4 and CD8. DP cells undergo positive and negative selection to successfully generate single-positive (SP) T cells that express either CD4 or CD8.

Several studies have demonstrated that calcium and calcineurin signals are involved in the regulation of thymocyte proliferation and the development of immature

DN thymocytes into mature SP cells [117]. In early experiments, the inhibition of calcineurin activity with cyclosporine A led to an impaired DP-to-SP transition and defects in negative selection [118]. On the other hand, more recent experiments performed using mice with a selective gene knockout of the catalytic subunit of calcineurin in T cells showed a defect in the number of SP thymocytes, indicating the involvement of calcineurin in positive selection [119].

More direct evidence of the importance of NFAT proteins for thymocyte development has come from the analysis of NFAT-deficient mice. In particular, defects in T cell development in NFATc3$^{-/-}$ mice include a reduction in the number of SP cells, which correlates with an increase apoptosis of DP precursors [120]. The activation of NFATc3 could be also a crucial control in positive selection and SP cell survival.

T cell activation and T_H-cell differentiation:

The importance of NFAT proteins in T cell activation is underscored by genetic data. In two human families, the inability to activate NFAT proteins was associated with severe immunodeficiency [121], while deficiency in both NFATc2 and NFATc1 in mice is associated with impaired T cell activation. These data confirm that the activation of NFAT proteins is essential for T cells to carry out many of

their effector functions; moreover, analysis of gene expression has clearly shown that the activation-induced expression of most genes in T cells is blocked by calcineurin inhibitors. In particular, NFATc1 and NFATc2 functions in T cells are associated with the production of many cytokines, including IL-2, IL-4, IL-10, IFN-γ, granulocyte/macrophage colony-stimulating factor and TNF [122]. Another important aspect is that NFAT might regulate the cell cycle; NFATc2 downregulates cyclin-dependent kinase 4 (CDK4), inducing exit from the cell cycle and re-entry into G0. To this regard, NFATc2-deficient mice show defects in CDK4 downregulation and increased levels of several cyclins [123].

The NFAT proteins can also regulate gene expression inducing the transcription of lineage-specific genes that commit T cells to one or other lineage, in particular to T_H1 or T_H2 lineage. The evidence supports a model in which NFAT proteins act together with signal transducer and activator of transcription (STAT) factors to determine the T_H1/T_H2 lineage choice. In particular, STAT1 and STAT4 downstream of IL-12 and IFN-γ, respectively, for T_H1 cells; and STAT6 downstream of IL-4 for T_H2 cells. The synergistic action of these two families of transcription factors elicits the expression of lineage-specific family of transcription factors that lead to changes in chromatin structure. The net result is that the inappropriate locus is silenced so that NFAT proteins bind specifically to the

promoters of IFN-γ or IL-4 genes in T_H1 or T_H2 cells [124-125]. Whether individual NFAT proteins have selective roles in T_H1/T_H2 cell differentiation has been controversial, despite some studies indicate that NFATc1 is required for T_H2-cell differentiation, while NFATc2 and NFATc3 promote T_H1-cell differentiation.

T cell tolerance:

For many years, NFAT has been considered to be a key regulator of T cell activation through its interaction with proteins of the AP1 family. The discovery that NFAT proteins can also form transcriptional complexes with other partners has introduced the possibility of defining new roles for NFAT proteins in T cells.

TCR engagement in the absence of co-stimulatory signals leads to the activation of NFAT, but in the absence of the full induction of other pathways that imply a poor concomitant activation of AP1. This results in the initiation of a transcriptional program that culminates in T cell anergy [126]. NFAT proteins have been shown to be crucial for the induction of anergy-inducing genes in T cells. In the absence of AP1, it is possible that homo- or heterodimer complexes of NFAT proteins are responsible for the transcriptional activation of these genes, although interactions of NFAT with other transcription factors, such

as myocyte enhancer factor 2 (MEF2), might also be involved [127].

Recent studies have also documented a role for the NFAT proteins as transcriptional partners of FOXP3 in inducing Treg cell differentiation [128-129]. These works has provided clear biochemical and functional evidence for a cooperative interaction of NFAT and FOXP3. Moreover, they demonstrate that whereas NFAT-AP1 complexes induce IL-2 expression, NFAT-FOXP3 complexes inhibit this process, but induce the expression of two surface receptors expressed by Treg cells, cytotoxic T lymphocyte antigen 4 (CTLA4) and CD25 [130].

NFAT proteins functions in other immune cells:

NFAT was originally identified as a major transcriptional regulator in naïve and differentiated effector T cells. However, over the years it has also become apparent that NFAT transcription factors have important roles in various other cell of the haematopoietic system.

<u>B cells:</u>

Several recent studies have shown that engagement of the B cell receptor (BCR) and MHC class II on B cells can activate NFAT and induce NFAT-dependent gene transcription [131].

A further study showed that Ca^{2+}-NFAT signaling has an important role in B-1a cell development. B-1a cells are a phenotipically and functionally distinct population of B cells that are long-lived and express CD5, CD43 and high levels of surface IgM, but low levels of CD45 and surface IgD. This study demonstrated that both splenic and peritoneal B-1a cells are essentially absent in NFATc1-deficient mice [132]. Another study showed that the number of B-1a cells are markedly reduced in mice with B cell-specific deletion of the regulatory b1 subunit of calcineurin (Cnb1). These mice also reported reduced plasma cell development and antibody production; moreover, B cells presented a proliferation defect downstream of the BCR [133].

Innate immune cells:

Besides the NFAT role in the cells of adaptive immune system, several studies have shown that exposure to inflammatory stimuli induces the activation of NFAT family also in innate immune cells, in particular in classical DCs, neutrophils, mast cells, natural killer T (NKT) cells and, under pathological circumstances, macrophages. Since NFAT members appeared only in vertebrates, it has been proposed that the appearance of the NFATc family also in the cells of innate immunity may have contributed to the adaptation process required for the transition to a higher

level of complexity of immune responses in vertebrate [134].

Over the years, two signal transduction pathways, the one initiated by Dectin-1 and the one initiated by CD14, have principally been characterized for their ability to induce NFATc activation in innate immune cells.

The first evidence for NFATc activation in innate immune cells came from a study demonstrating that dectin-1 (also known as CLEC-7A) signaling modulates gene expression by the activation of NFAT. This study showed that after stimulation by *C.albicans* and zymosan, dectin-1 mediated NFAT activation regulated the expression of IL-2, IL-10 and IL-12 in DCs [97]. Dectin-1 is a C-type lectin receptor with an intracellular immune-receptor tyrosine-based activation motif (ITAM) that has been shown to have a crucial role in the detection of zymosan and pathogenic fungi by macrophages and DCs. It has been demonstrated that upon ligand binding, the dectin-1 ITAM motif is phosphorylated by Src family kinase (SFK) members, leading to the recruitment of the tyrosine kinase Syk. Syk then activates PLC-γ2, that lead to the release of $InsP_3$, to the increase of cytoplasmic calcium levels and, finally, to the activation of NFATc members [135-136].

In inflammation driven by β-glucan bearing fungi, the NFATc pathway activated downstream of C-type lectin receptors, such as Dectin-1, has the primary function of producing cytokines. In particular, it has been discovered

that neutrophils have the ability to produce IL-10 in a NFATc-dependent manner in response to *C.albicans* in vitro, whereas their strictly innate functions, such as phagocytosis, ROS and NO production, are NFAT-independent. To this regard, it has been proposed that neutrophil-derived IL-10 could play an anti-inflammatory role preventing deleterious inflammatory response and excessive tissue damage [137].

Besides DCs and neutrophils, recent studies have also been reported a role for NFAT transcription factors for mast cells function and survival. Mast cells are resident in many different tissues and have a central role in the development of allergic diseases. It has been reported that NFATc2 is a major transcriptional regulator of IL-13 in mast cells [138]. Another study also showed that both NFATc1 and NFATc2 regulate IL-13 and TNF expression in mast cells, while degranulation and IL-6 expression are independent of NFAT activity [139]. More recent studies have also demonstrated that the Ca^{2+}-NFAT signaling pathway also regulates the expression of hypoxia-inducible factor 1α (HIF1α) and the expression of protein A1, which is fundamental for mast cells survival after stimulation with the high affinity Fc receptor for IgE (FcεRI) [140-141].

A recent study showed that calcineurin-NFAT signaling is essential for the development of another class of cells, the NKT cells. NKT cells are a distinct subset of lymphocytes

that co-express the TCR αβ and markers of the NK cell lineage. These cells constitute a crucial first line of defence against infectious agents and against tumors. It has been demonstrated that selective deletion of Cnb1 in the thymus resulted in significantly compromised NKT cell populations. The study showed that the genes that encodes the transcription factor early growth response 2 (EGR2) is specifically required for the development of mouse NKT cells and that EGR2 is a target of NFAT [142]. Finally, the role of NFAT signaling pathway in macrophages is more controversial; NFAT seems to has inflammatory effect that leads to immunopathological consequences. Several studies have reported that NFAT members are negatively regulated in macrophages during acute inflammatory responses. For instance, the leucine-rich repeat kinase 2 (LRRK2) has been identified as a negative regulator of NFAT activation in macrophages [143]. LRRK2 and the non-coding RNA repressor of nuclear factor of activated T cells (NRON) complex have the abilty to direct inhibit NFATc nuclear translocation [144]. Any deregulation of NFAT activation, such as LRRK2 deficiency, leads to the NFAT hyper-activation in macrophages which contribute to the development and/or maintenance of a chronic inflammatory state. In agreement with this studies, LRRK2-deficient mice are more susceptible to the development of severe colitis, a phenomenon that is reversed by the inhibition of

calcineurin and NFAT traslocation, and NFATc hyper-activation in macrophages has also been found in other chronic disorders, such as rheumatoid arthritis [145].

1.3.3 The CD14-NFAT pathway and its role in DCs

Recently, it has been described a novel signal transduction pathway induced by smooth LPS that exclusively relies on CD14 for activation of the transcription factor NFAT in DCs [146]. The lipopolysaccharide (LPS) is a highly expressed component of Gram-negative bacteria outer membrane. The general structure of LPS is conserved and is composed of three separate regions, the lipid A mojety, which is highly hydrophobic and is largely responsible for the endotoxic activity of the whole LPS, the core and the O-chain [147]. LPS induces assembly of the ligand-binding complex consisting of the TLR4, MD-2 and CD14 on the plasma membrane [15]. CD14 is a 55-kDa glycoprotein expressed on the surface of myelomonocytic cells as a glycosylphosphatidylinositol (GPI)-anchored receptor or secreted in a soluble form [148]. CD14 is well known as a co-receptor that assists TLR4 in its signaling functions. This molecule is required for LPS presentation to TLR4, allowing cellular responses to low-doses of LPS [149].

Several studies have demonstrated that CD14 is absolutely required for a full response to LPS [150].

Moreover, Granucci and co-workers [146] provided a definitive demonstration that CD14 can act independently of TLRs as a transducing receptor (Figure 8). CD14 is responsible for the induction of a rapid and transient influx of Ca^{2+} ions in LPS-stimulated DCs and the consequent increase in the cytosolic Ca^{2+} levels triggers the activation of calcineurin and NFAT. In this regard, the experiments performed on DCs derived from TLR4, MD-2, MyD88 and TRIF-deficient mice clearly revealed that activation of NFAT by LPS occurs without contribution of TLR4 or other TLRs, but that instead CD14 is necessary. Although the exact molecular mechanisms through which CD14 induces the Ca^{2+} influx remains to be fully elucidated, it is known that the GPI-anchor of CD14 and its location in lipid rafts on the plasma membrane of DCs are strictly required for NFATc activation. Indeed, CD14 appears to signal NFAT activation through its GPI-anchor, rather than by presenting LPS to an unknown transmembrane protein. Soluble CD14 is able to restore sensitivity to low doses of LPS in CD14-deficient DCs only in terms of pro-inflammatory cytokine production, but not in terms of NFATc activation. Furthermore, disruption of lipid raft integrity using a cholesterol-depleting agent abolishes the ability of wild-type DCs to induce NFAT activation after LPS exposure. According to the findings on another GPI-

anchored protein CD59 [151], these results suggest that LPS-induced CD14 clustering on the plasma membrane of DCs promotes the aggregation and consequent activation of other lipid raft-associated signaling proteins, such as SFKs (possibly Lyn), which in turn activates PLC-γ2. PLC-γ2 then acts by hydrolyzing its substrate into DAG and $InsP_3$, which in turn triggers the opening of ion channel receptors on the cell surface, resulting in a single wave of extracellular calcium influx that ultimately promotes calcineurin activation and dephosphorylation of NFAT. The mechanism through which Ca^{2+} increase is obtained upon LPS stimulation in DCs deserves further investigation. Calcineurin activation in T cells is typically operated by a two-step Ca^{2+} mobilization system that involves the opening of specific ion channel receptors localized on the endoplasmic reticulum and then an additional opening of ion channels on the plasma membrane (see 1.3.1). In the case of DCs, LPS induces instead a single and transient influx of intracellular Ca^{2+}, with no contribution from intracellular Ca^{2+} stores, which is sufficient to activate NFAT. This data suggest that $InsP_3$ release by PLC-γ2 may directly trigger the activation of functional plasma membrane IP_3Rs, as has already been observed in B cells [152].

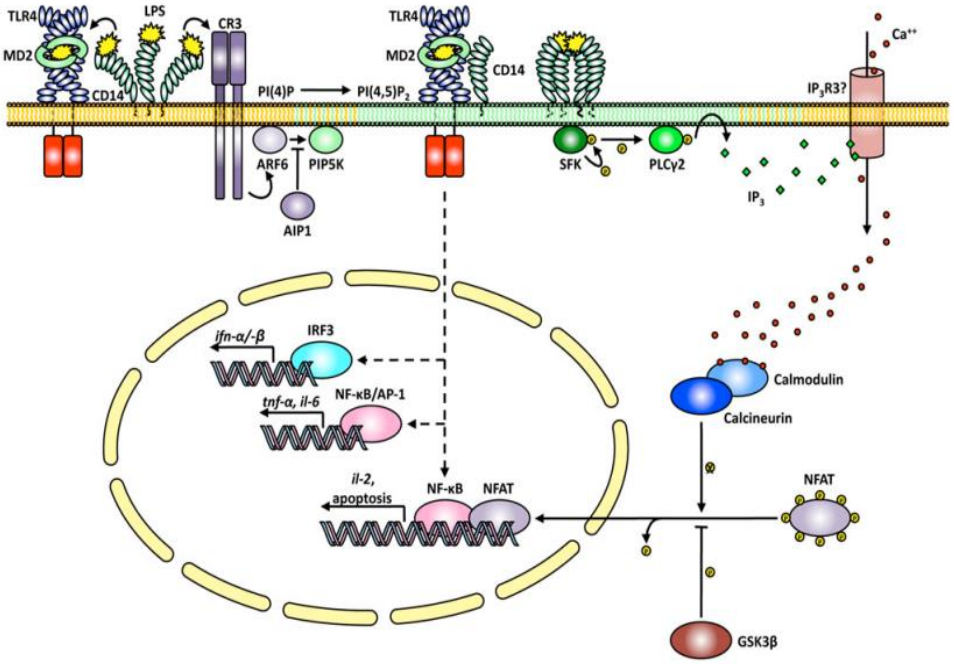

Figure 8: CD14-dependent NFAT activation in DCs.

In addition to its role in LPS recognition and presentation to TLR4, CD14 has autonomous signaling functions. Upon LPS-induced clusterization, CD14 recruits and activates a SFK members which, in turn, activates PLC-γ2. Subsequently, PLC-γ2 catalyzes the release of DAG and $InsP_3$, that directly tiggers a single wave of Ca^{2+} influx through plasma membrane. The increase of intracellular Ca^{2+} levels then triggers the activation of calcineurin and the dephosphorylation of NFAT, leading to its nuclear translocation.

Once into the nucleus, NFATc in DCs could exert both pro- and anti-inflammatory effects.

A recent study on the role of NFATc in classical DCs has evidenced its fundamental role in edema formation through generation of prostaglandin E2 (PGE2) [153], a

member of prostanoids (see 1.1.2). One of the first events occurring during inflammation is indeed local swelling, which lead to the edema formation, fundamental to favor antigen transport to the draining lymph nodes. Antigens arrival to the draining lymph nodes is mediated by two successive waves; in the first one antigens freely diffuse through lymphatic vessels and in the second are delivered by DCs. Edema formation mediates an increase in the interstitial pressure at the inflamed tissues, forcing the fluid into lymphatic vessels and favoring the entry of free antigen into the lymphatic and their arrival to draining LNs. The study demonstrated that DCs are the major regulators of edema formation in an in vivo model of LPS-induced inflammation and that DCs exert this function through their ability to activate the CD14-NFAT pathway. Moreover, the study revelead that NFAT in DCs controls the transcription of Ptges-1 that encodes mPGES-1, an enzyme which coordinates the synthesis of PGE2 together with cPLA2 and COX-2, leading to local swelling formation.

The inflammatory process is beneficial only if tightly regulated to avoid host tissue damaged by the inflammatory mediators. The immune system has adopted different solutions to maximize protection and limit damage of host tissues. One of them is the rapidity of activation of innate immunity combined with a rapid shutdown of the immune responses in order to allow tissue regeneration [5]. It has also been demonstrated that

NFATc signaling pathway participate in the control of switching off events. In this regard, the study of Granucci and co-workers [146] also showed that, at later time points, NFAT members (in particular NFATc2 and NFATc3) promote the expression of several genes with a pro-apoptotic function that induces DCs death. Among these genes, Nur77 was principally identified as an NFAT-dependent regulator of DCs apoptosis. In this study, it has also reported that macrophages, which do not undergo LPS-induced apoptosis, do not show a Ca^{2+} response or activation of NFAT after LPS exposure.

Finally, there is evidence that the activation of NFATc signaling pathway in DCs is important for the crosstalk between innate and adaptive immune cells and that may be fundamental for T cell activation. DCs have indeed the peculiar ability to decide whether and how a T cell response should be activated or suppressed; several studies have reported the fundamental role of IL-2 expression by DCs in this process. Using microarray gene expression analysis, it has been demonstrated that murine DCs are able to express IL-2 after stimulation with LPS and that DCs-produced IL-2 results as a key cytokine for T cell activation [154]. Similar results have been obtained in humans. Bielekova and colleagues demonstrated that activatory DCs are able to produce both IL-2 and the α-chain of IL-2 receptor, CD25, in the first few hours after interaction with T cells. Furthermore, the selective

blockade of CD25 by daclizumab, a humanized monoclonal antibody currently used in clinic, inhibits T cell activation [155]. More importantly, this study showed that DCs are able to trans-present IL-2 to T cells at the immunological synapse using CD25 expressed on their surface and that this complex represents a very efficient system for T cell priming in vitro. Since NFATc in DCs controls the expression of both IL-2 and CD25, it is clear its fundamental importance in T cell priming and activation. In this regard, the NFAT pathway may also have a role in DC-mediated peripheral T cell tolerance. Several studies have also reported that DC-derived IL-2 produced in inflamed pancreatic LNs is required for the differentiation of Treg cells in autoimmune-resistant NOD congenic mice [156] and for the deletion of autoreactive $CD8^+$ T cells in diabetic mice [157].

1.4 Transplant rejection

Transplantation has emerged as a viable therapeutic modality for the treatment of a variety of ailments and diseases and it has been an effective remedy for failing organs. However, organ transplantation from a genetically disparate donor induces an immune response in the recipient by donor antigens.

Once graft rejection occurs, it can be classified into three type of rejection, either hyperacute, acute and chronic. Hyperacute rejection occurs usually within the first 24 hours after transplantation. It is caused by pre-existing host antibodies that bind to antigens present in the graft endothelium, resulting in the activation of the complement system. On the other hand, acute rejection usually begin after the first week of transplantation and it is caused by mismatched MHC complexes that are present on all cells. Finally, chronic rejection is a third type of rejection and occurs months to years following transplantation. It is probably caused by multiple factors including allo-antibodies production and lymphocyte activation.

In particular, acute rejection of allografts is the result of a complex series of interactions involving coordination between both the innate and adaptive immune system with T cells being central to this process. The cascade of acute rejection begins with the presentation of donor

alloantigens to recipient T cells through antigen presenting cells or by a direct mechanism.

Nowadays, acute allograft rejection is as an impediment to long-term allograft survival, increasing the risk of developing chronic rejection and decreasing allograft half-life. For these reasons, acute allograft rejection remains the major barrier to successful transplantation.

Thanks to the discovery of immunosuppressive drugs, such as cyclosporine A and tacrolimus, early graft loss due to acute rejection has considerably decreased. However, the use of immunosuppressive drugs has not reduced the rates of graft loss due to chronic rejection and perhaps it has increased the risk of developing serious complications, such as life-threating infections and cancer [158].

1.4.1 Mechanisms of transplant rejection

Innate immunity and transplant rejection

Transplantation involves physical procedures of removing and re-implanting tissues or organs, which lead to mechanical injury and stress responses, such as the ischemia-reperfusion injury during primary vascularized organ transplantation. These process result in changes in gene and protein expression and folding within the donor tissue that have a profound influence on the immunological response of the recipient [159]. Local tissue damage generates many potential DAMPs, including reactive oxygen species (ROS), heat shock proteins (HSP), heparin sulfate and HGMB1 that can bind PRRs expressed on the surface of innate immune cells. For instance, the increasing production of ROS due to the ischemic injury leads to activation of caspases, finally resulting in cell apoptosis [160]. In addition, ROS induce activation of chaperoning proteins usually secreted from stressed or damaged cells, such as HSP70 and HGMB1, which are ligands of TLRs. It has been demonstrated that these proteins are able to bind TLR4 or TLR2, resulting in activation of immature DCs and /or vascular endothelium [161]. Oxidative injury also facilitates signaling through adaptor molecules, such as MyD88 and TRIF, which have shown to play a role during acute allograft rejection. In this regard, studies using MyD88- or TRIF-deficient allografts

demonstrate impaired donor-derived DCs migration and less graft cell damage [162].

DAMPs can also activate the complement cascade through the alternative or lectin pathways. Activated complement components constitute a proteolytic cascade present in the plasma that generates a range of effector molecules that can damage the graft in their own right, target the graft for destruction by phagocytes, facilitate antigen presentation and T cell activation and promote vasodilation and chemotaxis [163].

In conclusion, the sensing of DAMPs by PRRs result in the production of inflammatory mediators and pro-inflammatory cytokines such as IL-1, IL-6, TNFα and type I IFNs. These events identify the transplant as a site of injury and inflammation modifying the permeability of endothelial cells nearby the transplant and triggering the release of soluble molecules, in particular antigens from the graft. Most importantly, the pro-inflammatory environment of the transplant stimulates the migration of donor-derived antigen presenting cells, in particular DCs,

from the transplant to recipient lymphoid tissues (Figure 9).

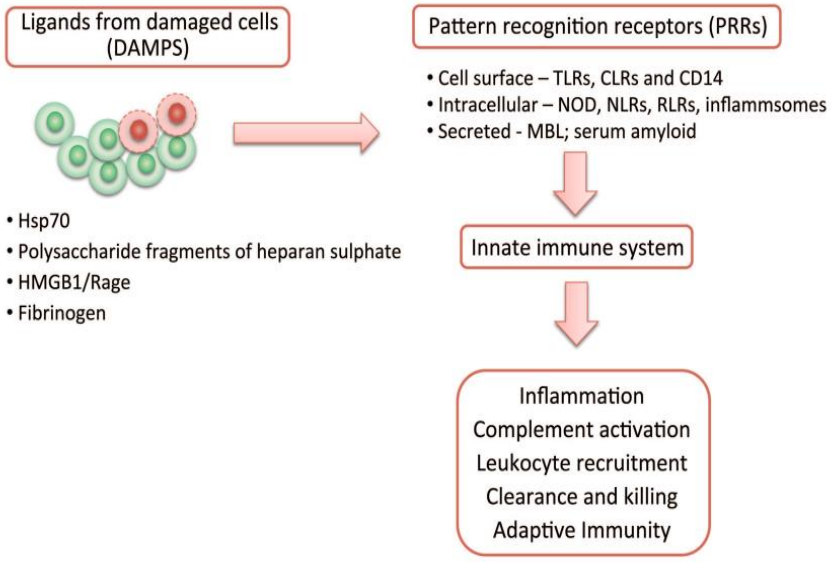

Figure 9: Innate immunity sets the scene for rejection [164]

Transplantation process cause injury to the graft cells, which start to produce several DAMPs. These molecules are then recognized by PRRs expressed by innate immune cells triggering their activation. The activated leukocytes will rapidly release inflammatory mediators, which recruits other leukocytes and mediate the migration of donor-derived APCs to the LNs. DAMPs can also activate the complement cascade resulting in the release of vasoactive molecules and causing further damage to the graft.

Allorecognition and T cell activation

Antigens that activate the immune system against the allograft, named alloantigens, are both major and minor histocompatibility antigens.

The major histocompatibility complex (MHC), called human leukocyte antigen (HLA) in human beings, are polymorphic molecules responsible for eliciting the strongest of responses to allogeneic tissues. In humans, the HLA complex encompasses more than $3,5*10^6$ base pairs of DNA on the short arm of chromosome 6 and it encondes polymorphic cell-surface glycoproteins-class I molecules (HLA-A,B,C) and class II molecules (HLA-DP, DQ, DR) which determine the recognition of antigens by T lymphocyte [165]. HLA class I molecules are constitutively expressed by most cells and tissues, while HLA class II molecules are expressed only by B lymphocytes, macrophages, monocytes and dendritic cells [166].

One of the reasons why transplantation induces such a dynamic immune response is the high precursor frequency of T cells able to respond to non-self MHC molecules (about 10%). This high level of reactivity results from a combination of specific recognition of alloantigens or alloantigenic peptides by T cells and through cross-reactivity of T cell specific for other peptide-MHC complexes with alloantigen [167]. Although MHC molecules are the most important alloantigens for

triggering rejection, transplant between individuals carrying identical MHC molecules are still vulnerable to rejection. In these settings, rejection is the result of T cell recognition of other polymorphic non-MHC molecules called minor histocompatibility antigens (miH) [168].

miH are proteins that are expressed in some individuals in the population but not others, thereby creating potential antigenic differences between donors and recipients. In theory, a polymorphism of any protein between donor and recipient can potentially elicit an anti-graft response. Any non-MHC gene that encodes epitopes capable of binding MHC class I or class II and inducing T cell responses can be considered a miH. For instance, proteins encoded on the Y chromosome of males (H-Y) are able to induce an anti-Y responses in females after transplantation [169]. Recently, both $CD8^+$ and $CD4^+$ T cell specific for miH isolated from humans and rodents have been shown to play an important role in the rejection of solid organs as well as causing graft-versus-host disease after bone marrow transplantation [170-171].

Transplantation is a unique immunological situation in which priming of recipient T cells with antigen can occur by two distinct pathway, named direct and indirect allorecognition. The direct pathway of allorecognition describes the ability of recipient T cells to “directly” recognize intact non-self MHC molecules present on the surface of donor cells. In order for recipient T cells to

directly respond to allo-MHC molecules, cells from within the graft must migrate out to make direct contact with T cells in the LNs. In this regard, mounting evidence suggest that the structural similarity between certain MHC molecules is close enough to allow T cell receptor ligation and to trigger T cell activation. Alloreactive T cells are thought to recognize allogeneic MHC regardless of the peptide bound to it [172]. However, there is also evidence to support the concept that peptide binding facilitates T cell response. It is possible that recognition may occur regardless the peptide when donor MHC is structurally very different from recipient MHC, whereas if the donor MHC is similar to the recipient ones, recognition may occur through peptide/MHC complex [173]. In contrast to the direct pathway, the indirect allorecognition describes the ability of T cells to recognize donor antigens that are processed and presented as peptides by self-MHC molecules. Three mechanisms of antigen delivery can occur via this allorecognition pathway. First, antigens from the graft are released into circulation and engulfed by recipient DCs that reside within secondary lymphoid tissue. Second, donor cells can migrate to LNs where they are engulfed by recipient DCs and third, recipient APC can migrate into the graft, pick up antigens, and then migrate to the LNs [174]. However, because there are only a finite number of donor APC transferred within a transplanted organ, the role of the direct pathway in allograft rejection

diminishes with time. Importantly, the indirect pathway is available for antigen presentation for as long as the graft remains in situ, and therefore becomes the dominant pathway of long term allorecognition.

At the end, specific immune response to a graft occurs in two main stages. In the first, APC migrate to draining LNs and present donor antigens to recipient alloreactive T cells, which become activated, proliferate and differentiate. In the second stage, the effector T cells are recruited into the transplanted organ where they can mediate inflammation and elicit tissue destruction (Figure 10).

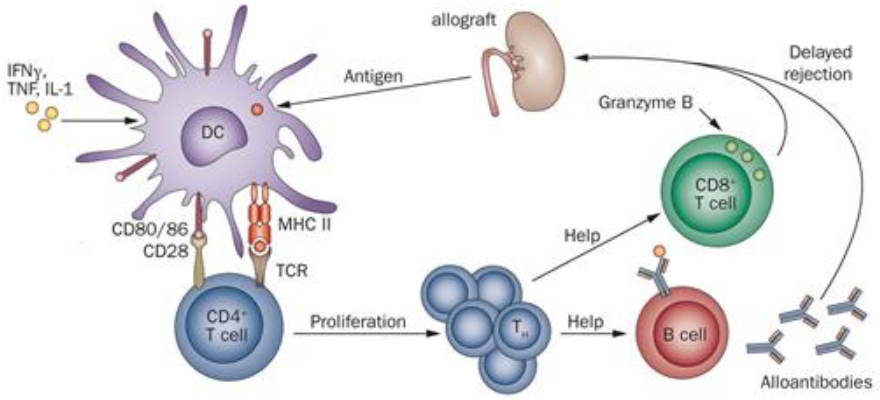

Figure 10: Mechanism of alloreactive T cell activation and graft rejection [175].

After transplantation, recipient T cells recognize donor alloantigens expressed by donor graft passenger DCs. Once activated, $CD4^+$ T cells initiate to proliferate and differentiate into Th effector cells which provide help to $CD8^+$ T cells and B cells. Effector mechanisms, such

as production of pro-inflammatory cytokines, antibodies and granzyme B induce then graft injury.

Several studies performed both in mice and humans have demonstrated that T cells are indispensable and the major responsible of allograft rejection. In this regards, animals that lack T cells are unable to reject fully mismatched transplants, whereas adoptive transfer of T cells to these animals is able to restore allograft rejection [176]. In clinical transplantation, therapies that deplete peripheral leukocytes , including T cells, are effective in preventing and reversing episodes of acute rejection and improve patient outcomes [177].

Once primed in the secondary lymphoid tissues, activated T cells can migrate into transplant tissue and mediate graft rejection. Some studies have demonstrated that $CD4^+$ T cells alone are sufficient to mediate complete graft destruction [178], while other studies have suggested that $CD8^+$ T cell activation is required to result in acute rejection [179]. However, tissue destruction occurs due to both direct $CD8^+$ T cell-mediated lysis of graft cells and $CD4^+$ T cell-mediated activation of accessory cells, such as B cells, which in turn produce alloantibody. In this regard, it has been demonstrated that activated $CD8^+$ cytotoxic T cells can migrate to the graft site where they are able to identify their target cells and to release granules containing cytotoxic molecules, such as perforin and granzyme B. Moreover, they are able to upregulate cell

surface expression of Fas ligand and secret soluble mediators such as TNFα [180]. On the other hand, alloantigen-specific $CD4^+$ T cells also express cytokines and co-stimulatory molecules that allow them to provide help for other cells, such as for B cell proliferation, differentiation, antibody class switching and affinity maturation. B cells produce then alloantibodies able to recognize both mismatched MHC molecules and miH and to activate complement cascade, allowing late graft loss. Moreover, B cells not only damage grafts by producing anti-graft antibodies, but they have recently been shown to infiltrate graft and present graft-derived antigens to alloreactive T cells via the indirect pathway of allorecognition [181].

Finally, after primary antigen exposure, long-lived antigen-specific memory T and B cells are generated that are able to deliver a more rapid and higher magnitude immune response, if the same antigen is encountered on a subsequent occasion. Although the generation of immunological memory is beneficial for protection against infectious pathogens, in transplantation the presence of allospecific memory produces an accelerated rejection response. In this context, clinical transplantation evidence of previous sensitization to donor antigens is associated with increased risk of acute rejection episodes and premature graft failure [182].

1.4.2 Therapies

The development of immunosuppressive strategies during the past four decades reflects enormous progress in understanding the cellular and molecular mechanisms that mediate allograft rejection. The immunosuppressive agents currently used in clinic are needed to prevent rejection of the transplanted organ, which may occur even though HLA matched donors are used. The immunosuppressive agents initially used in transplant for nearly two decades were corticosteroids, azathioprine and cyclosporine. However, several new agents have been introduced during the past few years, such as mycophenolate, tacrolimus and sirolimus. However, all or most of the immunosuppressive agents work blocking lymphocyte activation, maturation and proliferation.

Although the success of transplantation between unrelated donors and recipients can be attributed to the use of these agents, there is no method that will suppress the host's response to antigens of the graft and at the same time maintain other immune responses. Indeed, the immunosuppressive agents depress both innate and adaptive immunity, rendering the recipient more susceptible to infections and malignancy.

Inhibitors of inflammation or T cell proliferation

Corticosteroid and azathioprine remained the mainstay of immunosuppression for almost thirty years and they are still widely considered important components of most immunosuppressive regimens and are almost universally used as first-line treatment for acute allo-graft rejection.

Corticosteroids have a variety of anti-inflammatory and immunomodulatory effects; the two main corticosteroids used for the prevention of allograft rejection are prednisolone and prednisone. Corticosteroids effects include suppression of prostaglandin synthesis, reduction of histamine and bradykinin release and lowering of capillary permeability. Moreover, they are able to induce a decrease in the inflammatory response through reduced production of cytokines, such as IL-1, IL-2, IL-6 and TNFα. They also impaired macrophages function and decrease the number of circulating $CD4^+$ T cells [183]. However, the side effects of steroid treatment are numerous, including diabetogenesis, hypertension, adrenal suppression, osteoporosis and other problems.

Azathioprine is another chemical compound often used in combination with corticosteroids, which is able to halt DNA replication and block de novo pathway of purine synthesis. This latter effect confers specificity on lymphocytes, which lack a salvage pathway for purine synthesis. Recent evidence suggests that azathioprine also interferes with

CD28 co-stimulation of alloreactive T lymphocytes [184]. The principle side effect of this drug is dose-related bone marrow suppression, but it can also cause occasional liver impairment. More recently, mychophenolate mofetil is introduced in the treatment of graft rejection, since it has a similar but more effective mode of action to that of azathioprine.

On the other hand, sirolimus belongs to the group of immunosuppressive agents called mTOR inhibitors. This drug has the ability to bind the intracellular immunophilin FK506 binding protein, inhibiting in this way mTOR activation. Inhibition of mTOR pathway has a profound effect on the cell signaling pathway required for cell-cycle progression and cellular proliferation. The net effect is blockade of T cell activation by preventing progression of the cell cycle. However, sirolimus' side effects comprise metabolic, haemoatological and dermatological effects [185].

Monoclonal antibodies to lymphocyte and to cytokine receptors

Co-stimulatory blockade offers selective but long-lasting, graft-specific immunosuppression with the possibility of inducing tolerance. The first pathway targeted was the CD28 pathway. In order to do that, a soluble fusion protein was developed that consists of the extracellular binding

domain of CTLA4 fused with the Fc domain of human immunoglobulin (Ig)G1, creating Abatacept. Abatacept binds to both CD80 and CD86, blocking CD28 engagement and T cell activation [186]. However, although transplantation studies in rodents demonstrated efficacy, studies in nonhuman primates showed a low rate of efficacy and no induction of tolerance.

Targeting other co-stimulatory pathways, such as the CD154/CD40 pathway, are very appealing because of the potent ability to block T cell activation as well as antibody production. Initial studies in nonhuman primates demonstrated long-term kidney allograft survival using anti-CD154. However, anti-CD154 treatments in humans and nonhuman primates resulted in thromboembolic complication [187].

Prevention of graft rejection has also been approached by inhibiting cytokines from interacting with their receptors. For instance, chimeric or humanized murine anti-IL-2R α chain (CD25) antibodies (Daclizumab and Basiliximab) have been developed for clinical use [188]. The advantage of these monoclonal antibodies directed against CD25 is that such molecule is present only on activated T cell, which means that the main effect is on T cells possibly activated by graft antigens. More recently, it has also been demonstrated that Daclizumab is also able to block T cell activation binding the CD25 express on DCs surface [155]. However, these monoclonal antibodies are not useful

when the IL-2 production raised to high levels because cells are still activated by the low affinity IL-2R in the absence of CD25.

Calcineurin inhibitors

The main action of calcineurin inhibitors (cyclosporine A and tacrolimus) is that they prevent the synthesis of IL-2 and other cytokines that might activate alloreactive T cells. Through its hydrophobicity, cyclosporine A enters cell membranes to gain access to and bind the cytoplasmic isomerase protein cyclophilin. The complex then inhibits calcineurin, preventing NFAT activation and transcription of the IL-2 gene. In addition, it also blocks the synthesis of other cytokines and thereby interfers with activated $CD4^+$ T_H cell function. As a consequences, T cell proliferation and differentiation are blocked [189].

On the other hand, tacrolimus binds to a cytoplasmic isomerase protein in the same way of cyclosporine A, but it binds to the FK506 binding protein. The complex formed inhibits calcineurin, blocking T cell activation. Tacrolimus thus inhibits synthesis of IL-2, IL-3, IFN-γ and other cytokines and it was found to be more potent than cyclosporine A as an immunosuppressive agents [190].

Although calcineurin inhibitors are the most used and effective immunosuppressive agents, they are still associated with a range of side effects, many of which are

dose dependent and relate to the sites where calcineurin concentrations are highest, notably in the brain and the kidney. Besides nephrotoxicity and neurotoxicity, the side effects of these two agents include diabetogenesis and other metabolic effects.

1.5 Scope of the thesis

The family of nuclear factor of activated T cells (NFAT) consists of five proteins, with transcriptional activity, that can be subdivided into two groups according to their functional domains: the NFAT5, regulated by osmotic changes and the NFATc family regulated by calcineurin.

Although NFATc functions are well established in lymphocyte development, activation and tolerance, several studies have provided evidence for new roles of NFATc members in cells of the innate immune system, such as conventional dendritic cells (DCs) and macrophages. It has been proposed that NFATc member activation in innate immune cells might be implicated in the collaboration between innate and adaptive immunity.

DCs are professional antigen-presenting cells and are strong activators of primary T cell responses. It has been demonstrated that one of the reason of the high DC efficiency in T cell priming is the production of interleukin (IL)-2 after the exposure to inflammatory stimuli [154]. Recent works have proven that upregulation of CD25 and IL-2 production by DCs at the immune synapse are both fundamental to activate T cells [155]. Since IL-2 and CD25 are both controlled by NFATc members, we predict that the NFAT signalling pathway in DCs could play a

fundamental role in the activation of alloreactive T cell responses during acute transplant rejection.

In this context, the selective and specific inhibition of NFATc pathway in DCs could be crucial to block the activation of alloreactive T cells and to induce tolerance to the transplant. We, therefore, set up an experimental skin transplant model to test the role of the NFATc signalling pathway in DCs in alloreactive T cell priming.

We have generated a new NFAT inhibitor acting specifically in innate myeloid cells as a tool to study the contribution of the NFATc signalling pathway during acute transplant rejection.

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Chapter 2

Role of NFATc activation in innate immune cells in acute transplant rejection

Department of Biotechnology and Biosciences
University of Milano Bicocca

NFATc is consider an attractive target for the therapeutic modulation of immune responses in view of its importance for T cell-mediated immunity and for the control of different functions of innate immunity. In this context, inhibitors of calcineurin, such as cyclosporine A and tacrolimus, have been extensively used as immunosuppressive agents to improve graft survival and to treat autoimmune diseases [1-2]. Although highly successful, these inhibitors act by blocking calcineurin enzymatic activity and are therefore not selective NFATc inhibitors [3]. In fact, cyclosporine A and tacrolimus can affect the activity of any other target dephosphorylated by calcineurin, leading to nephrotoxicities and neurotoxicities [4-5]. Moreover, inhibition of NFATc using cyclosporine A and tacrolimus does not allow the specific study of the inhibition of this signaling pathway in innate immune cells.

The study of the role of the NFATc signaling pathway has also several problems due to the redundancy of the functions of NFATc members. Indeed, the NFATc family comprises four members that, in turn, present two or more alternatively spliced forms [6]. For instance, NFATc2 and NFATc3 are both involved in the regulation of apoptotic death in DCs [7] and present a redundant pro-apoptotic role even in T cells. On the other hand, NFATc1 has a non-redundant differentiation role in T cells [8]. Moreover, all the three members NFATc1, NFATc2 and NFATc3 are involved in IL-2 transcription in DCs.

For these reasons, the development of more specific inhibitors of NFAT function which allows the inhibition of all or different NFATc members in single or multiple immune cell types is required for the comprehension of the role of this pathway.

To do that, several studies concentrate on the identification of the docking-sites for the NFAT-calcineurin interaction in order to inhibit this process. Effective phosphate removal from NFAT by calcineurin requires its docking with the DNA-binding factor. Interactions between NFAT and calcineurin occur at a specific motif in the N terminus regulatory domain of NFAT, which has the consensus sequence Pro-X-Ile-X-Ile-Thr (PXIXIT), where X denotes any amino acid [9-10]. This motif is conserved among different NFAT family members and constitutes the main docking site for calcineurin on NFAT. A peptide with a high-affinity form of the PXIXIT sequence, Pro-Val-Ile-Val-Ile-Thr (VIVIT), was selected from a randomized peptide library. This peptide binds calcineurin with high affinity and effectively competes with NFAT for calcineurin binding, thus blocking NFAT dephosphorylation. Indeed, when expressed in cells as a green-fluorescent protein (GFP)-fusion protein, VIVIT also selectively blocked NFAT-dependent transcription [11]. A cell-permeable version of the VIVIT peptide has also successfully used to notably prolong graft survival in an experimental system of

islet-cell transplantation [12] and to ameliorate experimental colitis in mice [13].

However, the therapeutic use of peptide inhibitors is still limited by difficulties associated with delivery and product stability. The use of new delivery tools like nanoparticles (NPs) could help to overcome these problems. In fact, the main characteristics of NPs are to allow an efficient and specific drug delivery and to increase the in vivo half-life of the drug.

In collaboration with the Nanobiolab of the University of Milano-Bicocca, we have generated iron-oxide NPs conjugated with the VIVIT peptide (patent n°: PCT / IB2013/055943). In this way, VIVIT is protected from degradation and is delivered only to innate immune cells. Thanks to the use of NPs conjugated with VIVIT, we have been able to study the role of the NFATc signaling pathway in innate immune cells (Figure 1) and in particular in the context of acute transplant rejection.

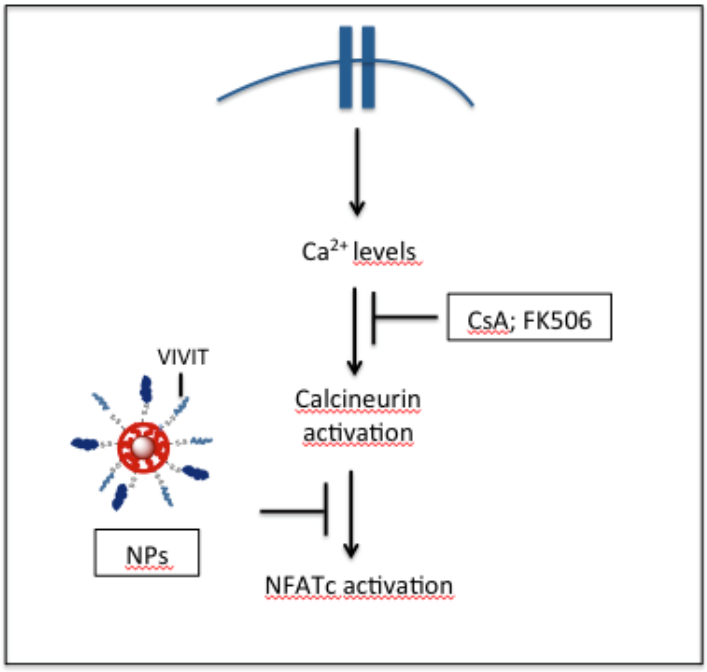


Figure 1: Generation of new specific NFAT inhibitor.

Cyclosporine A and tacrolimus are immunosuppressive drugs currently used in clinic able to block calcineurin activation. Iron-oxide NPs conjugated with the VIVIT peptide are also shown. The VIVIT peptide competes with calcineurin for the binding to NFAT, blocking its nuclear translocation.

2.1 Nanoparticles are only internalized by innate immune cells in vitro and in vivo

Commercially available iron oxide NPs coated with hydrophobic long-chain surfactants with uniform sizes ranging from 3 to 50nm were coated with an amphiphilic polymer, PMA, previously dissolved in chloroform [14]. Sodium borate buffer was added obtaining a clean NPs dispersion that was concentrated and reacted with 2,2-(ethylenedioxy) bis (ethylamine) (EDBE). After washing, the NPs were shaken for 4 hours in the presence of N-succinimidyl-3-[2-pyridyldithiol]-propionate (SPDP), concentrated and washed [15]. Then, VIVIT peptide and PEG-SH (500Da) were added and the mixture was shaken for 2 hours. Finally, the NPs suspension (MYTS) was concentrated and the final concentration determined by UV measurement (Figure 2).

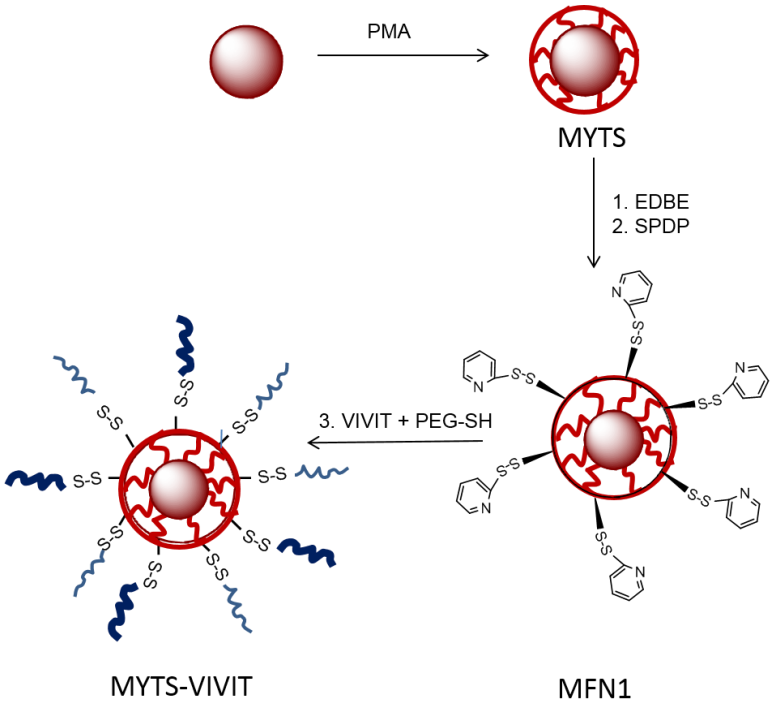


Figure 2: Nanoparticles synthesis

Commercially available iron oxide NPs were coated with an amphiphilic polymer, PMA. Then, VIVIT peptide and PEG-SH (500Da) were added through a disulfide bond. Finally, the NPs suspension (MYTS) was concentrated and the final concentration determined.

To investigate whether only innate immune cells were able to internalize MYTS NPs, we performed a kinetic analysis of the uptake of MYTS NPs both in vitro and in vivo. For in vitro studies, bone marrow-derived DCs (BMDCs) from wild-type (WT) mice were incubated at different time-points (10, 30, 60 and 90 minutes) with MYTS NPs at 37°C. To evaluate the uptake from BMDCs, we used MYTS-PEG NPs conjugated with the fluorocrome

fluorescein isothiocyanate (FITC) and MYTS-PEG NPs as control. Finally, results were analyzed by flow cytometry. BMDCs were able to internalize the MYTS-PEG FITC nanoparticles already after 10 minutes of incubation (Figure 3a). However, the NPs internalization increased over time, reaching a plateau after 90 minutes of incubation. Similar results were obtained repeating the in vitro uptake at 4°C to exclude the possibility that NPs could bind the BMDCs in a nonspecific way (Figure 3b).

Then, we evaluated NPs uptake after in vivo administration. To do that, MYTS-PEG FITC NPs or MYTS-PEG NPs as control were administered i.p. (100µg) to WT mice once or for two weeks every other day. Then, mice were euthanized and spleen and lymph nodes were recovered after administration and analyzed by flow cytometry. When injected in vivo, we observed that MYTS-PEG FITC NPs were only internalized by cells of the innate immune system (Figure 3c-3d) and in particular by phagocytes. We clearly observed that only DCs ($CD11c^+$), macrophages ($CD11b^+$) and neutrophils ($Ly6G^+$) were able to internalize the MYTS-PEG FITC NPs, whereas T ($CD4^+$) cells were not (Figure 4). Finally, NPs uptake was also evalulated in a non-lymphoid organ, the skin, after two weeks of i.p. treatment every other day. Skin was analyzed by flow cytometry after having obtained single cell suspensions. For the FACS analysis, cutaneous cells of hematopoietic and non-hematopoietic origin were

identified as $CD45^+$ and $CD45^-$, respectively. As shown in Figure 3e, NPs were only internalized by phagocytes (DCs and macrophages), even in the case of a non-lymphoid organ.

These results demonstrated that MYTS-PEG NPs are a useful tool to specific target innate myeloid cells, thanks to the phagocytic ability of these cells. On the contrary, cells of the adaptive immune system, like T and B cells, are not able to internalize the MYTS-PEG NPs. Given these results, MYTS-PEG NPs allow the study of NFATc signaling pathway only in innate immune cells and in particular in DCs.

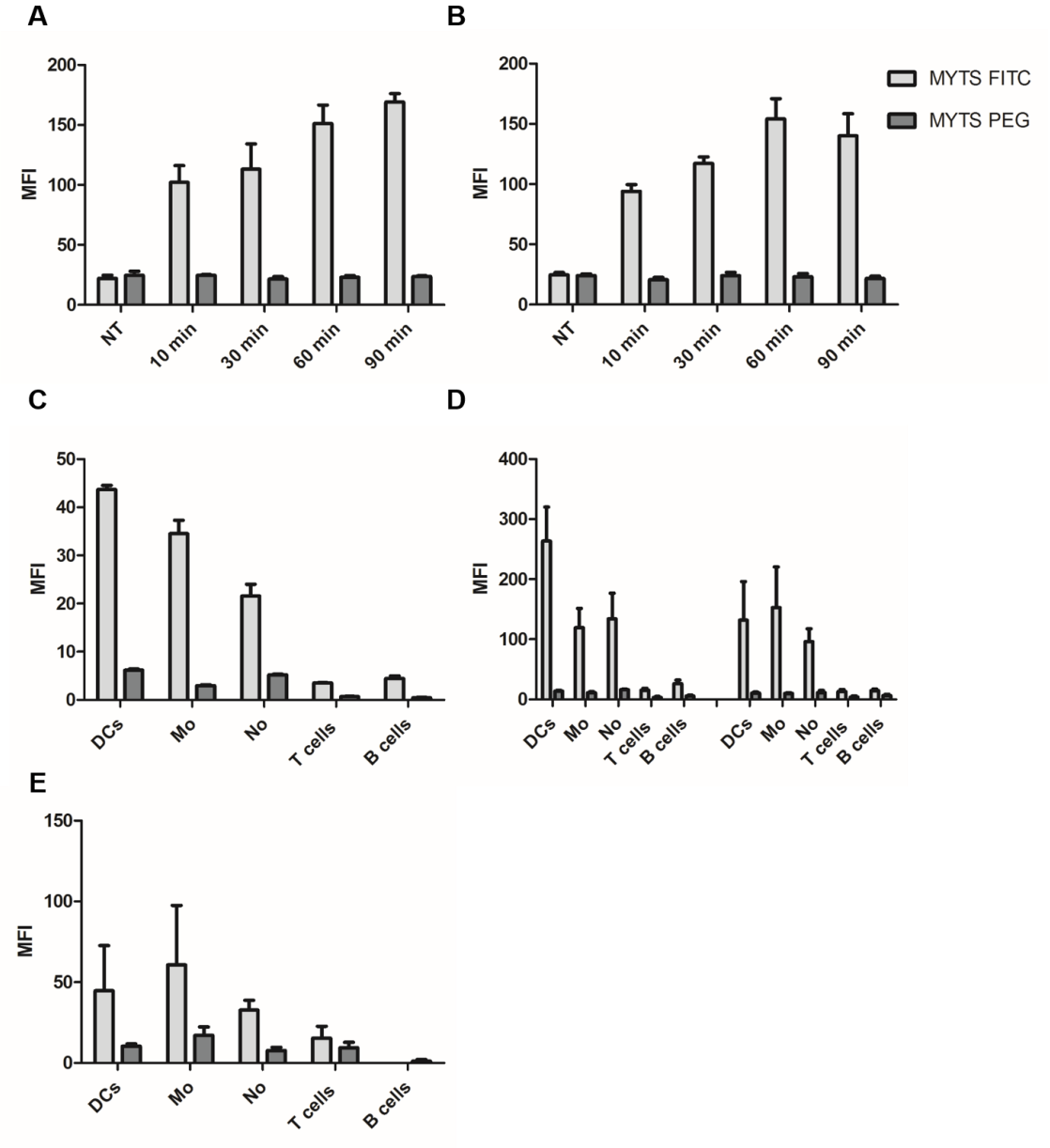


Figure 3: In vitro and in vivo nanoparticles uptake

BMDCs from WT mice were incubated at different time-points (10, 30, 60 and 90 minutes) with MYTS-PEG FITC (50ug/ml) NPs or MYTS-PEG (50ug/ml) NPs as control, at 37°C. (A) NPs uptake by BMDCs at 37°C and (B) 4°C. MYTS-PEG FITC or MYTS-PEG NPs were

administered i.p. (100ug) to WT mice once or every other day for two weeks. After 90 minutes spleen was recovered and analyzed by flow cytometry for the internalization of NPs. (C) NPs uptake by the indicated cell types in vivo. After two weeks, spleen and lymphnodes (D) or skin (E) were also recovered in order to evaluate NPs uptake. Values represent means of 2 indipendent experiments performed in triplicate + SD.

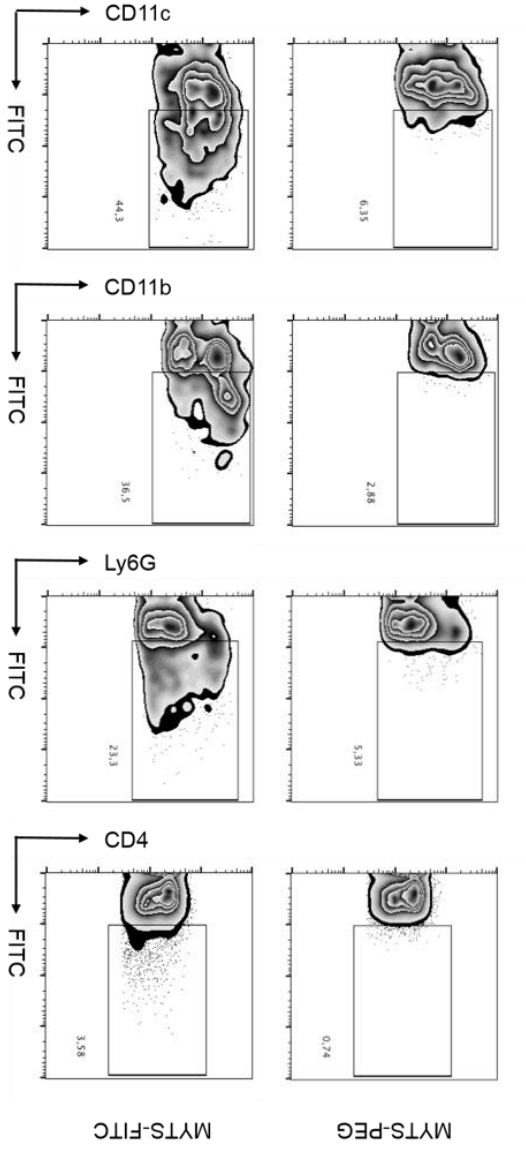


Figure 4: MYTS-PEG FITC or MYTS-PEG NPs were administered i.p. (100ug) to WT mice. After 90 minutes spleen was recovered and analyzed by flow cytometry for the internalization of nanoparticles. DCs: $CD11c^+$, macrophages: $CD11b^+$, neutrophils: $Ly6G^+$, T: $CD4^+$ cells.

2.2 NPs functionalized with VIVIT peptide are able to block NFATc translocation and IL-2 production

In order to test the efficacy of the NPs in blocking NFAT translocation, in vitro and in vivo experiments were performed.

After exposure to LPS, DCs can produce different pro-inflammatory cytokines, such as TNFα and IL-2. These cytokines are produced after activation and translocation of different transcription factors; in particular, TNFα production is dependent on the activation of the NFκ-B signaling pathway, whereas IL-2 production is strictly dependent on the activation of NFATc signaling pathway. Therefore, TNFα and IL-2 can be considered as markers for the activation of the NFκ-B and NFAT pathway, respectively. BMDCs from WT mice or a mouse DC-line, D1 [16], were pre-treated with different concentrations of MYTS-VIVIT NPs and then stimulated with LPS in order to induce cytokine production. After 18 hours, the amount of released cytokines was measured by ELISA. The results clearly showed that MYTS-VIVIT are able to block IL-2 production in a dose-dependent manner, while TNFα is not affected, demonstrating that MYTS-VIVIT NPs can inhibit the NFATc signaling pathway (Figure 5).

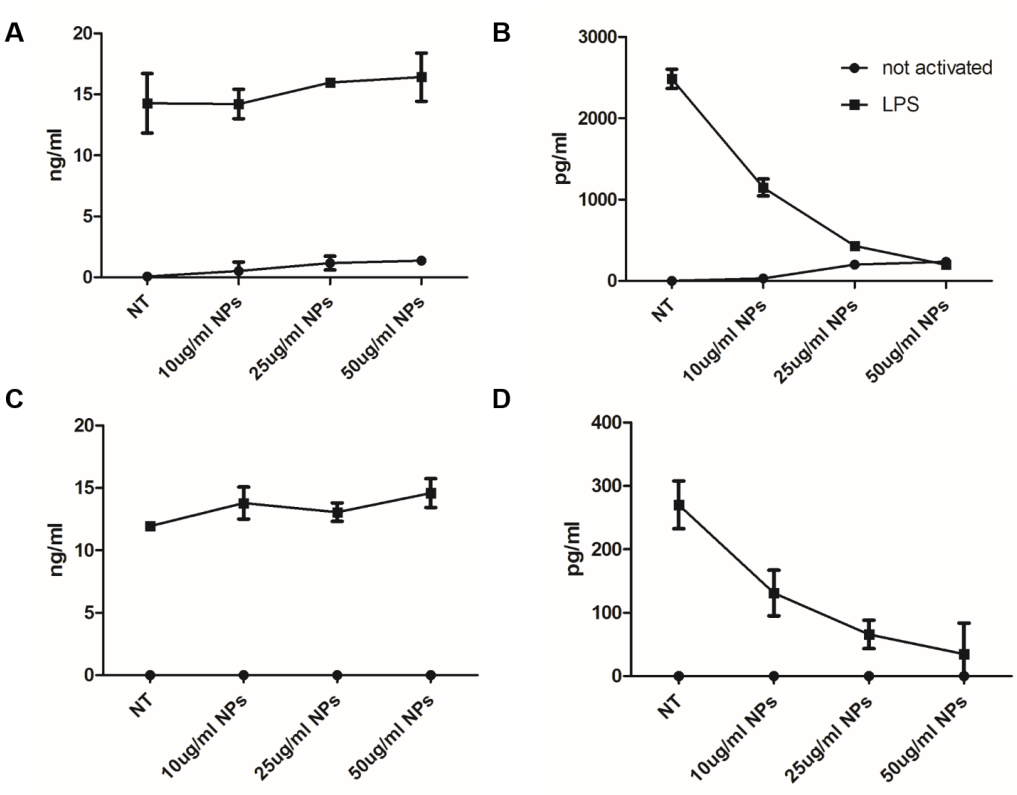


Figure 5: MYTS-VIVIT NPs are able to block IL-2 production in a dose-dependent manner

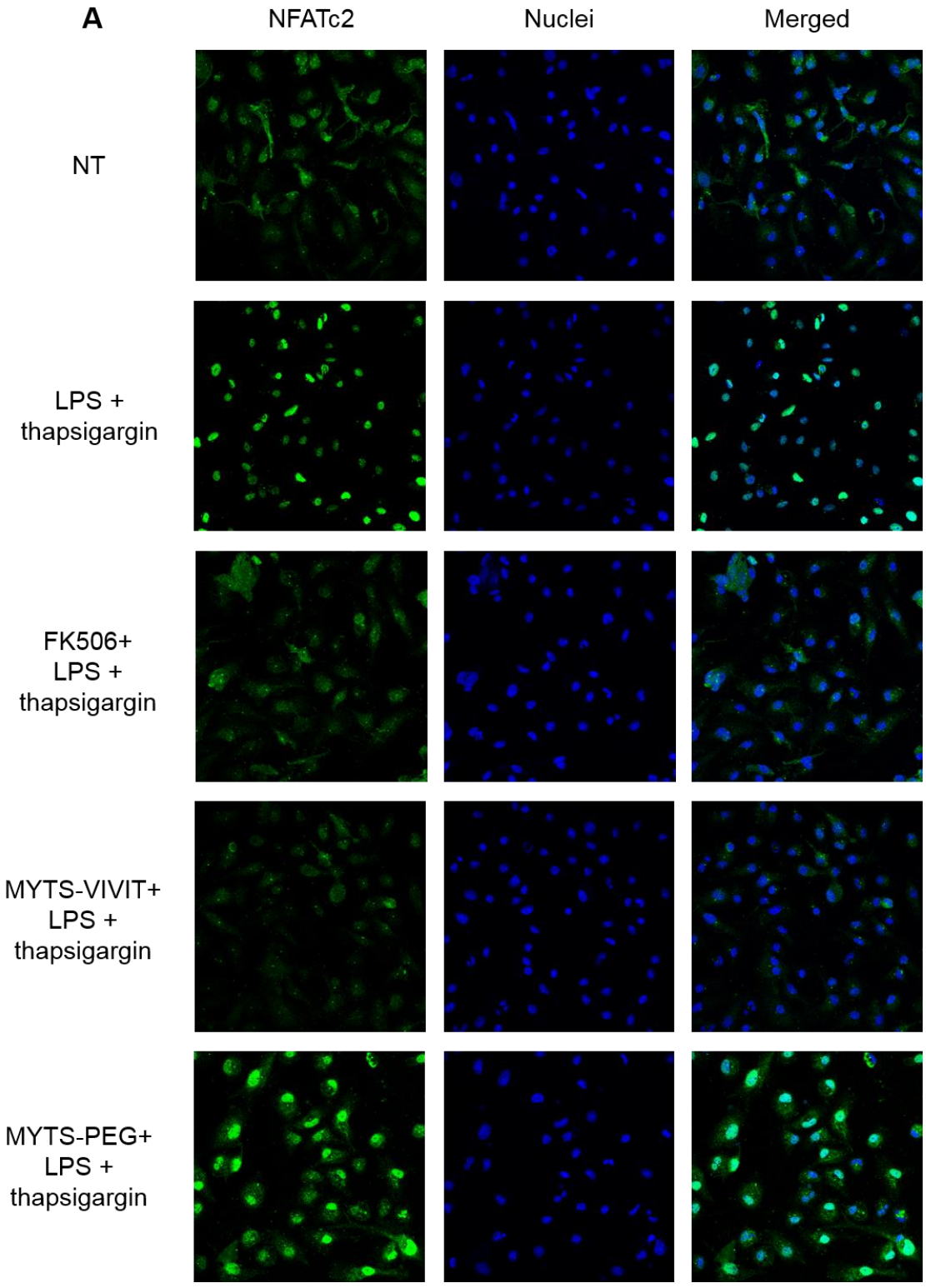
D1 cells (A-B) or BMDCs (C-D) were pre-treated for 2 hours with MYTS-VIVIT NPs. Then, they were stimulated with LPS (1µg/ml) for 18 hours. TNFα (A-C) and IL-2 (B-D) productions were measured by ELISA. Values represent means of at least 3 indipendent experiments performed in duplicate + SD.

Moreover, we also decided to direct investigate whether MYTS-VIVIT NPs were able to block the nuclear translocation of NFAT using immunofluorescence

analysis. To do that, BMDCs from WT mice were pre-treated with MYTS-VIVIT NPs, MYTS-PEG or tacrolimus (FK-506) as control for 2 hours. Then, they were stimulated with LPS and thapsigargin for 40 minutes in order to stimulate the nuclear translocation of NFAT. NFAT or NFκB nuclear translocation was assessed using confocal microscopy analysis. As shown in Figure 6, MYTS-VIVIT NPs could block the nuclear translocation of NFAT in a similar manner to FK-506. Furthermore, the NFAT inhibition was directly dependent on the presence of the VIVIT peptide on the surface of the MYTS NPs. Indeed, when MYTS-PEG NPs were used, the NFAT nuclear translocation was not blocked. Consistent with the previous data on cytokine production, MYTS-VIVIT NPs were able to block the NFAT translocation and activation, without affecting other pathways, as demonstrated by the confocal images on the nuclear translocation of NF-κB.

In summary, we demonstrated that MYTS-VIVIT NPs are able to target directly innate immune cells and, in particular, phagocytes like DCs and macrophages. Moreover, once internalized by DCs, the MYTS-VIVIT NPs block NFAT nuclear translocation without affecting other pathways.

Thanks to their characteristics, MYTS-VIVIT NPs are an optimal tool for the study of the role of the NFATc signaling pathway in innate immune cells, and in particular in DCs.


A
NFATc2
Nuclei
Merged
NT
LPS +
thapsigargin
FK506+
LPS +
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MYTS-VIVIT+
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LPS +
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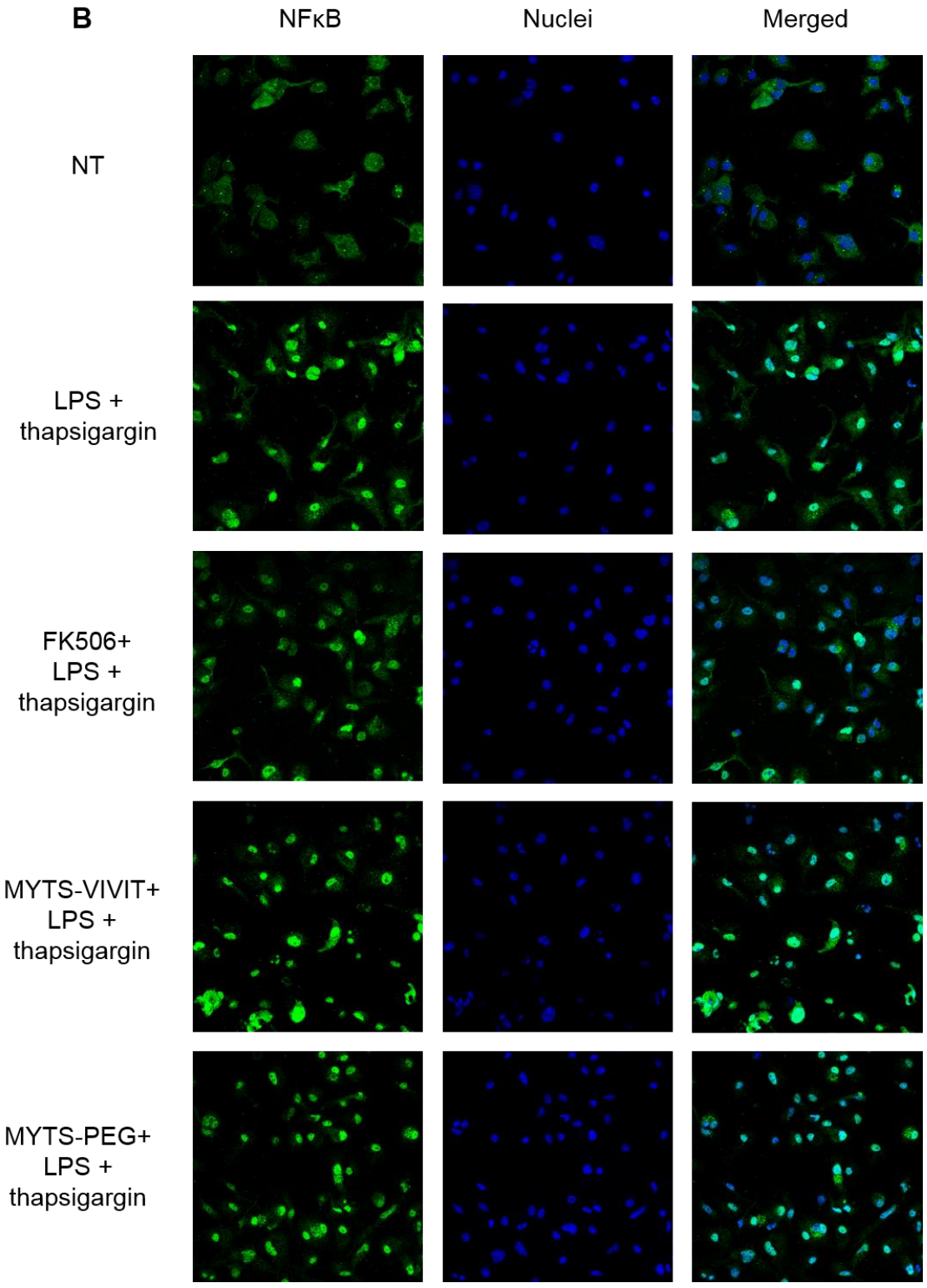

B
NFκB
Nuclei
Merged
NT
LPS +
thapsigargin
FK506+
LPS +
thapsigargin
MYTS-VIVIT+
LPS +
thapsigargin
MYTS-PEG+
LPS +
thapsigargin

Figure 6: MYTS-VIVIT NPs are able to block the nuclear translocation of NFAT, without affecting NF-κB pathway.
BMDCs (100000 cells) were pre-treated for 2 hours with MYTS-VIVIT NPs, MYTS-PEG NPs or FK-506 as control. Then, they were stimulated with LPS (1µg/ml) plus thapsigargin (50nM) for 40 minutes. Nuclear translocation of NFAT (A) or NF-κB (B) were then assessed using confocal microscopy.
Green: NFAT or NF-κB; Blue: nuclei (DRAQ5)

2.3 Treatment of skin transplanted mice with MYTS-VIVIT NPs is able to inhibit graft rejection

DCs have the peculiar ability to decide whether and how a T cell response should be activated or suppressed [17] and It has been demonstrated the fundamental role of IL-2 produced by DCs in this process [18-19]. Since NFATc activation in DCs controls the expression of IL-2, we predict that the NFATc signaling pathway could also play a crucial role in in the activation of alloreactive T cells in a context of acute transplant rejection. In this regard, MYTS-VIVIT NPs, allowing specific inhibition of NFAT in innate immune cells, are a useful tool to understand the involvement of this signaling pathway in the activation of alloreactive T cells.

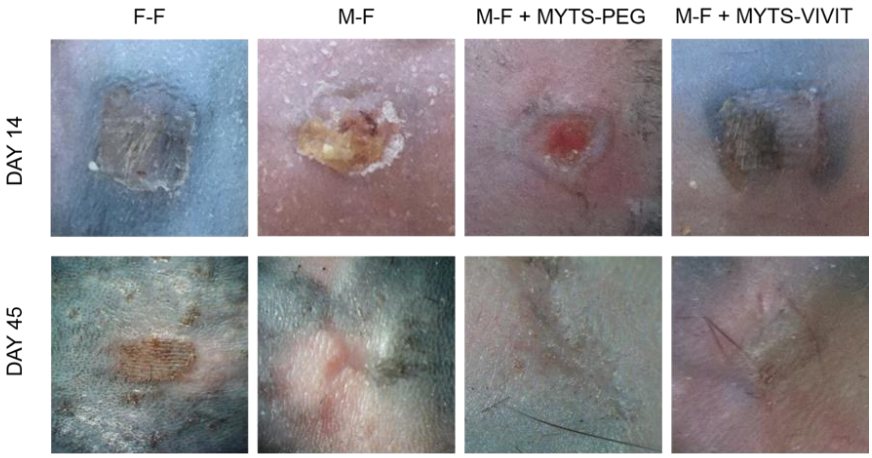
In order to investigate our hypothesis, we set up a skin transplant model in which donor and recipients mice are identical for the MHC and differ only for the miH antigens [20-21]. Sections of donor skin for grafting were taken from the tail and transplanted onto the dorsum of recipient mice. While female in female transplants are accepted, male in female are rejected in 10 days. Transplanted female mice receiving the donor male skin were further divided into four groups: 1) not treated; 2) treated with FK-506 (M-F + FK506); 3) treated with MYTS-VIVIT NPs (M-F + MYTS-VIVIT); 4) treated with control NPs conjugated with a scrambled inactive control peptide (MYTS-VEET NPs) [13]. Mice were treated every other day and monitored for skin graft rejection for the entire duration of the experiments (50 days). As shown in Figure 7a, skin graft rejection was obtained when donor male mice were used. Erosion of the graft occurred already two weeks after transplantation, while at day 50 only the remaining scar could be observed. On the contrary, when donor female mice were used, the grafts were accepted. Furthermore, 75% of mice treated with MYTS-VIVIT NPs were able to accept the transplant for the entire duration of the experiments, while the 80% treated with MYTS-VEET NPs rejected the graft fourteen days after transplantation. Mice treated with the control FK-506 were able to accept the graft during the first fourteen days, but they started to reject slowly the transplant already thirty days after

transplantation. More interestingly, 45% of mice treated with FK-506 undergoes rejection at day 50, while the ones treated with MYTS-VIVIT NPs still maintain the skin graft (Figure 7b).

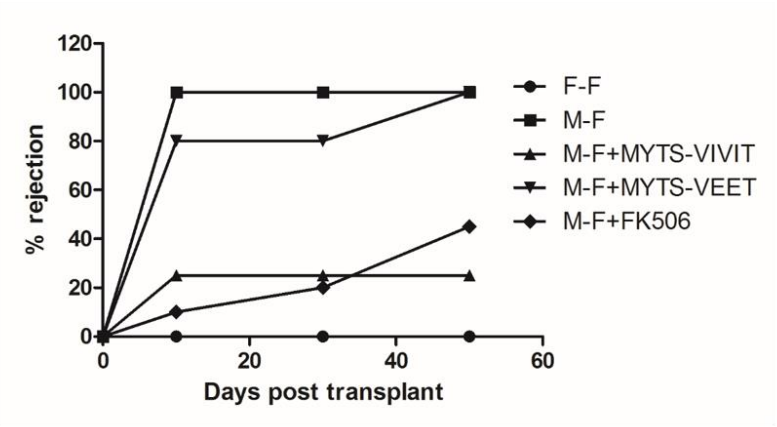
One of the most relevant difficulties regarding the use of immunosuppressive drugs is that the transplanted patient are dependent on the treatment for the entire life. We decided, therefore, to investigate the effect of treatment interruption. Treatments were stopped after 50 days and the graft status inspected for other 20 days. As expected, mice treated with FK-506 rejected the skin transplant immediately after interruption of the treatment. Diversely, mice treated with MYTS-VIVIT NPs maintained the transplant even at day 70 after surgery (Figure 7c).

These results suggest that treatment with MYTS-VIVIT NPs, and the consequent inhibition of the NFAT pathway in phagocytes (in particular DCs), can efficiently inhibit allo-responses in vivo. Furthermore, the treatment results effective even when interrupted, suggesting that a potential mechanism of tolerance to the transplant could be induced.

A


F-F
M-F
M-F + MYTS-PEG
M-F + MYTS-VIVIT
DAY 14
DAY 45

B


% rejection
Days post transplant
F-F
M-F
M-F+MYTS-VIVIT
M-F+MYTS-VEET
M-F+FK506

C

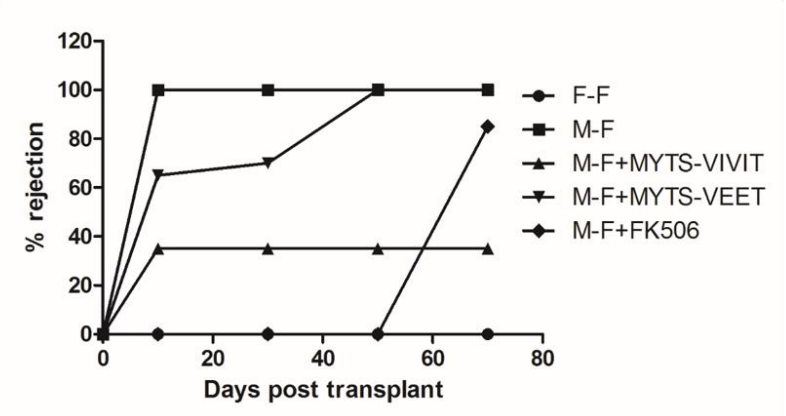

% rejection
Days post transplant
F-F
M-F
M-F+MYTS-VIVIT
M-F+MYTS-VEET
M-F+FK506

Figure 7: Treatment with MYTS-VIVIT NPs reduces skin graft rejection

Recipient C57/BL6 WT female mice were transplanted with the tail skin of a donor C57/BL6 WT male (graft rejection) or female (graft success) mouse. They were further divided into four groups and they were treated i.p. (100ug) every other day with MYTS-VIVIT NPs, MYTS-VEET NPs or FK-506, respectively. Skin graft acceptance or rejection at day 14 and 50 (A). Percentage (%) of skin graft rejection during (B) and after (C) treatment.

2.4 Inhibition of NFATc pathway also delay the rejection of a second skin transplant and may induce tolerance to the graft

Results indicates that treatment with MYTS-VIVIT NPs can inhibit the activation of allo-reactive T cells and may also be effective regarding the long-term survival of the transplant. In order to investigate this hypothesis, we performed a second-set skin transplant. When a recipient has previously rejected a graft, the second one is usually rejected more rapidly (6-8 days) [22]. In order to perform the experiments, transplanted female mice were re-grafted with the skin of a donor male C57/BL6 WT mouse 100 days after the first transplant. During the second-set transplant, mice were not treated to assess the effect of the initial treatment with MYTS-VIVIT NPs. As expected (Figure 8), mice not treated during the first setting also

rejected faster (10 days) the second graft. On the other hand, 70% of mice previously treated with MYTS-VIVIT NPs accepted the second skin graft at day 10 and rejected the graft only 35 days after the transplant.

Therefore, we are able to conclude that the inhibition of NFAT pathway in innate immune cells is also able to delay the rejection of a second transplant, indicating that the treatment with MYTS-VIVIT NPs is able to confer a long-term survival of the graft.

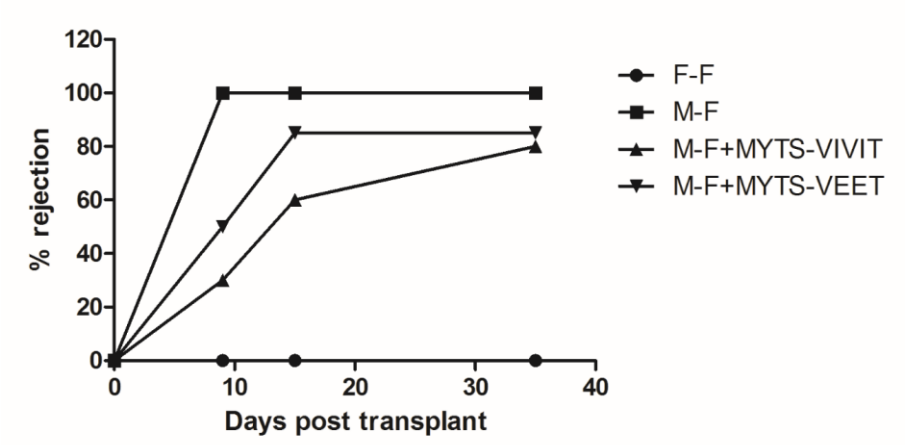


Figure 8: Treatment with MYTS-VIVIT NPs during the first transplant setting also delay the rejection of a second skin graft

100 days after the first transplant, recipient female mice treated with MYTS-VIVIT NPs or MYTS-VEET NPs and not treated controls received a second skin graft on the other side of their dorsum. Mice were not treated during second transplantation and the skin grafts were inspected at different time-points (day 9, 15 and 35). Percentage (%) of skin rejection is shown.

Transplanted female mice treated with MYTS-VIVIT NPs maintained the skin graft even when treatment was stopped and they showed delay rejection of a second skin graft. In order to explain our findings, we hypothesized that the blocking of NFAT pathway in innate immune cells may leads not only to an inhibition of effector allo-reactive T cells. Our results could be dependent by the generation of inducible peripheral Treg cells, which in turns mediate tolerance to the transplant. To assess our hypothesis, we developed a more specific model in order to evaluate the possible generation of inducible Treg cells. In this model, recipient C57/BL6 WT female mice received the skin of donor K5-mOVA mice. In particular, K5-mOVA mice expressed a membrane-bound form of the ovalbumin (OVA) peptide under the control of the keratin 5 (K5) promoter in the epidermal and hair follicular keratinocytes [23]. At the same time of the transplant, recipient female mice were also injected with $CD4^{+}Foxp3^{-}$ OTII cells. These cells were purified from DEREG OT-II mice, previously treated with diphtheria toxin (DT). The DEREG mice expressed the green fluorescent protein (GFP) and the diphtheria toxin receptor (DTR) under the control of the Foxp3 promoter, allowing us to deplete all the $Foxp3^{+}$ Treg cells [24]. After transplantation, recipient female mice were divided into three groups and they were treated with MYTS-VIVIT NPs or MYTS-VEET NPs for 50 days. Not treated recipient mice rejected the transplant already at

day 10. On the other hand, 90% of mice treated with MYTS-VIVIT NPs accepted the skin graft during the first 30 days after the transplant (Figure 9a), demonstrating the efficacy of the treatment even in a more specific model of acute transplant rejection.

To verify that MYTS-VIVIT NPs treatment promote the long-term survival of the transplant also in this experimental setting, treatments were stopped 50 days after transplantation and then mice were inspected at day 70. Consistent with our previous data, 80% of mice receiving MYTS-VIVIT NPs still maintained the graft 20 days after interruption of the treatment (Figure 9a).

Finally, we also performed a second-set skin graft. To this purpose, the transplanted female mice were re-grafted with the skin of donor K5-mOVA mice. Then, grafts were inspected for rejection at day 10 and 40 after the second transplant. As previously shown, 60% of mice receiving the MYTS-VIVIT treatment during the first setting also accepted the second skin graft (Figure 9b). On the other hand, control groups soon rejected the second graft at day 10.

From these results, we were able to demonstrate the efficacy of NPs treatment even in a more specific model. In this setting, we injected the transplanted mice with $CD4^+$ OTII T cells able to recognize the OVA peptide expressed by donor skin and to stimulate a strong reaction against the graft. However, mice treated with MYTS-VIVIT NPs are

still able to maintain the transplant. Clearly, more experiments are required to understand the underlying mechanisms. Moreover, $CD4^+$ OTII cell populations injected into transplanted mice were previously depleted from $Foxp3^+$ Treg cells. As we used DEREG OT II mice, the next step will be recover LNs from the transplanted mice at different time-points in order to assess whether $Foxp3^+GFP^+$ Treg cells could be generated.

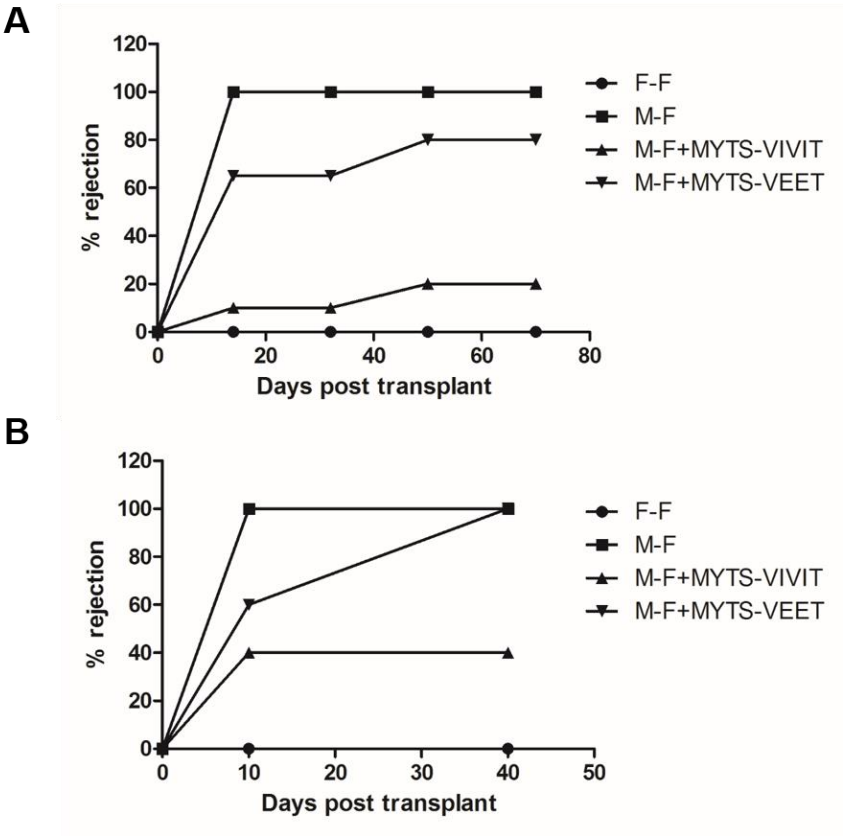


Figure 9: Treatment with MYTS-VIVIT NPs may induce tolerance to skin transplants

Recipient female WT mice were transplanted with the skin of donor male K5-mOVA mice. At the same time of the transplant, they were injected with Foxp3^{-}CD4^{+} OTII cells. Then, transplanted female mice were treated with MYTS-VIVIT NPs or control NPs. After 70 days, mice were re-grafted with the skin of a donor male K5-mOVA mouse. Percentage (%) of skin rejection during the first (A) and second (B) transplant are shown.

2.5 Donor dendritic cells (DCs) and donor DCs-derived IL2 are fundamental for skin transplant rejection

It is widely known that tissue resident dendritic cells (DC), including DC resident in transplanted tissues, are able to traffic to the secondary lymphoid organs of recipients, where they present alloantigens to T cells. This event is the basis for the process of direct allorecognition, in which recipient T cells directly recognize intact allo-MHC molecules on tissue-resident DC [26]. Despite ample evidence demonstrating the central role of tissue/dDC in alloimmune responses, characterization of dDC and their specific contributions to transplant rejection *versus* tolerance remain poorly described [27].

In order to assess the role of dDCs in our model, donor male mice were treated with MYTS-VIVIT NPs 10 days

before the transplant, every other days. Then, recipient female mice received the transplant from not treated (M-F) or treated donor (M+ MYTS-VIVIT –F) and were monitored for 50 days. Furthermore, treatment of donor male mice was also compared to the one of recipient female mice (M-F +MYTS-VIVIT). As shown in Figure 10, treatment of donor mice with MYTS-VIVIT NPs delayed skin graft rejection at least of 15 days. In this period, all the mice receiving the skin of treated donor mice accepted the transplant, suggesting that the contribution of dDCs is fundamental in the initial phases of rejection. After 15 days, the role of recipient DCs became more evident and mice started to reject the transplant. Indeed, only recipient mice treated with MYTS-VIVIT NPs (M-F +MYTS-VIVIT) maintained the skin graft.

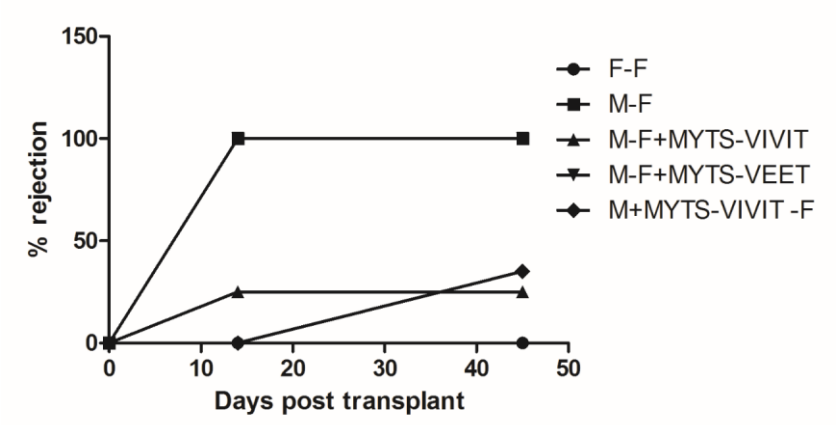


Figure 10: Treatment with MYTS-VIVIT of donor male mice is able to delay the rejection of the graft

Donor male mice were treated i.p. with MYTS-VIVIT NPs (100ug) every other days, starting 10 days before the transplantation. Then, recipient C57/BL6 WT female mice were transplanted with the skin of the tail of the treated (M+ MYTS-VIVIT –F) or not treated male mice (M-F). Treatment of recipient female mice (M-F +MYTS-VIVIT) was shown as control. The percentage (%) of skin graft rejection is shown.

These results suggest that NFAT signaling pathway is fundamental to raise rejection not only when activated in recipient innate cells, but also when activated in donor tissues. We postulated that NFATc activation is fundamental for DCs to produce IL-2 that in turns support the activation of alloreactive T cells. To investigate our hypothesis, skin transplant were performed using IL-2 deficient or DC deficient donor mice (DOG mice). DOG are transgenic mice expressing the diphtheria toxin receptor (DTR) under the control of the CD11c promoter [28]. Therefore, DOG mice allow the depletion of $CD11c^{hi}$ DCs through repeated injection of diphtheria toxin (DT), not only in the spleen, but also in the skin (Figure 11). Thus, recipient female mice were grafted with the skin of donor IL2 deficient male mice or the DT-treated DOG (DOG DT) mice (or not treated as control); recipient mice were monitored for 80 days. Recipient mice that have received the skin from either IL2 deficient or DOG DT donor mice had a delayed graft rejection (Figure 12). At day 20, mice receiving skin from IL2 deficient donor mice completely

accepted the transplant (Figure 12a). Instead, 70% of mice grafted with tail of DOG DT mice accepted the donor skin (Figure 12b). As treatment with DT is only able to deplete the $CD11c^{hi}$ DCs and not all the DCs population, this difference between the two groups may be due to the remaining DCs after treatment with DT. However, mice started to reject the transplant 20 days later, indicating that recipient T cells anyway activated at later timepoints. Taken together, these data provide a demonstration that dDCs and in particular dDC-derived IL-2 are fundamental for graft rejection during the first days after transplantation.

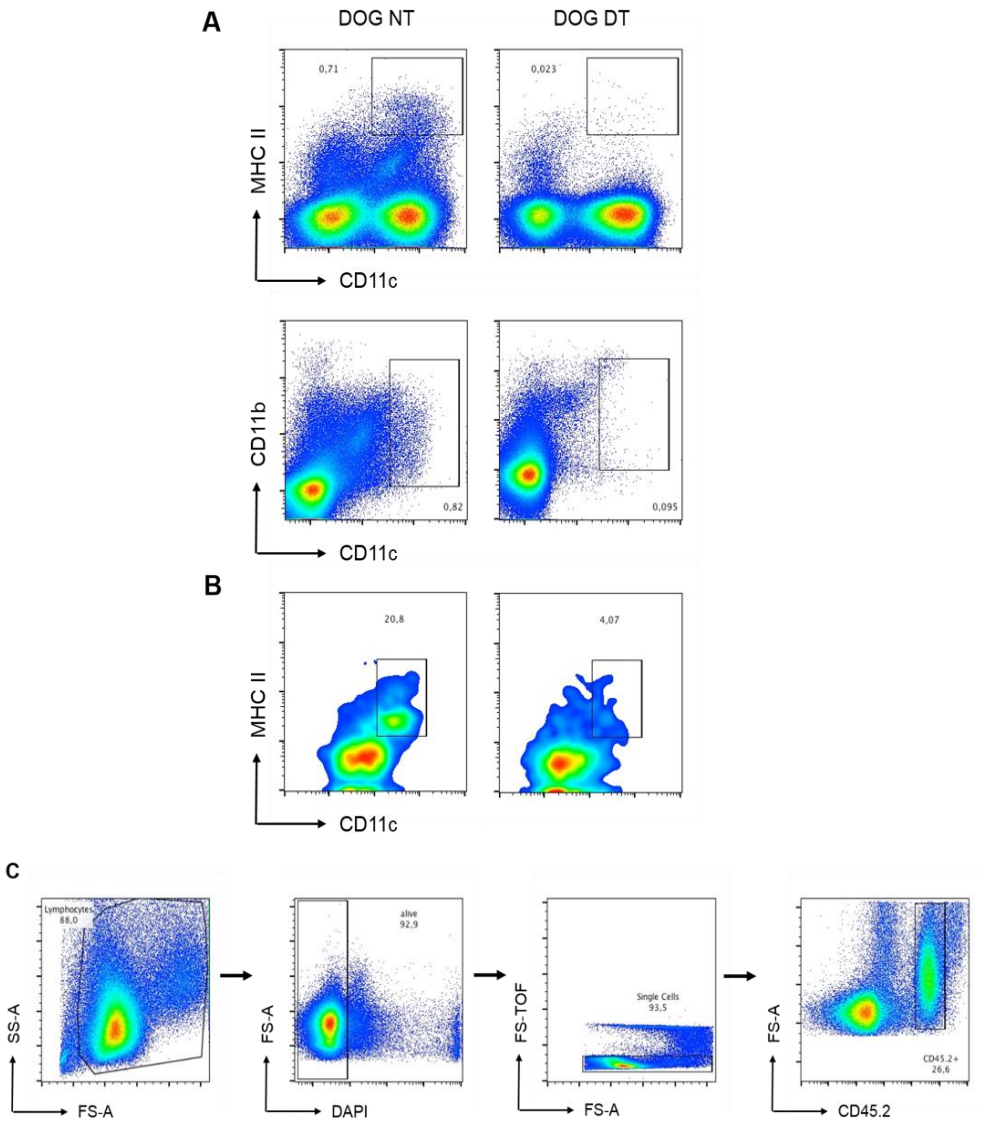


Figure 11: DC depletion from DOG mice

DOG mice were treated i.v. with DT (16ng/g) for three consecutive days. Before transplant, spleen (A) and skin (B) from not treated and treated DOG mice were recovered and DC depletion was analyzed by flow cytometry. Gating strategy for skin cell populations (C).

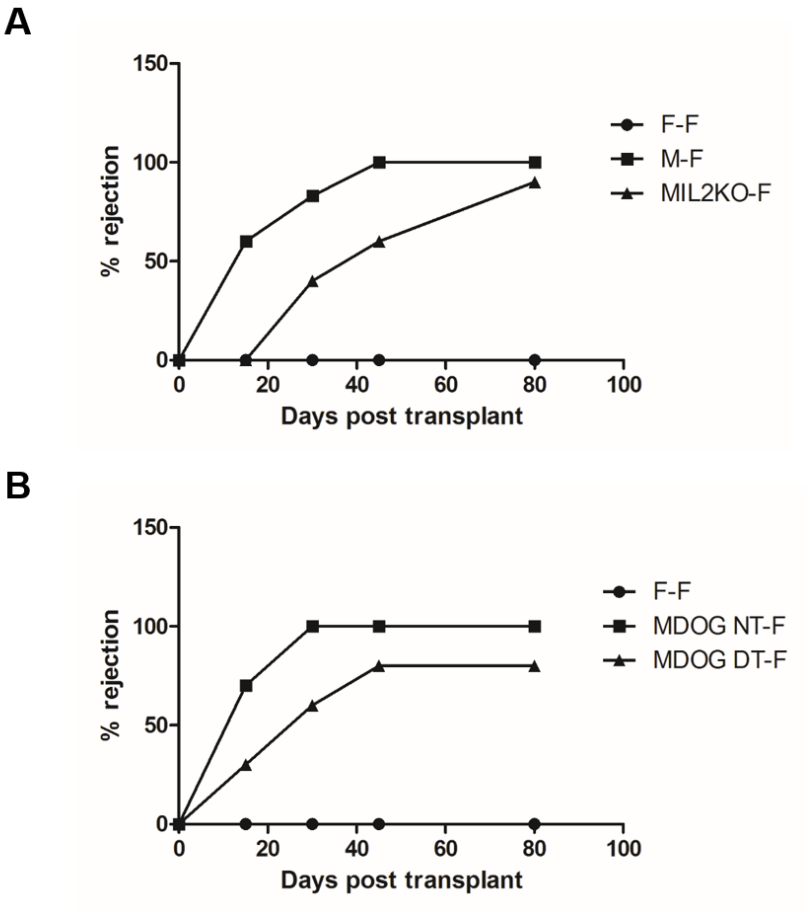


Figure 12: Donor DC-derived IL-2 is involved in allograft rejection
Recipient C57/BL6 WT female mice were transplanted with the skin of donor $IL2^{-/-}$ male mice (A). Recipient C57/BL6 DOG female mice were transplanted with the skin of DT-treated DOG male mice (not treated DOG male mice were used as control) (B). Skin graft rejection (%) was monitored for 80 days at different time-points.

2.6 Activation of the NFATc signaling pathway in donor cells is required for skin graft rejection

We thus evaluated the contribution of NFATc signaling pathway in dDCs, using a donor NFATc2$^{-/-}$ mouse (donor WT female or male mice were used as control). Consistent with the previous data, mice transplanted with the skin of donor NFATc2$^{-/-}$ mice slowly started to reject the transplant (Figure 13a).

In a similar manner to mice receiving the skin from donor male IL2$^{-/-}$ or DOG DT mice, 90% of mice grafted with skin of NFATc2$^{-/-}$ accepted transplants at day 15. Then, they started rejection at least two weeks after control mice.

To evaluate whether this result is due to a defect in the number of myeloid cells present in the skin of NFATc2$^{-/-}$ mice, we analyzed the cells population of NFATc2$^{-/-}$ and WT mice by flow citometry. As shown in Figure 13b, innate immune cells, like DCs (CD11c^{+}) and macrophages (F4/80^{+}), in the skin of NFATc2$^{-/-}$ mice did not show difference compared to WT mice.

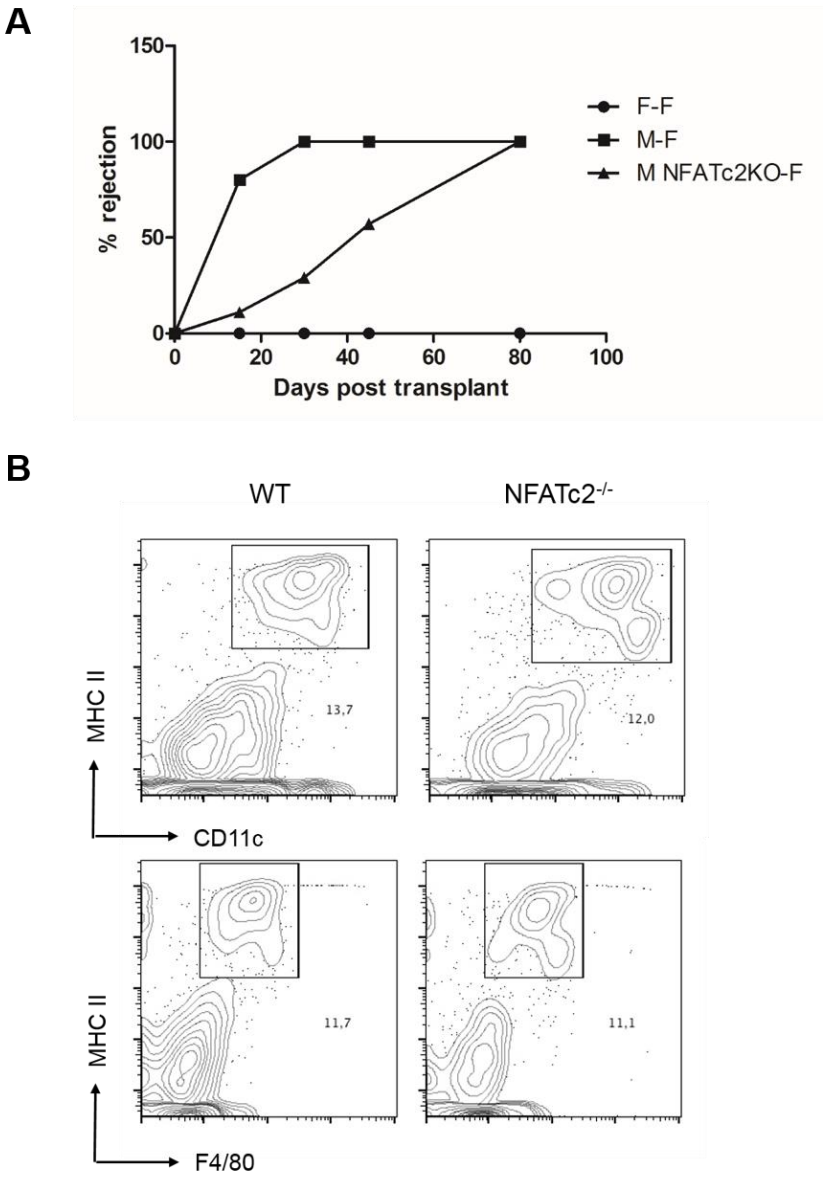


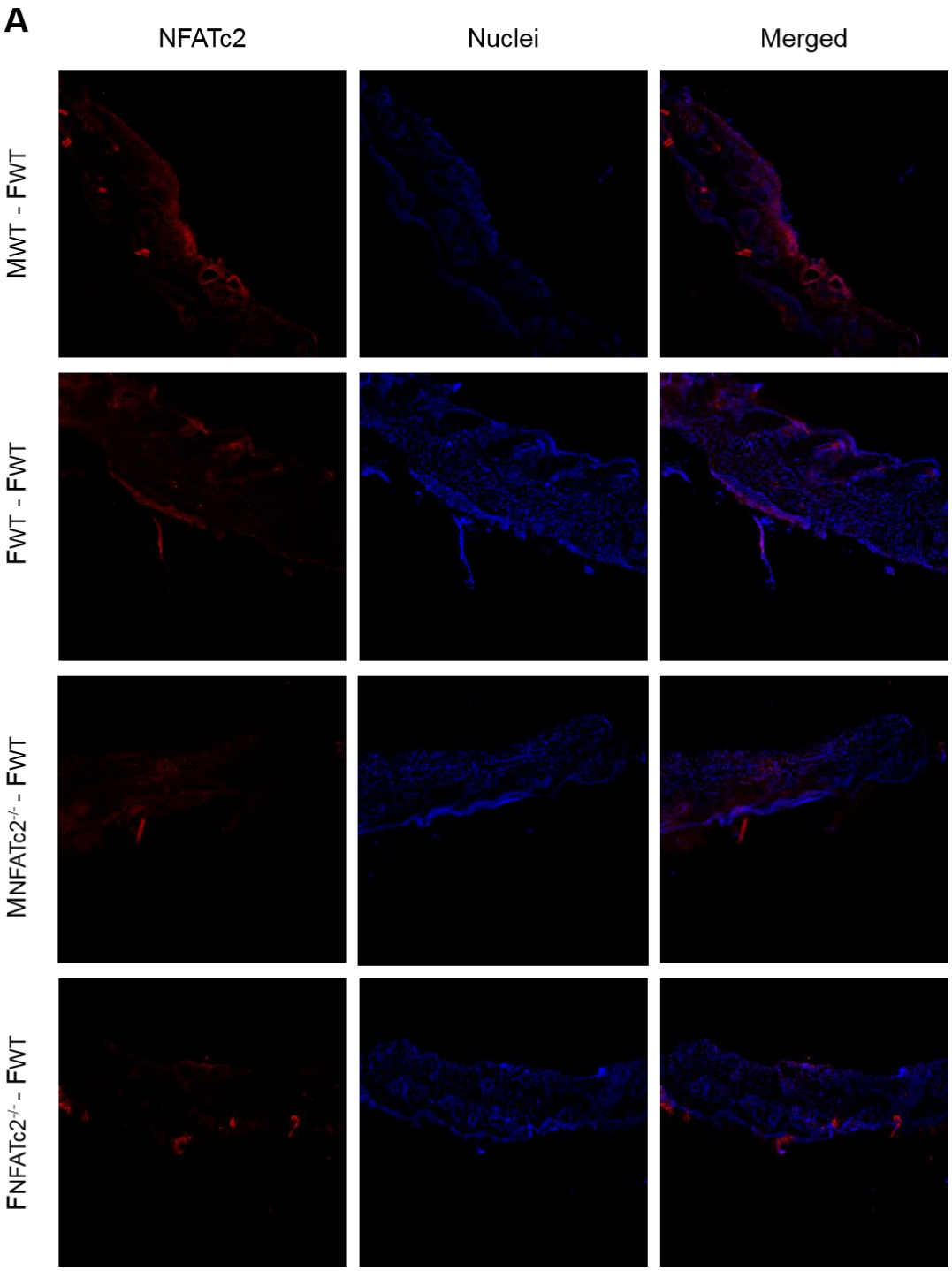
Figure 13: Transplantation of donor NFATc2$^{-/-}$ mice skin delays the allograft response

Recipient C57/BL6 WT female mice were grafted with the skin of donor C57/BL6 NFATc2$^{-/-}$ male mice (donor female or male WT mice were used as control). Then, mice were inspected for skin graft

rejection (%) at different time-points for 90 days (A). Skin cell populations of NFATc2$^{-/-}$ mice compared to WT mice (B). DCs: CD11c^{+}MHC II^{+}. Mo: F4/80^{+}MHC II^{+}.

To evaluate the NFATc activation and translocation in donor cells during transplantation, skin of the donor WT or NFATc2$^{-/-}$ male and female mice were also recovered three days after transplant and immunohistochemical analysis were performed. As shown in Figure 14a, NFATc2 is upregulated in the cells of donor male mice compared to the ones of donor female mice. Moreover, NFATc upregulation and activation in donor male mice was apparent in the dermis, especially in the areas near the recipient skin (Figure 14b). On the contrary, NFATc2$^{-/-}$ was not upregulated or activated in the skin of donor female WT mice and donor NFATc2$^{-/-}$ mice. In particular, absence of NFATc2 activation in the skin of male NFATc2$^{-/-}$ mice indicate that neither recipient cells are able to infiltrate the skin of the donor three days after transplant (Figure 14a).

These results confirmed that the events that are at the basis of acute transplant rejection occurred already in the first days after the transplantation. Moreover, we were also able to show that NFATc activation in donor cells has a fundamental role in initiating these events.


A
NFATc2
Nuclei
Merged
MWT - FWT
FWT - FWT
MNFATc2-/- - FWT
FNFATc2-/- - FWT

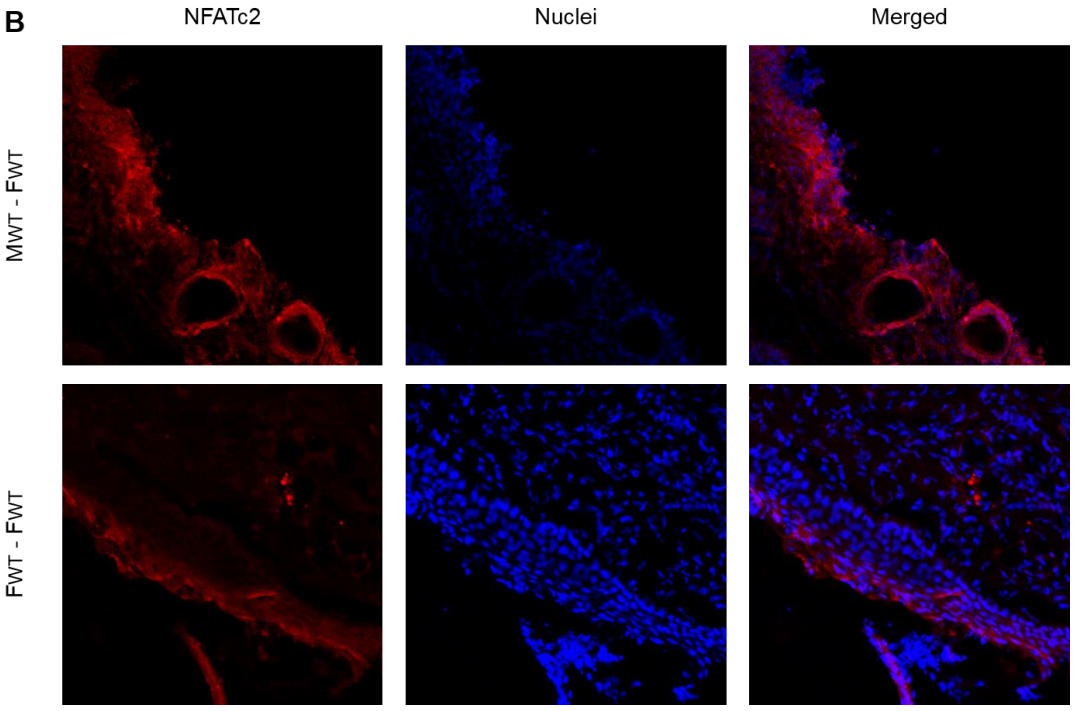


Figure 14: NFATc activation in donor skin cells occur early after transplantation

Recipient C57/BL6 WT female mice received the skin of donor WT or NFATc2$^{-/-}$ mice. Three days after transplantation, skin grafts were recovered and frozen. Then immunohistochemical analysis were performed (A). NFATc2 activation and translocation in the skin of donor male WT mice compared to donor female mice (B).

Red; NFATc2. Blue; DRAQ5

2.7 Tslp and Ly6G are actively regulated during skin graft and may influence transplant rejection

As we observed NFATc2 activation and translocation in the skin of donor male mice already three days after the transplant, we decided to investigate the early events that contribute to the initiation of graft rejection and may be related to NFATc activation. Recipient female WT mice received the skin of donor male or female WT mice and donor skin grafts were recovered three days after. Then, microarray gene expression analysis was performed to identify differentially expressed genes between the two groups of transplanted mice.

From the results, we obtained 617 differentially expressed genes, most of them related with the immune system (Figure 15). In particular, we focused on two genes differentially expressed between the two groups, thymic stromal lymphopoietin (Tslp) and lymphocyte antigen 6 complex (Ly6G).

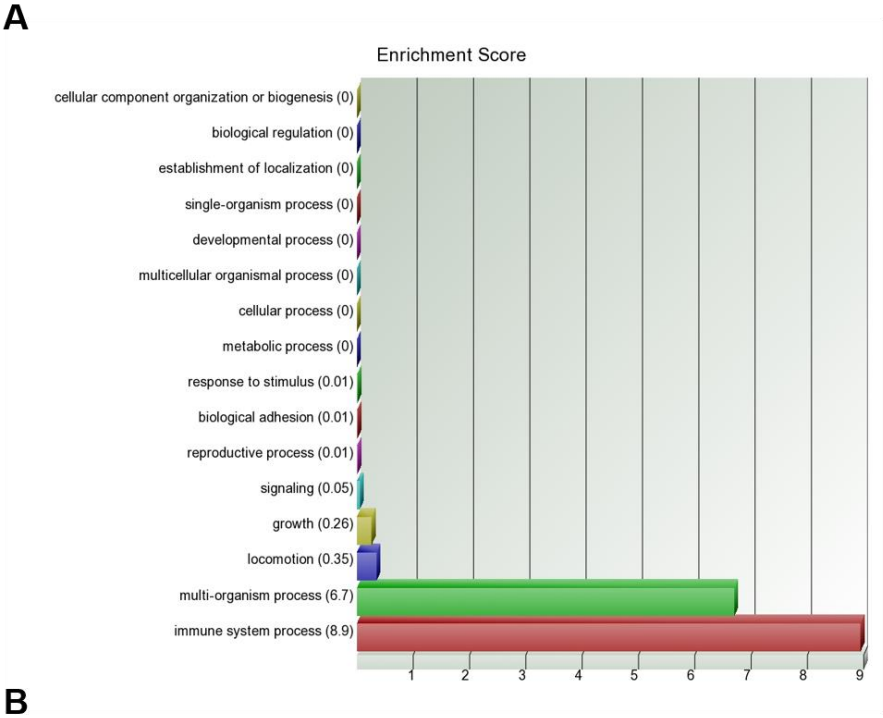
Thymic stromal lymphopoietin (Tslp) is upregulated in the group of mice receiving the skin from donor male mouse. Tslp is a cytokine produced by different cell types, including epithelial cells, that can induce release of T cell-attracting chemokines. Furthermore, several studies also

reported the function of Tslp in inducing maturation of DCs [30].

To validate the results obtained from gene expression analysis, quantitative real-time PCR (qPCR) were performed in order to quantify Tslp expression during transplant of donor WT mice at different time-points (day 1, 2 and 3). Tslp expression is upregulated immediately after transplant (day 1) in both groups of mice compared to control, not transplanted mice (Figure 16a). However, Tslp expression resulted significantly upregulated in mice receiving the skin of donor male WT mice compared to the other group for all the three days (day 1 and day 2; p-value < 0.05; day 3; p-value < 0.001).

As several studies reported that the NFAT pathway may regulates Tslp production [30-31], we decided to investigate Tslp expression also in mice receiving the skin from donor NFATc2$^{-/-}$ mice. As shown in Figure 16b, Tslp expression is upregulated compared to control, not transplanted mice, but unlike WT mice, there were no significantly differences between the two groups of mice.

A


Enrichment Score
cellular component organization or biogenesis (0)
biological regulation (0)
establishment of localization (0)
single-organism process (0)
developmental process (0)
multicellular organismal process (0)
cellular process (0)
metabolic process (0)
response to stimulus (0.01)
biological adhesion (0.01)
reproductive process (0.01)
signaling (0.05)
growth (0.26)
locomotion (0.35)
multi-organism process (6.7)
immune system process (8.9)
1
2
3
4
5
6
7
8
9

B

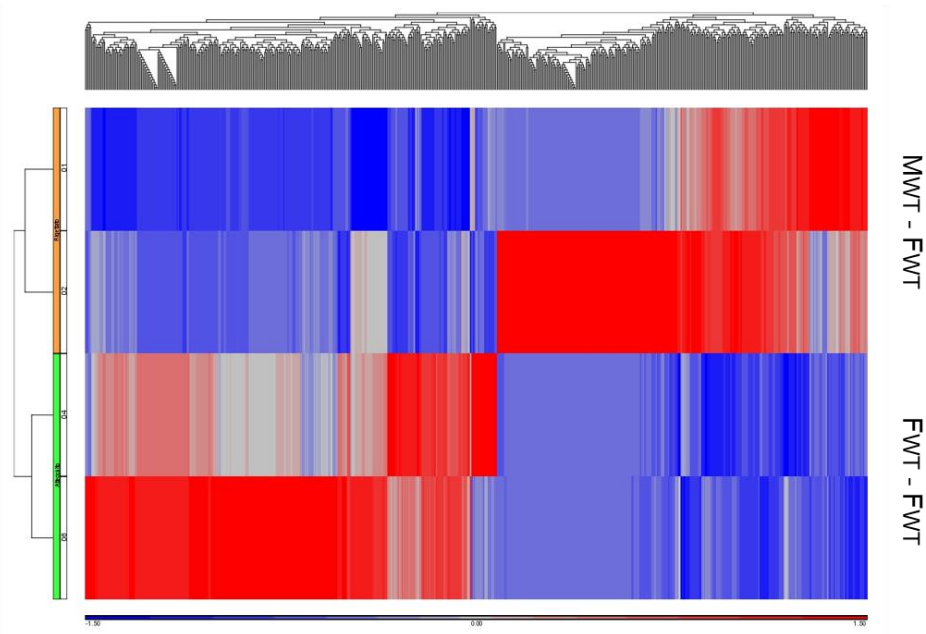

MWT - FWT
FWT - FWT

Figure 15: Differential expressed genes obtained by gene expression analysis.

Recipient female WT mice received skin of donor male or female WT mouse. Then, donor skin grafts were recovered three days after transplantation. RNA was extracted and microarray gene expression analysis was performed. Data obtained were analyzed for enrichment score (A) and differential expressed genes (DEGs) between the two groups of transplanted mice were calculated (fold change=2) (B).

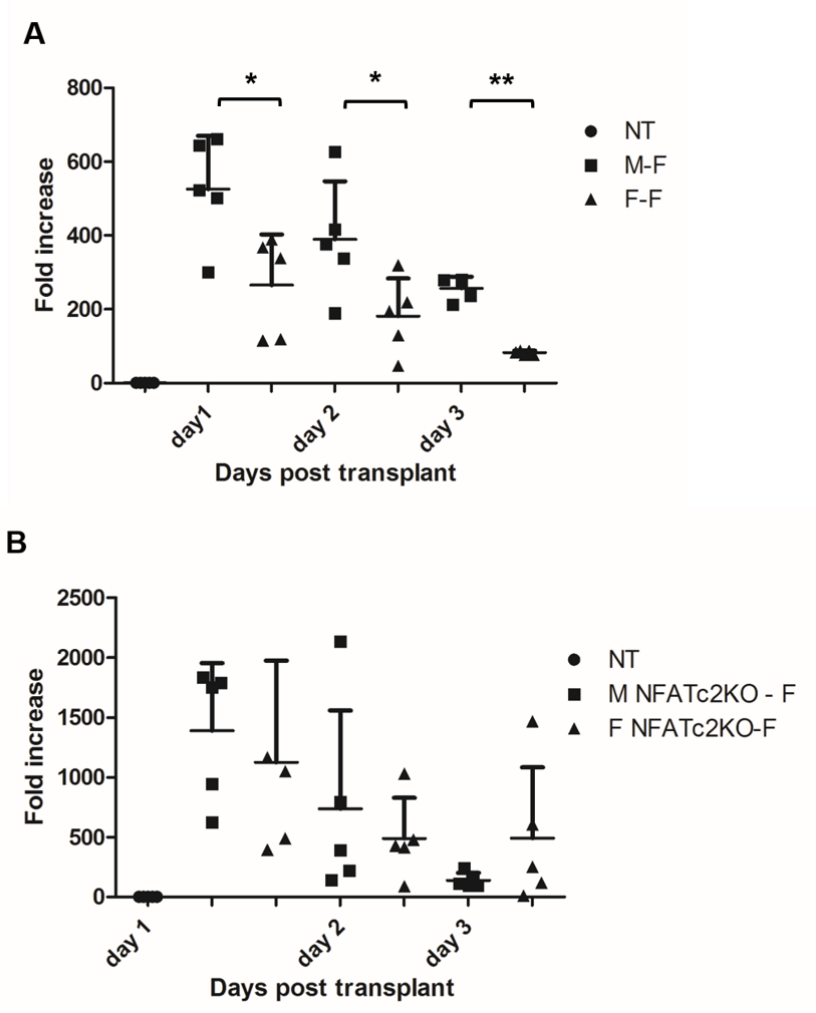


Figure 16: Tslp expression is upregulated during transplant rejection

Recipient female WT mice received the skin of donor male or female WT (A) or NFATc2$^{-/-}$ (B) mice. Donor skins were recovered at different time-points after transplantation (day 1, 2 and 3) and qPCR for Tslp expression were performed. Fold increase between transplanted and not transplanted mice were calculated. Values represent means of one experiment performed using 5 animals for groups. * = p-value < 0.05; ** = p-value < 0.001.

These results indicate Tslp as one of the potential cytokine that are involved in the first events that lead to transplant rejection and suggest a correlation between Tslp expression and NFAT pathway.

We also focused our attention on lymphocyte antigene 6 complex (Ly6G), which is upregulated in mice receiving the skin from donor female mouse. Ly6G is a 21-25kD glycosylphosphatidylinositol (GPI)-linked differentiation antigen that is expressed by myeloid-derived cells in a tightly developmentally-regulated manner in the bone marrow. Monocytes express Ly6G transiently during bone marrow development, while Ly6G expression in granulocytes and peripheral neutrophils directly correlates with the level of cell differentiation and maturation [32]. Moreover, Ly6G is also expressed by a recently discovered cell subset, the myeloid derived suppressor cells (MDSCs) [33].

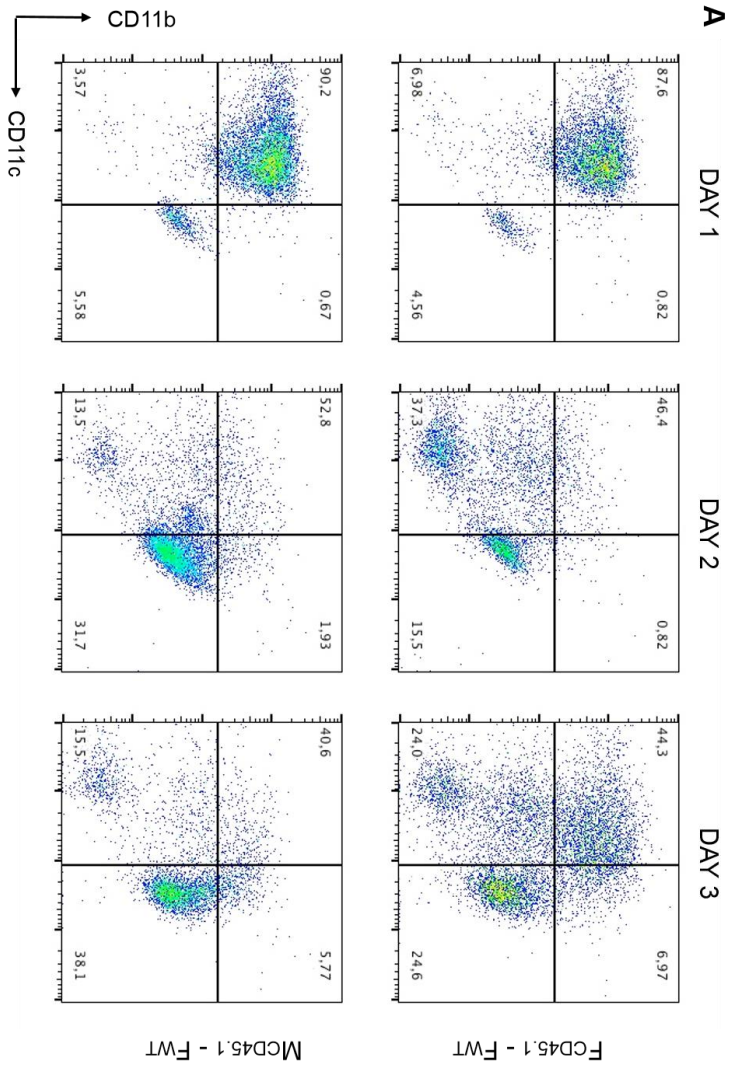
Thus, we evaluated cell populations that migrate into the skin graft during the first days after the transplant. To this

purpose, recipient female CD45.2$^+$ WT mice received the skin of donor CD45.1$^+$ WT male or female mice. Donor skin transplants were recovered at different time-points (day 1, 2, 3). Skin was analyzed by flow cytometry after having obtained single cell suspensions. For the FACS analysis, cutaneous cells of donor or recipient origin were identified as CD45.1$^+$ and CD45.2$^+$, respectively. At day 1, we observed the presence of a cell infiltrate derived from the recipient mice (CD45.2$^+$) characterized by the expression of CD11b$^+$Gr1$^+$ markers. Since there is no difference between the two groups at day 1, we postulated that recruitment into the graft of CD11b$^+$Gr1$^+$ cells is probably due to the surgical procedure that determines a local inflammation (Figure 17b).

However, at day 2 and day 3 we observed a clear difference between cell populations recruited into the graft. In particular, donor male skin graft revealed the presence of significantly more recipient CD45.2$^+$CD11c$^+$ DCs compared with donor female skin graft (Figure 17a), confirming the fundamental role of DCs in inducing graft rejection.

On the contrary, at day 3 we recovered more CD45.2$^+$CD11b$^+$ cells in the skin of donor female mice compared to the skin of donor male mice (Figure 17a). Consistent with the microarray data on the expression of Ly6G at day 3, the CD45.2$^+$CD11b$^+$ cell population also expressed Gr1$^+$ markers (Figure 17b).

Although further analysis are required, our data suggest the presence of a cell population characterized by the expression of $CD11b^{+}Gr1^{+}$ markers in the skin of donor female mice that may favor graft acceptance.



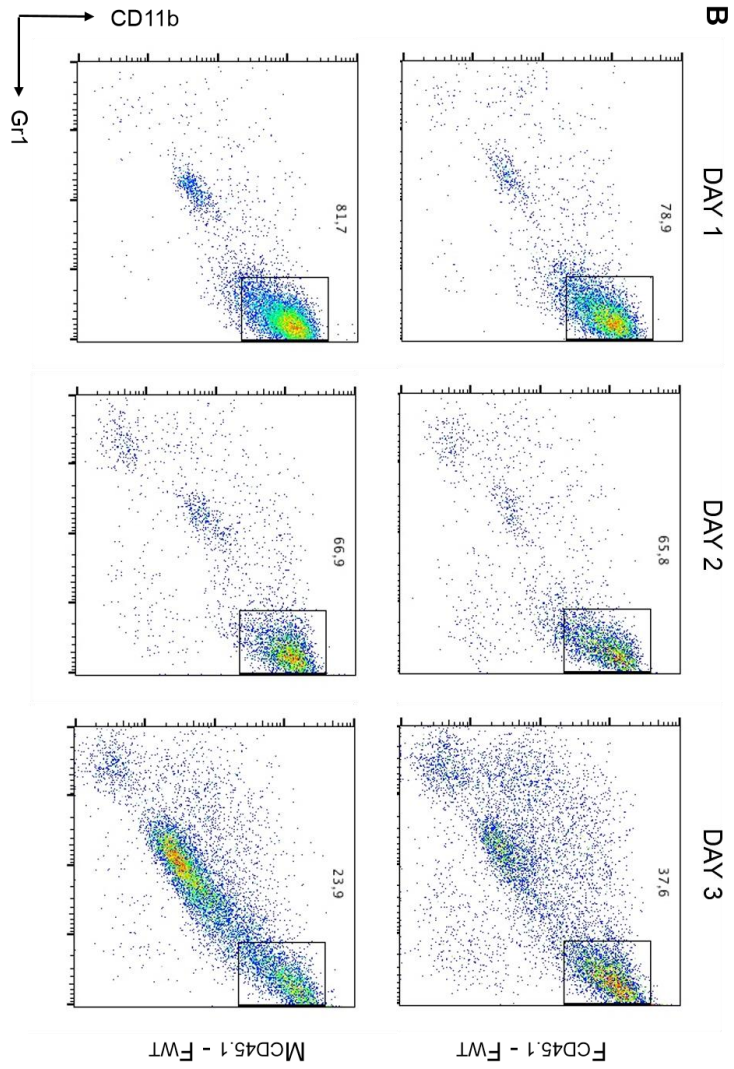


Figure 17: CD11b$^+$Gr1$^+$ cells are recruited in the skin of donor female mice during transplant

Recipient female CD45.2$^+$ WT mice received the skin of donor CD45.1$^+$ WT male or female mice. At different time-points (day 1, 2, 3), donor skin were recovered and analyzed by flow cytometry after obtaining a single cell suspension. CD11c$^+$CD11b$^+$ cells (A) and CD11b$^+$Gr1$^+$ (B) cells present in the donor skin graft.

2.8 IL2 and PGE2 may promote transplant rejection by increasing vasodilation

Although not well understood, vascular endothelial cells have a major role in the inflammatory process. The properties of endothelial cells change depending on various phases of the inflammatory responses in response to different inflammatory mediators. In particular, the inflammatory mediators act on endothelial cells in order to favor an increase in local blood flow and vasodilation, inducing leukocytes extravasation [34]. In addition to the well-known mediators that can induce vasodilation, several studies have also reported the expression of the IL-2 receptor (IL-2R) by endothelial cells [35] and they have demonstrated a direct effect of IL-2 in increasing endothelial cells permeability [36]. In this context, we postulated that the production of IL2 following NFATc activation in DCs may help transplant rejection increasing vasodilation and promoting the extravasation of lymphocytes in the donor skin graft.

To investigate this hypothesis, we first decide to evaluate whether IL2 is effective in inducing vasodilation using an in vitro model. A human cell line of endothelial cells, the HUVEC cells, was used to evaluate the effects of IL2 on cell permeability. We also used PGE_2 as control. Indeed PGE_2 has well documented vasoactive functions and its expression in inflammatory conditions in DCs also depend

by NFATc2 activation [37]. Transendothelial electrical resistance (TEER) value was used to estimate permeability of endothelial cells in vitro, using an electrical cell sensor system. Increase in the TEER value was associated with cell confluence and decrease in permeability, whereas decrease in the TEER value was associated with increase permeability. Therefore, HUVEC cells were grown on transwell and TEER value was measured every day in order to evaluate cell grown. Once the TEER value reached the plateau, indicating cell confluence (time 0), HUVEC cells were incubated with different concentrations of IL-2 or PGE_2. Finally, TEER value was measured to monitor cell permeability at different time-points. We observed decreased TEER values when HUVEC cells were incubated with both IL-2 and PGE_2, especially after 10 hours from incubation (Figure 18). As expected, PGE_2 was more effective than IL-2 in increasing endothelial cell permeability, even at lower concentrations (250 and 125ng/ml) and earlier time-points (2-4 hours). However, our results showed that even IL-2 is slightly vasoactive for endothelial cells. Moreover, the effect of IL-2 was significant not only at higher concentrations (400U/ml), but also at lower concentrations (200U/ml).

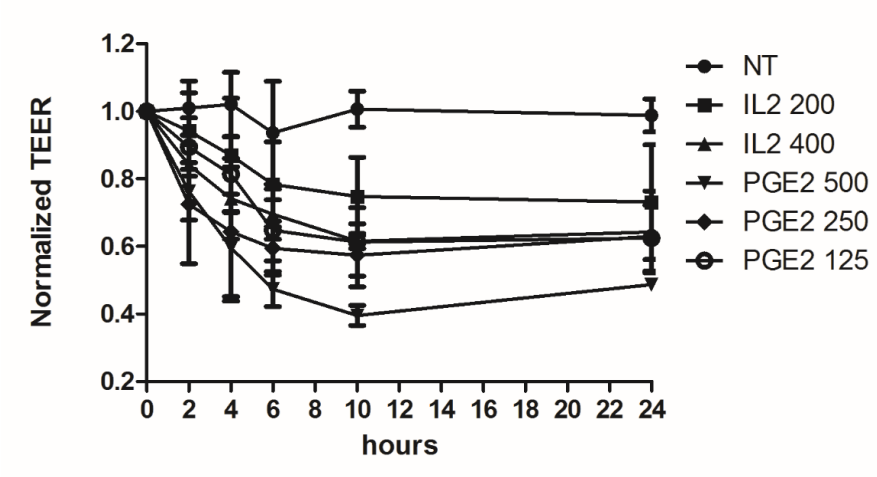
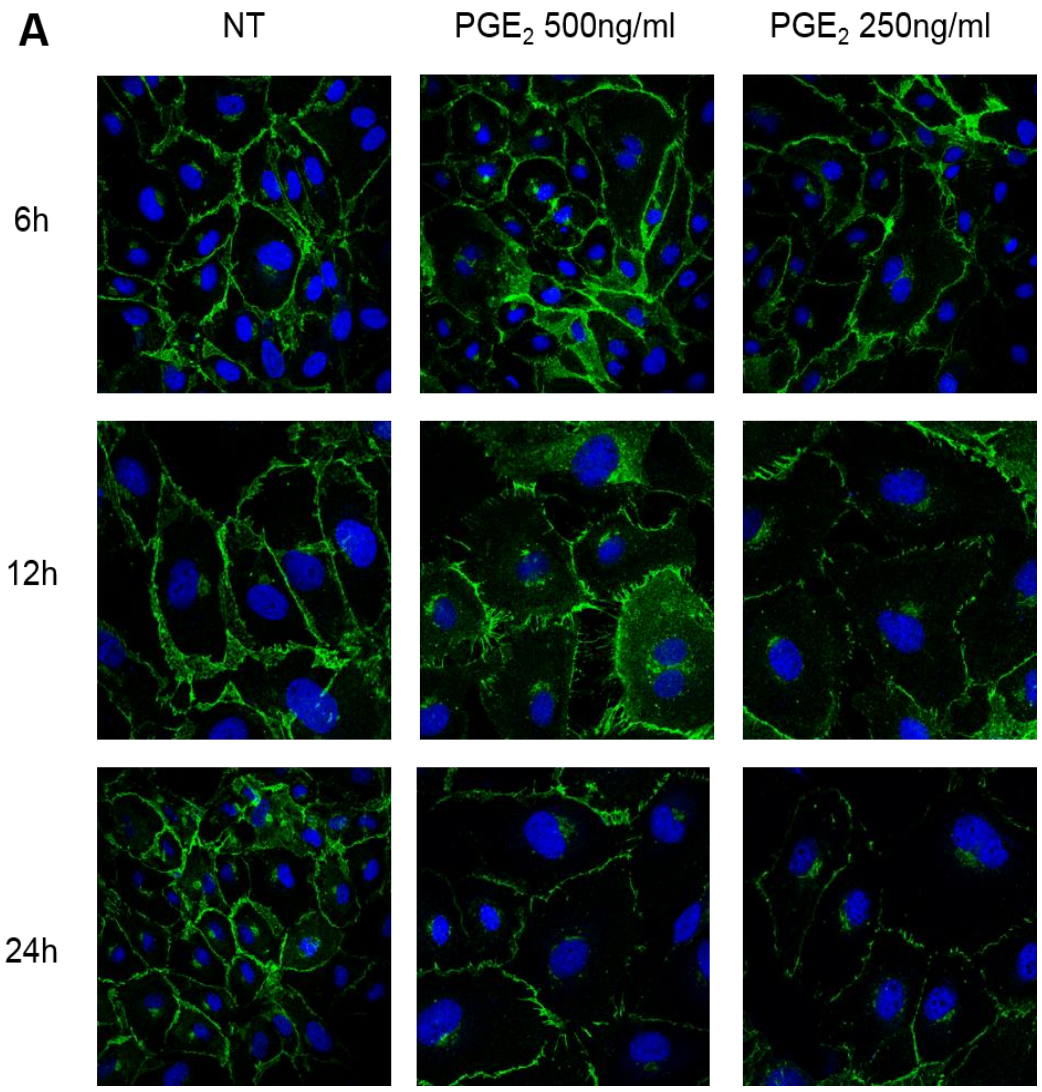


Figure 18: IL-2 and PGE_2 are able to induce cell permeability in vitro

HUVEC cells were grown on transwell filters and TEER was measured every day. Once TEER value reached the plateau (time 0), HUVEC cells were incubated with different concentrations of IL2 (400 and 200 U/ml) or PGE_2 (500, 250 and 125 ng/ml). Then, TEER values were measured at different time-points (2, 4, 6, 10 and 24 hours) in order to evaluate cell permeability. Values represent means of 2 indipendent experiments performed in triplicate + SD.

Since IL-2 is effective in increase endothelial cell permeability, we also decided to evaluate whether this cytokine could exert its effect promoting a change in adherent junctions. To this purpose, HUVEC cells were incubated at different time-points (6, 12 and 24 hours) with different concentrations of IL-2 or PGE_2 and the effect on VE-cadherin was analyzed using confocal microscopy. PGE_2 clearly modulated integrity of adherent junctions on cell membrane both at higher (500ng/ml) and at lower

(250ng/ml) concentrations. As shown in figure 19a, VE-cadherin progressively disappeared from the membrane of HUVEC cells; indeed, the effects are visible already 6 hours after incubation, but they become pronounced 12 and 24 hours after. Consistent with the previous data obtained measuring the TEER values, IL-2 slightly re-modulates the distribution of VE-cadherin on HUVEC cells. In particular, IL-2 effects on VE-cadherin were observed mainly 24 hours after the treatment (Figure 19b). Moreover, the re-distribution of VE-cadherin induced by IL-2 was dose-dependent. Since the structure of cytoskeleton is related to both adherent and tight junctions [38], we also evaluated the effects of IL-2 on actin filaments. From this analysis, we observed that the re-distribution of VE-cadherin dependent on IL-2 was also related with the generation of actin focal adhesion points, suggesting also an involvement of the cytoskeleton in the process (Figure 20).


A
NT
PGE2 500ng/ml
PGE2 250ng/ml
6h
12h
24h

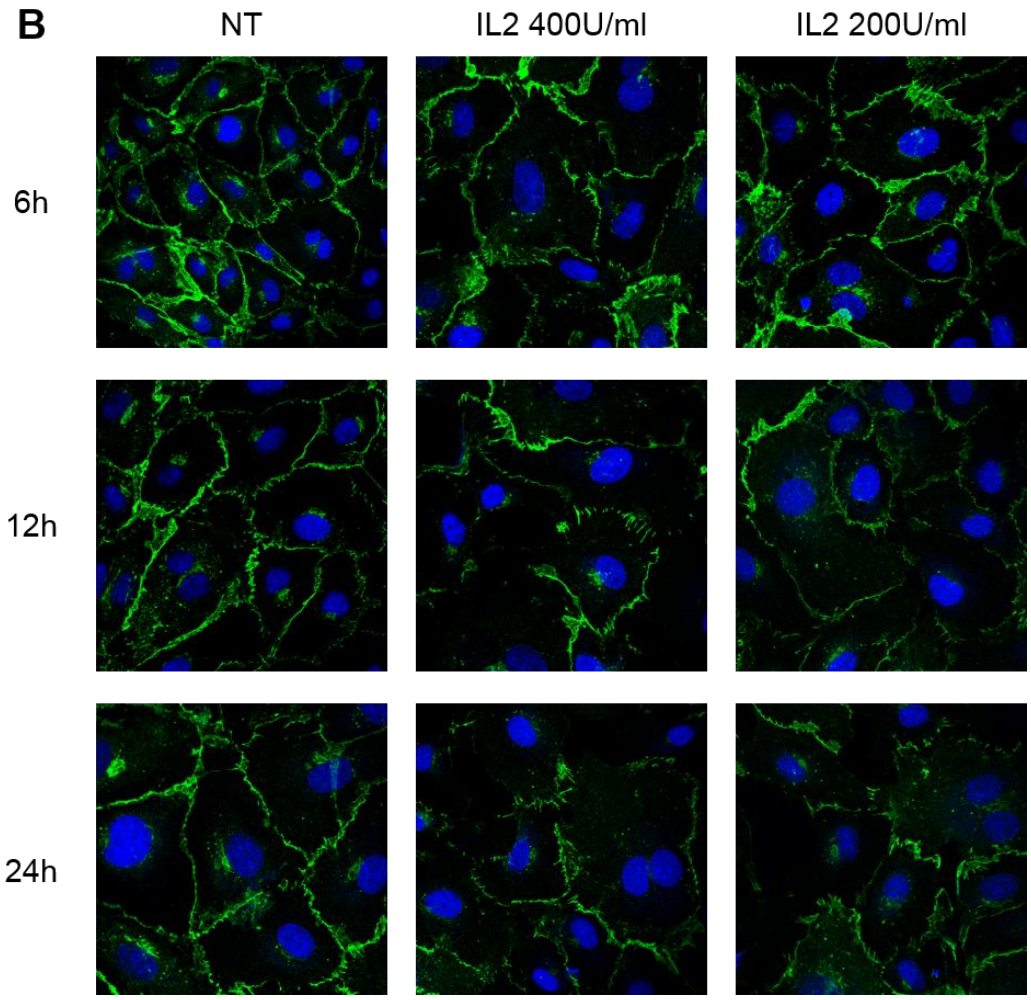


Figure 19: IL-2 and PGE_2 alter distribution of VE-cadherin in HUVEC cells

Effects of PGE2 (A) or IL-2 (B) on VE-cadherin. HUVEC cells (100000 cells) were incubated with different concentrations of PGE_2 (500 or 250 ng/ml) or IL-2 (400 or 200 U/ml) at different time-points (6-12-24 hours). Then, VE-cadherin (green) and nuclei (blue) were stained and confocal microscopy was performed.

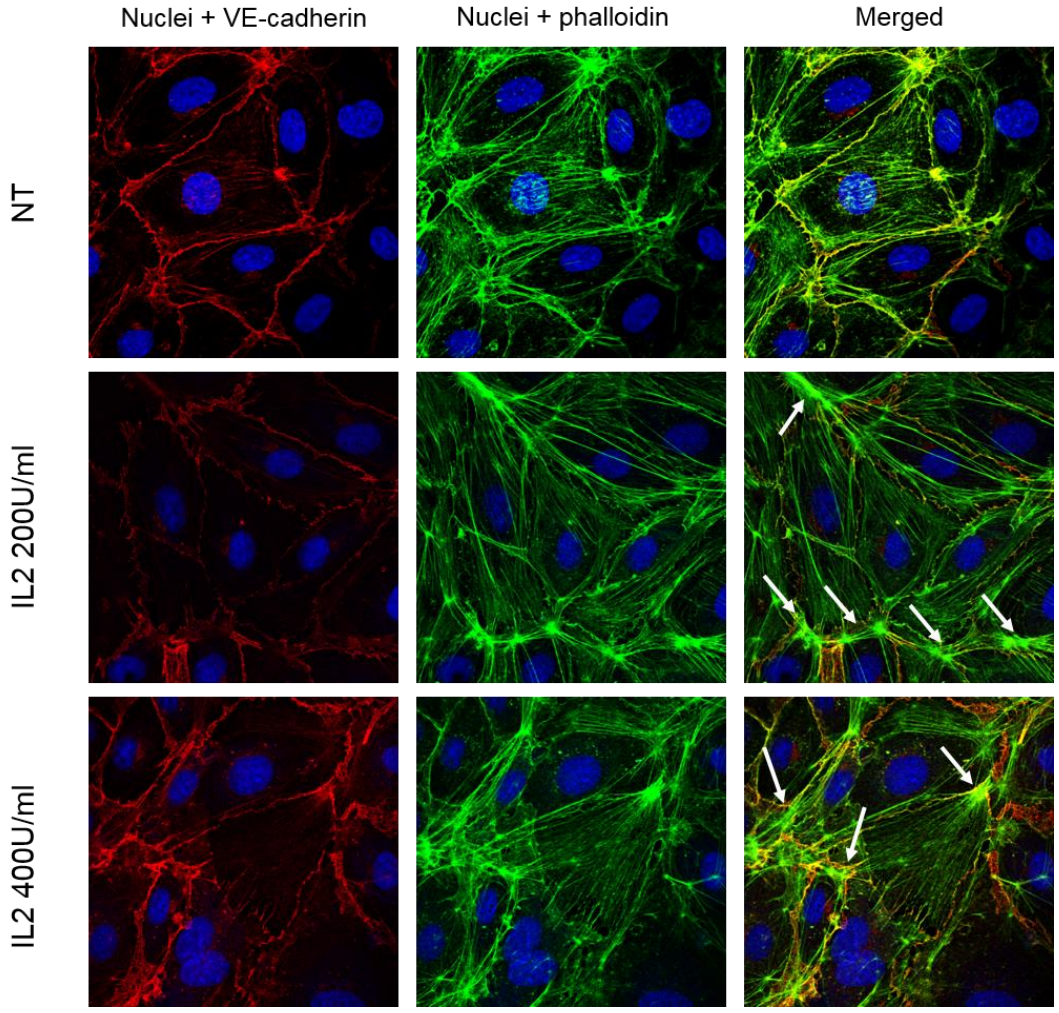


Figure 20: IL-2 alter distribution of cytoskeleton in HUVEC cells

HUVEC cells (100000 cells) were incubated with different concentrations of IL-2 (400 or 200 U/ml) for 24 hours. Then, VE-cadherin (red), phalloidin (green) and nuclei (blue) were stained and confocal analysis were performed. White arrow indicate focal adhesion points.

Finally, we also evaluated the blood vessels permeability in the transplanted mice. To this purpose, female recipient C57/BL6 WT mice received the skin of both donor male and female WT mice. After 6 or 10 days from the transplantation, mice were injected i.v. with Evans Blue dye [39] and euthanized in order to assess vascular permeability. As shown in figure 21, we observed increase vascular permeability (day 6) and angiogenesis (day 10) in recipient mice receiving the skin of donor WT male mice compared to the control group, conferming the fundamental role of blood vessels in transplant rejection.
Although our results suggest a role also for IL-2 in vascular permeability, further analysis are required in order to assess the effective contribution of this process in transplant rejection.

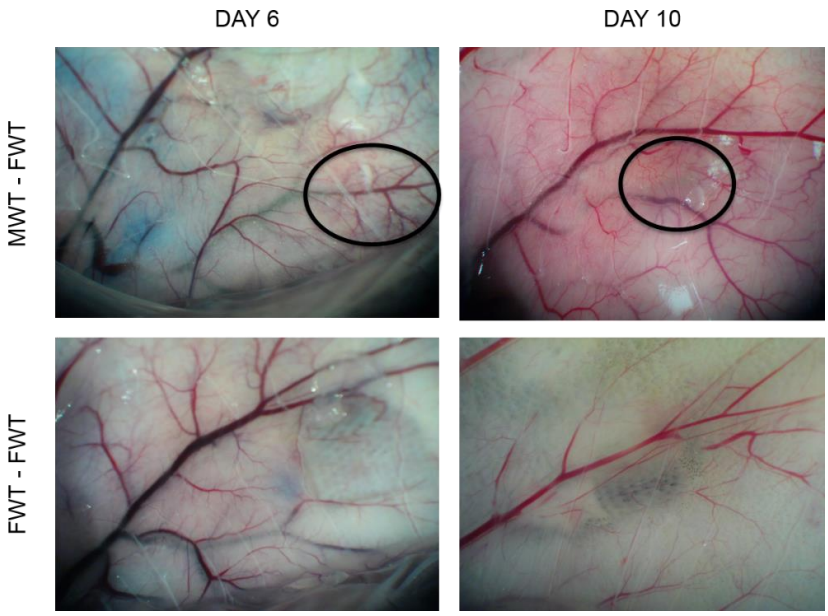


Figure 21: Recipient female mice receiving the skin of donor male mice showed increased vascular permeability and angiogenesis
Recipient female WT mice received the skin of donor male or female WT mice. At day 6 or 10, mice were injected i.v. with Evans Blue dye and then euthanized in order to determine blood vessels permeability. Black circle indicate the transplant areas.

2.9 Materials and methods

Cells. BMDCs were derived from BM progenitors of WT mice [7] and D1 cells were cultured as previously described [18]. HUVEC cells were purchased from Lonza and were cultured using EGM-2 endothelial cell growth media in presence of rhEGF (5ng/ml), VEGF, rhFGF, ascorbic acid (50ug/ml), L-Glutamine (10mM), hydrocortisone (1ug/ml), sulfate heparin (0,75 U/ml) and fetal bovine serum (2%).

Mice. WT CD45.2 and CD45.1 mice, K5-mOVA and OT-II transgenic mice were purchased from Harlan-Italy. E. Serfling and A. Schimpl (Institute of Virology and Immunobiology, Wurzburg, Germany) provided NFATc2-/- and IL2-/- mice, respectively. DEREG mice were obtained from T.Sparwasser (Twincore, Hannover, Germany) while DOG mice were provided by N. Garbi (Institute of Molecular Medicine and Experimental Immunology, Bonn, Germany). In DEREG and DOG mice, a specific $Foxp3^{+}$Treg or DC ablation (respectively) can be induced by diphtheria toxin injection [24-27]. All animals were housed under pathogen-free conditions, and all experiments were carried out in accordance with relevant laws and institutional guidelines.

Antibodies and chemicals. Antibodies for flow cytometry were purchased from Biolegend. Antibody against murine NFAT and NF-κB were purchased from Invitrogen, while antibody against human VE-cadherin were purchased from CellSignaling. Phalloidin was purchased from Invitrogen. TRL4-grade smooth LPS (E. coli,O55:B5) was purchased from Enzo Life Sciences. FK-506, thapsigargin, diphtheria toxin and Evans Blue dye were purchased from Sigma-Aldrich. PGE_2 was purchased from Cayman Chemical. F. Sallusto gently provided recombinant human IL2.

Nanoparticles synthesis. MYTS nanoparticles were gently provided by D. Prosperi (NanoBioLab, University of Milano-Bicocca, Milano, Italy). In brief, MNP were synthesized by solvothermal decomposition in octadecene from iron oleate precursor, as described previously [39]. MNP (10 mg) suspended in chloroform (5 mg mL^{-1}) were transferred to water phase by mixing with a 0.5 M solution of an amphiphilic polymer (poly(isobutylene-alt-1-tetradecene-maleic anhydride)) (PMA, 136 µL) in 5 mL of sodium borate buffer (SBB, pH 12) [40]. After activation of the carboxylate groups of the PMA by 0.1 M EDC (6.5 µL), 0.05 M 2,2-(ethylenedioxy)-bis(ethylamine) (EDBE, 2.5 µL) was added and stirred 2 h. Next, nanoparticle dispersion was concentrated and washed twice with water. The resulting PMA-coated nanoparticles (PMNP) were

dispersible in aqueous media. For PMNP suspension, PMNP as synthesized are concentrated in Amicon tubes (50 kDa filter cutoff) (Millipore Corporation, Billerica, MA) by centrifuging at 3000 rpm. Then, VIVIT peptide and PEG-SH (500Da) were added and the mixture was shaken for 2 hours. For uptake experiments, fluorescein isothiocyanate (FITC) was added to the mixture. Finally, the NPs suspension (MYTS) was concentrated and the final concentration determined by UV measurement.

In vitro and in vivo nanoparticles uptake. BMDCs were incubate with MYTS-FITC or MYTS-PEG NPs at 37°C or 4°C for 10, 30, 60 and 90 minutes. Then, cells were washed with PBS and FACS analysis were performed. For in vivo experiments, mice were injected i.p. once or for two weeks (every other day) with MYTS-FITC or MYTS-PEG NPs (100 µg/ml). Then, mice were euthanized and spleen, lymph nodes and skin were recovered and analyzed for flow cytometry.

ELISA assays. Concentrations of IL-2 and TNFα in BMDCs or D1 supernatants were assessed by ELISA kits purchased from R&D Systems and eBioscience, respectively.

Immunocytochemistry. BMDCs or HUVEC cells (100000 cells) were seeded on glass coverslips. BMDCs were first

incubated with MYTS-VIVIT, MYTS-PEG NPs (25 μg/ml) or FK-506 (10 ng/ml) for 90 minutes and then incubated with LPS (1μg/ml) plus thapsigargin (50nM) for 40 minutes. HUVEC cells were incubated with IL-2 (400 or 200 U/ml) or PGE_2 (500 or 250 ng/ml) for 6, 12 or 24 hours. Then, cells were fixed in paraformaldehyde 4% and then were permeabilized with 0.2% BSA 0.1% Triton X-100 in PBS. Successively, cells were kept in blocking solution (2% BSA in PBS) for 30 min. For BMDCs, rabbit anti-mouse NFATc2 or rabbit anti-mouse NF-κB were used. For HUVEC cells, rabbit anti-human VE-cadherin was used. All primary antibodies and secondary anti-rabbit AlexaFluor 488 or 555 antibodies diluted in blocking solution were added and incubated at room temperature. For HUVEC cells, phalloidin was added. Finally the samples were mounted in FluorSave™ Reagent (Calbiochem) and were imaged by Leica TCS SP2 confocal microscope. ImageJ software was used for image analysis and processing.

In vivo treatment with NPs or FK-506. For in vivo treatment, FK-506 was resuspended in 40% w/v HCO-60/ethanol at the dose of 40μg/ml. MYTS-VIVIT and MYTS-PEG NPs were diluted in sterile PBS at 100 μg/ml. Mice were injected i.p. with FK-506 or NPs the day before the transplant and every other day for 50 days. For the

donor mice treatment, mice were i.p. injected 10 days before the transplant every other days.

Skin grafting. Sections of donor skin for grafting were taken from the tail and transplanted onto the dorsum of recipient mice. Dressings were removed on day 14, and grafts were observed daily until rejection or the end of the experiment. Rejection was determined when the graft became erosive or scale-encrusted.

Foxp3$^+$Treg or DC depletion. Diphtheria toxin (40 ng/g) was daily administered to DEREG mice through an i.v. injection for 2 consecutive days, while 16ng/g of DT was daily administered (i.v.) for 3 consecutive days to DOG mice. Control mice were given PBS. Effective Foxp3$^+$Treg or DC depletion was assessed by FACS analysis.

Immunohistochemical analysis. Cryostat sections were fixed at room temperature for 15 seconds, air dried, and permeabilized with methanol for 3 minutes. Then, they were kept in blocking solution (PBS-BSA 0,1%) for 10 minutes. Rabbit anti-mouse NFATc2 and anti-rabbit AlexaFluor 555 diluted in blocking solution were added and incubated at room temperature. Samples were mounted in FluorSave™ Reagent (Calbiochem) and were imaged by Leica TCS SP2 confocal microscope. ImageJ software was used for image analysis and processing.

Quantitative real-time PCR in vivo. Transplanted skin was recovered, briefly washed in cold PBS, lyzed in TRIzol solution and mechanically disrupted using a TissueLyser (20 shakes for 10 minutes). Total mRNA was extracted with an RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions. A NanoDrop spectrophotometer (ThermoScientific) was used to quantify mRNA and to assess its purity, and 700 ng mRNA was retrotranscribed to cDNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Then 10 ng cDNA was amplified using the Power SYBR Green PCR Master Mix (Applied Biosystems) in a 7500 Fast Real-Time PCR System (Applied Biosystems), and data were analyzed using the built.in software. Primer pairs used were as follows: 5'-AGCATGGTTCTTCTCAGGAGC-3' and 5'-AGCCCCTTTCAAATCTCCAGT-3' (Tslp); 5'-CGAAAGCATTTGCCAAGAAT-3' and 5'-AGTCGGCATCGTTTATGGTC-3' (18S). 18S mRNA was used as an internal reference for relative quantification studies.

Sample preparation and array hybridization. Total mRNA was extracted from transplanted skin with an RNeasy Mini Kit (QIAGEN). An aliquot (100 ng) of RNA was used for the preparation of targets for Affymetrix®

Mouse Gene 2.0 ST arrays (Affymetrix, Santa Clara, CA), according to the Affymetrix WT Plus Reagent kit manual. Briefly, for the microarray experiment, 100 ng of total RNA was used for ssDNA synthesis, using the GeneChip® WT Plus Reagent Kit (Affymetrix), adding in the mix 2 µl of Poly-A RNA controls (*lys, phe, thr* and *dap*). The ssDNA was finally fragmented and labelled according to the manufacturer's protocol. Then, it was injected into Affymetrix Mouse Gene 2.0 ST microarray chips and the hybridization was performed under rotation at 45°C ± 16 hours. After the hybridization, the arrays were washed and stained with R-phycoerythrin streptavidin in the GeneChip® Fluidics Station 450. The images were scanned by Affymetrix GeneChip Command Console (AGCC) and analyzed with the Affymetrix GeneChip Expression Console.

TEER measurements. HUVEC cells (10000 cells) were seeded onto polycarbonate transwell inserts (6.5 mm diameter and 0,4 µm pore size) coated with 4 µg/ml fibronectin. TEER was measured daily using EVOM2 instrument. Once TEER values reached plateau, HUVEC cells were incubated with IL-2 (400 or 200 U/ml) or PGE_2 (500, 250 or 125 ng/ml) and TEER values were measured after 2, 4, 6, 10 and 24 hours.

Isolation of skin cells. Skin was isolated and digested for 90 minutes at 37°C in complete medium (RPMI 5% FBS, L-glutamine and antibiotics) plus 0.25 mg/ml of Liberase (Roche) and 0.2 mg/ml DNase I (Sigma). After the digestion, cells were passed through a 70 µm cell strainer and centrifuge. Then, FACS analysed were performed.

Flow cytometry. Single-cell suspensions of spleen, lymph nodes or skin were pelleted and resuspended with the appropriate amount of antibody in 200 µl of PBS, and incubated for 20 minutes on ice in the dark. The cells were than washed once with 1 ml of PBS. When required, secondary reagent incubation in 200 µl of PE-Cy5.5 conjugated streptavidin (diluted 1/500) was performed for 15 minutes on ice in the dark. For FACS analysis, the following antibodies were used: anti-CD11c APC, anti-CD11b FITC, anti-F4/80 biotin, anti-CD4 APC-Cy7, anti-CD8 PE, anti-CD45.1 PE and anti-CD45.2 PE-Cy7. Data were acquired using a Beckman-Coulter FACS Gallios and analyzed with FlowJo (TreeStar) software.

Statistical analysis. Means were compared by paired t tests. Data are expressed and plotted as mean ± S.D. values. Sample sizes for each experimental condition are provided in the figures and the respective legends.

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3. Final considerations

3.1 Summary

Nuclear factor of activated T cells (NFAT) is known as an inducible nuclear factor that could bind the interleukin-2 (IL-2) promoter in T cells [1]. The NFAT family of transcription factors comprises five members divided in two groups. The NFAT5 member [2], which is the most ancient form regulated by osmotic stress, and the NFATc family, that includes NFAT1, NFAT2, NFAT3 and NFAT4 (also known as NFATc2, NFATc1, NFATc4, NFATc3, respectively), regulated by the activity of the Ca^{2+} / calcineurin signaling pathway [3].

Many studies have now defined crucial regulatory roles for the NFAT family member in numerous developmental programs in vertebrates, both in immune and non-immune cell types [4-5]. However, the NFAT proteins function remains best understood in the immune system, especially in adaptive immunity and in particular for T cells development, activation and differentiation [6-7].

Recent studies have highlighted new roles for NFAT members in B cells and in innate immune cells, such as DCs, macrophages and mast cells. Importantly, two signal transduction pathways, the one initiated by Dectin-1 [8] and the one initiated by CD14, have been characterized for their ability to induce NFATc activation in DCs. In this

regard, Granucci and co-workers [9] showed that CD14 is a signaling receptor that acts independently of TLRs. After stimulation with LPS, the activation of CD14 in DCs is responsible for the induction of a rapid and transient influx of Ca^{2+} ions. Then, the consequent increase in the cytosolic Ca^{2+} levels triggers the activation of calcineurin and NFAT. Once into the nucleus, NFATc could exert both pro- and anti-inflammatory effects.

A recent study has evidenced the fundamental role of NFATc as regulator of edema formation in response to LPS-induced inflammation [10].

Moreover, the study showed that NFAT in DCs controls the local swelling formation by controlling production of prostaglandin E_2 (PGE_2), a member of prostanoids.

The studies of Granucci and co-workers [9] showed that NFAT also promotes the expression of pro-apoptotic genes at late time points after stimulation with LPS. Among these genes, Nur77 was principally identified as an NFAT-dependent pro-apoptotic gene.

Finally, there is evidence that NFATc pathway could be important for the crosstalk between innate and adaptive immunity and that may be fundamental for DCs-dependent T cell activation. DCs are APCs that have the peculiar ability to decide whether and how a T cell response should be activated. Several studies have reported the fundamental role of IL-2 produced by DCs in this process [11-12].

Since IL-2 is controlled by the NFATc family, our hypothesis is that NFATc activation in DCs could also be fundamental in a condition of sterile inflammation, like acute transplant rejection, where alloreactive T cell responses play a crucial role.

Acute grafts rejection is the result of a complex series of interactions involving coordination between both the innate and adaptive immune system with T cells being central to this process. The cascade of acute rejection begins with the presentation of donor alloantigens (both mismatched MHC complexes and miH antigens) presented by APCs, mostly DCs, to T cells [13].

For these reasons, NFATc is consider an attractive target for the therapeutic modulation of immune response. Cyclosporine A and tacrolimus (FK506) have been extensively used as immunosuppressive agents to improve graft survival [14]. Although highly successful, these inhibitors act by blocking calcineurin enzymatic activity, affecting other pathways and leading to nephrotoxicities and neurotoxicities. Moreover, they does not allow the specific study of NFAT inhibition in innate immune cells. The development of more specific inhibitors of NFAT function is thus required.

The study of the regulatory domain of NFAT activation has allowed the identification of a peptide with a high-affinity for the calcineurin-binding sequence PXIXIT [15-16]. This peptide, called VIVIT (Pro-Val-Ile-Val-Ile-Thr), is able to

bind with high affinity calcineurin and to effectively compete with NFAT for calcineurin binding, thus blocking NFAT dephosphorylation.

However, the therapeutic use of peptide inhibitors is still limited by issues regarding the stability and delivery to desired cells. The use of new delivery tools like nanoparticles (NPs) could, therefore, help to overcome these problems.

In collaboration with the Nanobiolab of the University of Milano-Bicocca, we were able to generate iron-oxide NPs conjugated with the VIVIT peptide (MYTS-VIVIT NPs).

We demonstrated that MYTS-VIVIT NPs are able to inhibit NFATc translocation and IL-2 production in DCs, without affecting other pathways, like the NFκB pathway. Moreover, we also showed that MYTS NPs were only internalized by phagocytes (DCs, macrophages and neutrophils) when administered in vivo.

Thanks to the MYTS-VIVIT NPs, we were able to study the role of the NFATc signaling pathway in innate immune cells in the context of acute transplant rejection.

3.2 Discussion and future perspectives

In order to evaluate the role of the NFATc signalling pathway in DCs during acute rejection, we set up a minor histocompatibility mismatched skin transplant model and we treated the grafted mice with the MYTS-VIVIT NPs. Our

results demonstrated that NPs treatment favours graft acceptance, suggesting a fundamental role of the NFATc pathway in innate cells during the process of acute transplant rejection. Interestingly, MYTS-VIVIT NPs resulted more effective than commonly used immunosuppressive drugs, like tacrolimus, to induce long-term survival of the transplant. Indeed, mice treated with tacrolimus still rejected the transplant. Furthermore, they were unable to maintain the graft when the treatment was interrupted and to accept a second skin transplant. On the contrary, mice treated with MYTS-VIVIT NPs accepted the graft even when the treatment was interrupted and they also rejected slowly a second skin graft. Taken together, our results demonstrated that activation of NFAT pathway in innate immune cells is fundamental to lead to transplant rejection. Furthermore, our results also suggested that MYTS-VIVIT NPs could induce a potential tolerance mechanism to the transplant. We proposed that inhibition of the NFAT pathway and IL-2 production in DCs could affect the fate of T cells in lymph nodes, preventing activation of alloreactive T cells and/or inducing T regulatory cells generation. Several studies have reported the key role of DC-produced IL-2 to determine T cell fate in a steady-state condition [17] or after encounter of an inflammatory stimulus [11]. In particular, a recent study has demonstrated that CD25 expression on DCs can serve to present IL-2 in trans to antigen-specific T cells

[12]. As antigen-driven proliferation of T cells was found to depend on CD25 expression by DCs, it has been proposed that DCs- dependent IL-2 trans-presentation presumably operate very early during T cell stimulation. IL-2 produced by DCs at the immunological synapse could, therefore, represent a fundamental signal for T cell activation.

Moreover, the fate of naïve T cells is also determined by three signals that are provided by mature DCs. In particular, the cytokine environment created by DCs during the course of T cell activation is one of the key determinants for the differentiation into distinct T cell subsets. For instance, interleukin-10 (IL-10) and TGF-β production are associated with the development of T regulatory cells and the induction of tolerance [18]. In this regard, it has been proposed that the secretion of IL-2 by DCs could be necessary to counteract the effect of IL-10. Indeed, it has been shown that IL-10 induces hyporesponsiveness of $CD4^+$ alloantigen T cells in the absence of IL-2 [19].

Therefore, the treatment with MYTS-VIVIT NPs may result in the formation of anergic or non-responsive T cells and/or in the generation of Treg that in turn induce tolerance to the transplant. However, more experiments are required to fully elucidate the mechanisms that underline this process and whether a Treg cell generation occur after treatment with MYTS-VIVIT NPs.

The fundamental role of the NFATc pathway and IL-2 production were also confirmed by results obtained from experiments on donor mice. Treatment of donor mice with MYTS-VIVIT NPs delayed rejection of the allograft of at least two weeks compared to control mice. Similar results were also obtained when we used IL2 deficient, NFATc2 deficient and DC-depleted mice as donor mice. Taken together, these data highlighted the central role of donor DCs in inducing allograft responses during the first days after transplantation. Once again, NFATc activation is crucial for donor DCs to promote alloreactive T cell activation and transplant rejection. In this regard, our data demonstrated that pre-treatment of donors lead to a better outcome of the graft and suggested that the combined treatment of donor and recipient could be effective to decrease acute transplant rejection.

As demonstrated by our results on NFATc translocation in donor tissues and on gene expression analysis, the process of inflammation at the basis of graft rejection starts very early after surgery. Indeed, transplanted mice displayed a different gene profile already three days after transplantation. Furthermore, NFATc2 activation and translocation was already visible in male donor tissues at this time. We hypothesized that early NFAT activation in DCs may favour local inflammation into the graft and that IL-2 could contribute to this process increasing vasodilation and favouring lymphocyte extravasion. The

potential ability of IL-2 to induce vasodilation is also suggested by the findings that high-doses IL-2 administration in carcinoma patients cause vascular leak syndrome (VLS). VLS is associated with increased vascular permeability, hypotension, pulmonary edema, liver cell damage and renal failure [20]. Recent data suggest that VLS is primarily mediated by the direct binding of IL-2 to endothelial cells that induces increased permeability [21]. Recently, several studies have also demonstrated that non-lymphoid cells, like fibroblast and endothelial cells can express component of the IL-2R. In particular, the function of IL-2R on pulmonary endothelial cells was demonstrated by the finding that IL-2 stimulation led to an increase in the levels of phosphorylated STAT5 and the production of high levels of nitrite in these cells [22]. One possibility is that the expression of IL-2R on non-lymphoid cells contributes to the control of IL-2 homeostasis in non-lymphoid organs. Alternatively, the trans-presentation of IL-2 bound to endothelial cells in inflamed blood vessels might provide survival signals to activated effector T cells that are extravasating to the site of an ongoing immune response, such as acute transplant rejection. In this regard, our data on HUVEC also showed an increased permeability in response to different doses of IL-2. Although our preliminary results suggest a role of IL-2 in endothelial permeability, further experiments are required to understand whether IL-2 could have a role in

promoting local inflammation that leads to accelerate graft rejection.

Finally, we performed a microarray analysis to evaluate differentially expressed genes during transplant acceptance or rejection. Our attention focused mainly on two genes, Tslp and Ly6G, which were upregulated in donor male and female tissues, respectively. In particular, thymic stromal lymphopoietin (TSLP) is an interleukin 7-like cytokine secreted by epithelial cells such as those of the airway, intestine and skin [23]. TSLP acts on different lineages of immune cells, like B cells, DCs, T cells, mast cells and eosinophils. In particular, TSLP can act via DCs to regulate the activation and differentiation of T cells [24-25]. Furthermore, it has demonstrated that TSLP could induce a pro-inflammatory phenotype in human $CD14^+$ monocytes [26-27] and promote activation of $CD11c^+$ DCs [28]. A recent study [29] also demonstrated that multiple activators of TLRs, including zymosan, LPS, and CpG, could each induce expression of TSLP in mouse bone marrow-derived DCs and splenic DCs. Moreover, several studies also reported that NFAT pathway regulates TSLP production [30-31]. Here, we demonstrated that although Tslp expression is increased in all transplanted mice, its expression is significantly upregulated in donor tissues that undergo rejection. Moreover, Tslp upregulation did not occur when we used the skin of NFATc2-deficient mice. Taken together, these data could indicate that DCs could

be a source of TSLP production during acute rejection that may participate to promote local inflammatory event and favour the recruitment of inflammatory cells. Although further experiments are required, this hypothesis seems to be confirmed by flow cytometry analysis on donor tissues. Indeed, the presence of increased number of $CD11c^{+}$ DCs is detected in donor male tissues 2 and 3 days after surgery. On the other hand, we found that donor female tissues express higher levels of lymphocyte antigen 6 complex (Ly6G) and have increased number of $CD11b^{+}Gr1^{+}$ cells 3 days after transplantation. Ly6G is usually expressed by granulocytes and peripheral neutrophils and directly correlates with the level of cell differentiation and maturation [32]. Moreover, Ly6G is also expressed by a recently discovered cell subset, the myeloid derived suppressor cells (MDSCs) [33]. The term MDSCs is used to define mouse cells elicited under pathological conditions that express CD11b and Gr-1 antigens and do not or weakly express other markers of mature myeloid cells [34]. Currently, two main MDSCs population have been characterized: monocytic MDSCs ($CD11b^{+}Gr1^{int}Ly6G^{-}Ly6C^{hi}$ cells) and polymorphonuclear MDSCs ($CD11b^{+}Gr1^{hi}Ly6G^{+}Ly6C^{low/int}$). MDSCs exploit a plethora of mechanisms to influence both innate and adaptive responses. In particular, they upregulate genes either related to the metabolism of amino acids or involved in production of reactive oxygen species. More

importantly, they have the potential to suppress immune responses of T cells both in vitro and in vivo and the potential to induce Treg cell generation [35].

Taken together, our data supported a model in which innate immune cells could contribute to create a pro-inflammatory environment during the early phases of acute transplant rejection. Moreover, we demonstrated that the activation of NFAT pathway and the IL-2 production in both donor and recipient DCs have a central role in this process. On the other hand, we postulate that transplant of syngeneic graft lead instead to the recruitment of suppressor cells, like MDSCs. Furthermore, we hypothesize that inhibition of NFAT pathway in DCs with MYTS-VIVIT NPs during transplant may block the activation of alloreactive T cells and allow the generation of Treg cell, which in turns mediate tolerance to the transplant.

In conclusion, although further studies are required, we demonstrated the central role of the NFAT pathway in innate cells during acute transplant rejection. Moreover, we proposed MYTS-VIVIT NPs as a useful tool to study the effect of NFAT inhibition

in myeloid cells and as a potential treatment for acute transplant rejection.

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