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Long-term graft tolerance induction by NFATc pathway inhibition in innate immune cells

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CHAPTER 1. GENERAL INTRODUCTION

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1.1 Introduction

Over a period of several hundred million years, multicellular organisms have survived by developing increasingly complex defense mechanisms to prevent disseminated infections, control repair and restore tissue homeostasis. All species have evolved with a facility to protect themselves against pathogens: the immune system. The immune system encompasses several members with peculiar roles, and it can be divided into: innate immunity and adaptive or acquired immunity (Parkin and Cohen 2001). The former stands out as the most ancient form of response to pathogens, providing defense pathways highly conserved among species, both invertebrate and vertebrate (Kimbrell and Beutler 2001). The adaptive immunity has specifically developed in vertebrates, more complex organisms in evolutionary terms. Indeed, they are provided with both the two interconnected arms of immunity, the innate and the adaptive, which synergistically collaborate to provide a more intricate and efficient degree of defense (Boehm 2012). Innate and adaptive immunity exhibit peculiar properties that intervene temporally and spatially distinctly.

1.2 Innate immunity

Innate immunity represents the rapid first line of intervention upon microbial infections, to provide both immediate defense to the invading pathogen and initiate adaptive responses that will establish antigen-specific immunological memory. Therefore, body surfaces and mucosal barriers belong to the innate arm of immunity, displaying chemical and physical properties to prevent ingress of exogenous organisms (Doran et al. 2013). To provide a higher degree of defense, the body surfaces directly exposed to the external environment are patrolled mainly by innate leukocytes acting like sentinels. As shown in Figure 1, several cell-types enrich the innate compartment: the mononuclear phagocyte system, composed by dendritic cells (DCs), monocytes and macrophages (Guilliams et al. 2015), granulocytes, as neutrophils, basophils and eosinophils, mast cells, natural killer cells (NKs) and for certain extents NKT cells and γδ T cells (Figure 1). In addition, innate immunity includes a humoral arm, composed by the complement system, pentraxins and naturally occurring antibodies (Shishido et al. 2012).

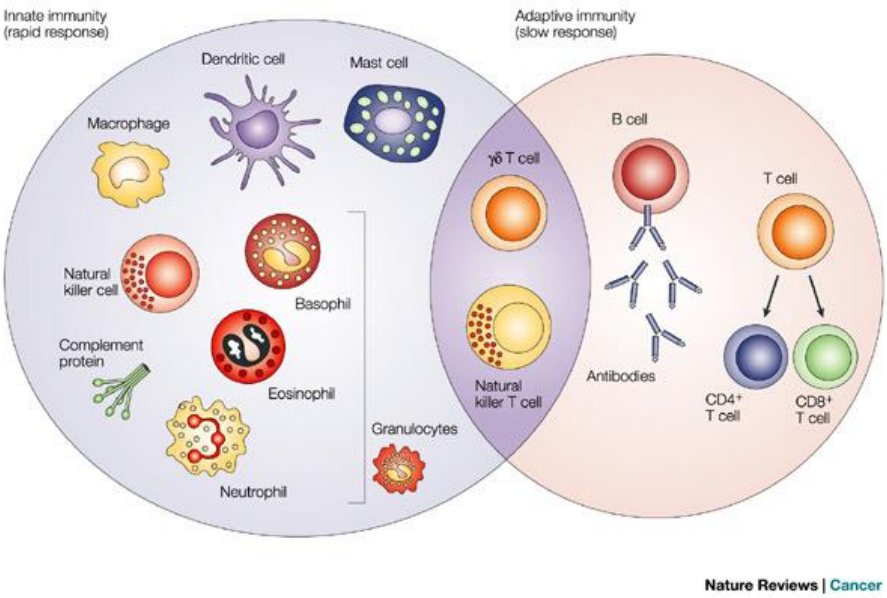

Figure 1: The immune system

The Figure displays the subdivision of the immune system into its two arms: the innate immunity, which encompasses granulocytes, monocytes/macrophages, dendritic cells, mast cells and others, and the adaptive immunity, which includes T lymphocytes, B lymphocytes. Natural killer T cells and γδ T cells share features with both the branches of the immune system. Adapted from Dranoff 2004.

1.2.1 How innate immunity senses external stimuli: Pattern Recognition Receptors (PRRs)

In 1989 Charles Janeway theorized that the capability of innate immune cells of discriminating between infectious non-self and non-infectious self (Infectious Non-self theory, INS theory) occurs via specific receptors and that their engagement leads to the activation of antigen presenting cells (APCs) and further antigen-specific responses orchestrated by T lymphocytes (Janeway 1992). This hypothesis paved the way for the discovery of a limited number of germline-encoded receptors in innate immune cells, required to sense the invading microorganisms: the pattern recognition receptors (PRRs) (Akira, Uematsu, and Takeuchi 2006). PRRs recognize highly conserved molecules, shared among pathogens always referred to as pathogen-associated molecular patterns (PAMP). PAMPs constitute molecules expressed by microorganisms necessary for their survival and thus difficult to eliminate or alter for the immune evasion. PRRs are categorized in 5 families, based on protein domain homology: Toll-like receptors (TLRs), C-type lectin receptors (CLRs), nucleotide-binding domain, leucin-rich repeat (LRR)-containing (or NOD-like) receptors (NLRs), RIG-I-like receptors (RLRs) and the AIM2-like receptors (ALRs). TLRs and CLRs localize at the plasma membrane to interact with extracellular microbial ligands and within endosomes, while

NLRs, RLRs and ALRs reside in the cytoplasm where they sense putative intracellular pathogens. This strict compartmentalization provides some elusive insights into the pathogen that infected the host and, in parallel, drives the type of response required. Indeed, upon engagement, receptors move to specific intracellular sites dictating the adaptor proteins that intervene and skewing the signaling cascade (Brubaker et al. 2015). TLRs can be considered as the prototype of PRRs, in terms of signaling cascade and final outcome (Figure 2). 11 human TLRs and 13 mouse TLRs have been identified. Each TLR recognizes distinct PAMPs derived from various microorganisms and can be distinguished also depending on the ligands they bind. TLR1, 2, 4 and 6 recognize lipids and lipopeptides, TLR5 and mouse TLR11 bind proteins while TLR3, 7, 8 and 9 sense nucleic acids (Kawai and Akira 2007). Generally, TLRs engagement culminates with the activation of the Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-κB) and Activated Protein 1 (AP-1) TFs, leading to the production of pro-inflammatory cytokines and upregulation of costimulatory molecules in DCs. The paradigm of TLRs pathway is well represented by TLR4 signaling. The main ligand of TLR4 is lipopolysaccharide (LPS), a component of the cell wall of Gram-negative bacteria. Truly, LPS binds to a complex of proteins: TLR4, its co-receptor CD14 and MD2. Upon engagement, the complex dimerizes and recruit the adaptor protein TIR domain

containing adaptor protein (TIRAP) to its TIR (Toll/Interleukin-1 receptor) homology domain, triggering Myeloid differentiation primary response 88 (MyD88) recruitment (Kagan and Medzhitov 2006). Finally, the signaling cascade culminates in the activation of NF-κB and AP-1 TFs, as already mentioned (Akira and Takeda 2004). After MyD88-mediated signaling has been induced, TLR4 is internalized via a process that specifically requires CD14 (I Zanoni et al. 2011). This different cellular localization leads to the initiation of another cascade that exploits the adapter TRIF-related adaptor molecule (TRAM) and TIR domain-containing adaptor protein-inducing interferon β (TRIF) and results in the activation of Interferon Regulatory Factor-3 (IRF3), mediating type I IFN transcription (Kagan et al. 2008; I Zanoni et al. 2011). Therefore, the recognition of microbial component and the PRRs engagement not only regulate the activation of innate immunity, but also instruct the subsequent acquired immunity, in order to mount the appropriate type of response.

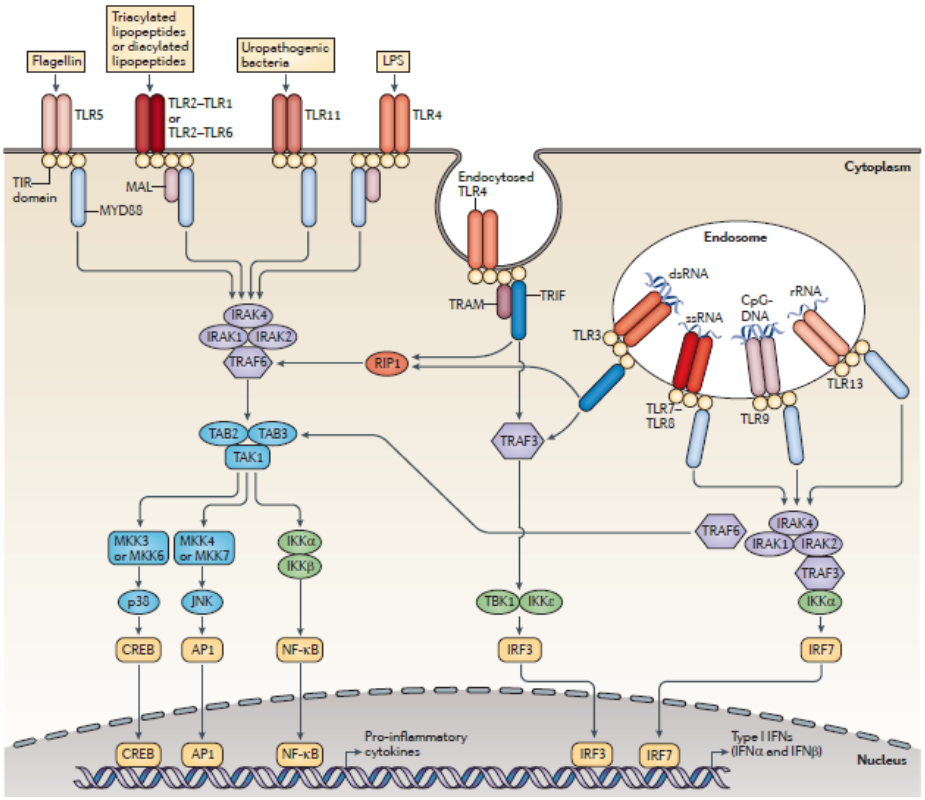

Figure 2: Toll-like receptors signaling cascades

Toll-like receptors belong to the repertoire of germline-encoded pattern recognition receptors innate immunity is provided with. TLR1, 2, 4, 5, 6 and 11 localize on the plasma membrane while TLR3, 7, 8 and 13 reside in endosomal compartment. The Figure displays the distinct adaptor proteins and TFs activated downstream each TLR engagement. Adapted from Neill, Golenbock and Bowie 2013.

The emerged role of PRRs confirmed the INS theory by Janeway, even though the immune responses observed and reported in other contexts such as tolerance against microbiota, immunity against tumors, autoimmunity and tissue injuries could not be efficiently explicated by this theory. Hence, in 1994, before the discovery of PRRs, Polly Matzinger proposed the so-called Danger theory (Matzinger 1994). According to Matzinger, immune responses are triggered by danger signals or alarm signals, expressed and hidden in all the body's own cells, that are released upon stress and injuries. These molecules have been defined damage-associated molecular patterns (DAMPs) and, like PAMPs, they are recognized by conserved receptors on APCs to induce adaptive responses to damage. Thus, in physiological conditions, PRRs and their endogenous ligands are localized in different cellular compartments, preventing their encounter and subsequent signaling cascade, while in pathological contexts they are released passively from injured tissues or dying cells. The two theories, Janeway's and Matzinger's, are not mutually exclusive and they complement each other for several aspects of immune responses. To corroborate the Danger theory, in the last years, several endogenous ligands have been proved to engage PRRs, in particular TLRs (L. Yu, Wang, and Chen 2010), inducing sterile inflammation. DAMPs so far identified can be categorized into: intracellular constituents as nucleic acids or proteins that remain

sequestered in a precise spatial distribution into specific organelles of cells in homeostatic conditions, but that are released upon stress, injury or damage and become exposed. The other category encompasses extracellular components that are structurally and chemically modified in stressed conditions. Among the intracellular molecules, the most characterized so far are Heath shock proteins (Hsp) and High mobility group box-1 (HMGB-1). Hsp act like intracellular chaperons of naïve and aberrantly folded proteins, in particular under stressful conditions. When Hsp are released in the extracellular space due to necrosis or cell damage, they are capable of binding specifically to TLRs inducing APCs activation and further T cell triggering (H. Fang et al. 2011). Similarly, HMGB-1 is a non-histonic protein dedicated to the stability of nucleosomes, thus localizing into the nucleus (Celona et al. 2011). Upon necrosis, cell membrane disruption results in the passive release of HGMB-1, which becomes ligand of TLR4 and promotes TNF-α production in MΦ (H. Yang et al. 2010). The other endogenous ligands for TLRs cited are extracellular matrix components as fibronectin, heparan sulphate, biglycan, fibrinogen, oligosaccharides of hyaluronan and hyaluronan fragments (Miyake 2007). Therefore, nowadays, both Janeway's and Matzinger's theories are, to some extent, concomitantly accepted, and still the contribution of invading pathogens and

tissue damage in engaging PRRs in complex contexts is still controversial.

1.3 APCs: key bridge between innate and adaptive responses

With the appearance of the adaptive immune system in jawed fish (Pancer and Cooper 2006), several tolerance mechanisms have evolved in parallel to suppress the generation of autoimmunity or allergic diseases. The capability of the immune system to discriminate between foreign antigen ("non-self") and endogenous molecules ("self") has always been of extreme interest for the scientific community. Initially, Burnet hypothesized that each lymphocyte exhibits a different receptor specific for the recognition of a precise foreign antigen, suggesting that to prevent autoimmunity T cells expressing receptor for self-antigens are rapidly eliminated in early life (Sir M Burnet 1959; Sir Macfarlane Burnet 1959). This hypothesis was furtherly confirmed by Medawar and co-workers that clearly demonstrated the tolerance against a skin transplant in adult mice that had been injected with donor cells as pups (Billingham, Brent, and Medawar 1953). Few years later, it appeared clear that the presence of non-self antigen does not suffice the elicitation of

adaptive responses but costimulatory signals are required as well (Lafferty and Cunningham 1975). In those years, the official discovery of dendritic cells (DCs) by Ralph M. Steinman and Zanvil A. Cohn paved the way for the final identification of DCs as the APCs par excellence, capable of providing the costimulatory signals, necessary for effective T cell elicitation (Banchereau and Steinman 1998).

1.3.1 Biology of Dendritic cells

Among innate immune cells, DCs represent the crucial bridge that link adaptive and innate immunity. Initially, a population of accessory adherent and non-lymphoid immune cells required for efficient lymphocyte activation was identified (Hartmann et al. 1970). This population was thought to be Metchninkoff's macrophages. Subsequently, further studies rose the possibility of the presence, among splenocytes, of the so-called A or 3rd cells that elicited adaptive immunity in vitro (Cosenza, Leserman, and Rowley 1971). In the meantime, Ralph M. Steinman and Zanvil A. Cohn discovered this population in mice spleen and, due to the highly frequent dendrites it exhibited, they named these cell-type DCs, described as "large stellate cell with distinct properties" from mononuclear phagocytes, granulocytes and lymphocytes (R. M. Steinman and

Chon 1973). DCs appeared to be adherent and non-proliferating cells that lack lymphocytic, both T and B, surface markers, clearly exhibiting less endocytic capacity when compared to macrophages without collagen-like molecules deposition capability (R. M. Steinman and Cohn 1974).

Further studies shed light on this novel population that initially met considerable skepticism. In the next years, Steinman and colleagues provided new insights on DCs, confirming their exquisite role in stimulating T cell proliferation in primary mixed leukocytes reactions (MLR) (R. M. Steinman and Witmer 1978), correlated to the notion that DCs express both MHC I and II molecules (Nussenzweig and Steinman 1980; B. R. M. Steinman et al. 1979) and are capable of processing protein antigens resulting in the elicitation of antigen-specific responses. These pioneering works paved the way for the further characterization and exploitation of DCs, being the unique cell-type at the interface of innate and adaptive immunity.

Nowadays, DCs represent the primary professional APCs and encompass several subtypes with different intrinsic capabilities and diverse tissue localizations. Historically, depending on their ontogeny and functionalities, DCs are classified into conventional DCs (cDCs) and plasmacytoid DCs (pDCs). The formers stand out as the most effective in promoting T cell responses, while the latter originate from a lymphoid progenitor

and exert anti-viral functions via the release of type I IFNs. In general, DCs localize both in lymphoid and non-lymphoid organs, especially at the body surfaces. Indeed, they patrol the physical barriers they reside in an immature state, characterized by extreme phagocytic and antigen-processing capabilities. Upon encounter of pathogens, due to the loss of barrier integrity and further micro-organisms invasion, DCs become fully mature. Indeed, the sensing of bacteria, fungi, viruses and parasites leads to the activation on highly conserved pathways that result in the maturation process of DCs. They began with the antigen proteolysis for further antigen load on the major histocompatibility complex (MHC) class I for intracellular peptides, and MHC class II for exogenous peptides. MHC class I mediates the presentation to $CD8^+$ cytotoxic T cells, while MHC class II to $CD4^+$ helper T cells. In parallel, DCs upregulate costimulatory molecules and CCR7, a chemokine receptor that specifically binds CCL19 and CCL21, expressed by the afferent lymphatic vessels, allowing DCs emigration from peripheral tissues to the draining lymph nodes. Here, they localize in the paracortex, also known as T cell area, increasing the chance to encounter the circulating cognate T cell clone and present the antigen (Figure 3). The efficiency of the adaptive response depends on the presentation of the antigens and requires 3 specific conditions: i) antigen loading, presentation on MHC class I or II and TCR engagement (referred to as "signal 1") ii)

high expression of costimulatory molecules, as CD80 and CD86 (referred to as "signal 2") iii) release of key cytokines to complete and skew the adaptive response (referred to as "signal 3"). This communication at the interface between DC and T cell with the interaction of ligands and receptors is defined immunological synapse (Grakoui et al. 1999). Some specific subsets of DCs display an intrinsic specialization in capturing exogenous antigen but presenting them via the MHC class I to $CD8^+$ T cells. This phenomenon is termed cross-presentation. While MHC molecules engage the TCR, costimulatory molecules can bind two modulatory receptors: CD28 and the Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4). CTLA-4 exerts its negative regulation via ectodomain competition with the positive costimulatory receptor CD28 for the binding of CD80 and CD86. Intriguingly, CTLA-4 exhibits higher affinity for DCs costimulation, hence its expression needs to be finely and temporally regulated to obtain T cell activation (Yokosuka et al. 2010). Furthermore, to promote survival and expansion of T lymphocytes, the crucial "signal 3" required for all types of T cell polarization is IL-2. Even though initially IL-2 was identified as prerogative of T cells that sustain their activation in an autocrine and paracrine manner, it is now evident that DCs-derived IL-2 is the real driver of T lymphocytes proliferation (Wuest et al. 2011).

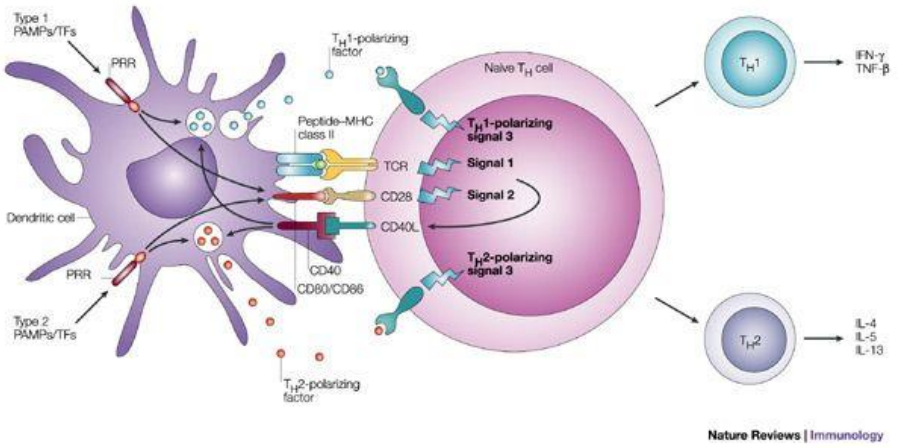

Figure 3: Antigen presentation by DCs

DCs are the primary professional antigen presenting cells that provide the 3 signals for optimal T lymphocytes activation: TCR engagement via peptide-MHC II complex (signal 1), costimulation through CD28 binding (signal 2) and polarizing factor release (signal 3). Adapted from Kapsenberg 2003.

1.3.2 Dendritic cells classification

DCs represent a versatile population of cells, divided into several subtypes that differ in ontogeny, localization, phenotype and functionalities. Their classification is evolving over time due to the increase of information researchers provide with distinct experimental models and advanced techniques for the human counterpart (Alcántara-Hernández et al. 2017; See et al. 2017; Villani et al. 2017). Despite their extreme heterogeneity, almost

all DCs originates from a bipotent progenitor in the bone marrow, known as macrophage and DC precursor (MDP) that can give rise to both monocytes and DCs (Fogg et al. 2006). MDP then differentiates into common DCs precursors (CDPs), which generate specifically cDCs and pDCs (S H Naik et al. 2007). The latter complete their developmental process in the bone marrow, while the former, at this phase named precursors of DCs (pre-DCs), egress from the bone marrow to terminally differentiate in the lymphoid or non-lymphoid tissue where they are supposed to reside, acquiring specific phenotypical and functional specializations (K. Liu et al. 2009; Shalin H Naik et al. 2006). The development and proliferation of DCs progenitors rely on two main growth factors: FMS-like tyrosine kinase 3 ligand (FLT3L) and Granulocyte-macrophage colony-stimulating factor (GM-CSF). FLT3L strongly induces expansion of both cDCs and pDCs (Waskow et al. 2009) and confers DC-related properties to Langerhans cells (LCs) and monocytes. Its fundamental role emerges in mice lacking FLT3L that exhibit deficiency in the DC compartment (Mckenna et al. 2000). In addition to FLT3L, GM-CSF appears to be critical for cDCs establishment in non-lymphoid tissues, in particular for $CD103^+$ cDCs if compared to $CD11b^+$ cDCs, probably because GM-CSF is implicated in CD103 expression (Greter et al. 2012; King, Kroenke, and Segal 2010; Kingston et al. 2009). The precise contribution of the two growth factors remains elusive, even if they seem to synergistically

cooperate for the promotion of DCs generation (Kingston et al. 2009).

1.3.1.1 Plasmacytoid DCs

pDCs have been defined plasmacytoid for their morphology, which exhibits an extremely developed secretory compartment (Reizis et al. 2011). pDCs arise from lymphoid progenitors even though they clearly display classical DC features as FLT3L-requirement for development and relatively limited potential to prime T lymphocytes after maturation (Kingston et al. 2009; Sapoznikov et al. 2007). Despite these general DC properties, pDCs specialization resides into their capability to rapidly produce abundant amounts of type I IFN upon viral infections (Nakano, Yanagita, and Gunn 2001), due to the constitutive expression of IRF7 TF (Honda et al. 2005). Another key TF involved in the generation of pDCs is the helix-loop-helix TF E2-2, also named TCF4 that prevents the skewing towards cDC by suppressing Id2 TF, known to be fundamental to generate this subset (Cisse et al. 2008; Ghosh et al. 2010). pDCs are mainly found circulating in blood and in peripheral organs and are emerging as critical components of pathological machineries driving autoimmune diseases, from Systemic Lupus Erythematosus (SLE) to psoriasis (Panda, Kolbeck, and Sanjuan 2017).

1.3.1.2 Conventional DCs

cDCs represent 1-5% of tissue cells depending on the organ they populate and exhibit a half-life of approximately 3-6 days. cDCs are fastly turned over via bone marrow precursors in a FLT3L-dependent manner (Mckenna et al. 2000). cDCs can be further discriminated between CD8α$^+$ cDCs, residing in lymphoid organs, and CD103$^+$ or CD11b$^+$ DCs, found in non-lymphoid tissues. CD8α$^+$ and CD103$^+$ cDCs appear to be conserved through evolution (Crozat et al. 2011). Indeed, they both rely on IRF8, Id2 and Batf3 TFs, differently from CD11b$^+$ cDCs that mainly depend on RbpJ and IRF4. Furthermore, it has been identified the human equivalent of these subsets of cells, marked by the expression of CD141 and XCR1. XCR1 has revealed to be the common signature shared by these highly conserved subsets, which share also an intrinsic predisposition to cross-presentation (Crozat et al. 2011; Dorner et al. 2009; Haniffa et al. 2012; Kroczek and Henn 2012; Roberts et al. 2016). As already emerged, the DCs heterogeneity scenario in mouse is relatively well representative of the human DCs lineages.

CD103$^+$ cDCs represent the 20-30% of all cDCs and exhibit the peculiarity of residing mainly in connective tissues. CD103$^+$ DCs lack the expression of macrophage markers as CD11b, CD115, CD172a, F4/80 and CX3CR1, but they may express CD8 in the Peyer's patches while they exhibit CD11b in the lamina propria

(LP) of the gut. In addition to the LP, other tissues are populated by CD11b$^+$ DCs: muscles and skin. CD11b$^+$ cDCs are spread among non-lymphoid and lymphoid tissues, where they represent the most abundant cDCs subtypes, except for the thymus. In contrast to the fine characterization of the other cDCs subsets, CD11b$^+$ cDCs display a higher degree of heterogeneity and their functionalities remain, to some extent, elusive. They exhibit inefficiency to cross-present and produce cytokines, like IL-12p70, as XCR1$^+$ cDCs do. Despite this, CD11b$^+$ cDCs have revealed to be superior in the induction of CD4$^+$ T cell adaptive responses, probably because of their massive expression of MHC class II via IRF4 (Vander Lugt et al. 2014; Schlitzer et al. 2013).

1.3.1.3 Skin-resident DCs

The skin embodies all the functional properties of the physical and chemical barriers of the body, thus it is enriched with various immune cells and among these, DCs. This tissue is provided with a unique DC population that exhibits peculiar capabilities: the Langerhans cells (LCs). LCs specifically localize in the most external layer of the skin, the epidermis. Their ontogeny differs from the classical observed for cDCs and, to some extent, they have been associated to tissue-resident macrophages as microglia. Indeed, LCs originates from precursors of yolk-sac and fetal liver during pre-natal life (Collin

and Milne 2016). Their turn over differs profoundly from the rapid replenishment of blood-borne cDCs and it is mediated by in situ self-renewal (Miriam Merad et al. 2002). Although they are considered part of the DCs population, LCs share a gene expression more similar to macrophages than cDCs (Miller et al. 2012). LCs extend their protrusions all along the epidermis guaranteeing an efficient defense against external threats. Even though they are sessile cells, upon encounter of pathogens LCs mediate phagocytosis and upregulate CCR7 to migrate to draining lymph node and elicit adaptive responses (Stoitzner et al. 2003). While LCs represent the only DC population in the epidermis, the dermis hosts multiple DC subsets. Indeed, in addition to LCs that are migrating to the draining lymph node and thus passing through the dermis, this connective tissue encompasses other 4 DCs populations: $CD103^{+}$ $CD207^{+}$, $CD103^{-}$ $CD207^{+}$, $CD207^{-}$ $CD11b^{+}$ and $CD11b^{-}$ $CD103^{-}$ $CD207^{-}$ cDCs (Henri et al. 2010). The peculiar specializations of every single subset remain elusive, even though they all exhibit classical DCs features: CCR7-dependent migration to draining lymph nodes (Henri et al. 2010; Ohl et al. 2004) and presentation of antigens via MHC class II (Bedoui et al. 2009). The contribution of the diverse subsets in the initiation of adaptive responses have highlighted some distinct intrinsic features that still reman poorly understood. LCs exhibit delayed kinetics when reaching the draining lymph node if compared to the dermal DCs

(Kissenpfennig et al. 2005). Moreover, some works have reported a tolerogenic profile of LCs that seem to display higher capacity of suppressing immune responses (Flacher et al. 2014; Igyarto et al. 2009; Kautz-Neu et al. 2011), but this intrinsic properties of LCs are controversial (Epaulard et al. 2014). Indeed, LCs appears to be fundamental for the generation of specific T cell responses, differently from other dermal subsets that are more prone to elicit other types of adaptive immunity (Igyártó et al. 2011). The diverse talents of DCs are currently emerging in the more disparate scenarios and will probably provide new insights for the pathogenesis of diseases as well as for the developmental strategies of vaccines (Gornati, Zanoni, and Granucci 2018).

1.4 DCs-mediated T cell polarization

The instauration of the more appropriate adaptive response is fundamental to correctly and efficiently eliminate the invading pathogen and restore homeostasis. The various PRRs of innate immunity provide information about the type of microorganism, its viability, virulence and replication and finally the localization of the infection. The mechanisms underlying the capability of the immune system to obtain and, to some extent, "understand" these cues is still elusive. Some have speculated that the extreme heterogeneity in DCs

compartment is evolutionarily required to counteract the variety of exogenous and noxious agents that can affect the host. Indeed, diverse DCs subtypes express different levels of PRRs and consequently are more prone to release cytokines that skew specific effector functions (M Merad et al. 2013). Upon engagement of the TCR via MHC class II, $CD4^+$ T cells differentiate into T helper cells (Th). Depending on the stimuli encountered by DCs, Th cells are polarized into Th1, Th2, Th17, Tregs and the recently discovered Th9 and Th22. Th cells share the capability of driving and influencing the immune activities of other effector cells, from macrophages to $CD8^+$ T cells. Conversely, the latter are activated by MHC class I-dependent antigen presentation and differentiate into cytotoxic T lymphocytes (CTLs). Generally, viral, bacterial, protozoan and fungal infections require the instauration of Type 1 immunity, including Th1, CTLs and Th17, while helminths, parasites, allergens and venoms promote Type 2 immunity, via Th2 polarization. Of note, all types of immunity require the intervention of regulatory T cells to prevent exacerbation of the responses.

1.4.1 Th1 polarization

Upon TLRs engagement, DCs activate NF-κB that mediates the production of pro-inflammatory cytokines and chemokines, including Interleukin (IL) -1β, IL-6, Tumor Necrosis Factor (TNF) -α and IL-12p40. The key factor that induces Th1 polarization is the active form of IL-12, IL-12p70, a heterodimer formed by IL-12p40 and IL-12p35. The latter is transcribed independently of the former and it is triggered by type I IFNs and IFN-γ, derived by DCs themselves and other effector cells, respectively (Trinchieri and Sher 2007). Thus, IL-12p70, but also IFN-γ, represent the "signal 3" for Th1 generation. Additionally, IL-18 and IL-27 have resulted to enforce differentiation or elicitation of Th1 cells, enriching the scenario of Th1 positive modulators (Chang et al. 2000; Pflanz et al. 2002), even if IL-27 effects exhibit controversies (Hunter and Kastelein 2012). Th1 functionalities rely mainly on the production of IFN-γ, a pleiotropic cytokine that promotes the classical activation of MΦ, enhancing their killing of intracellular microorganisms and contributes to antiviral responses. Furthermore, Th1 contribute to trigger B cell production of antibody, in particular IgG2a, for opsonization, neutralization of virus and complement fixation (Mahon et al. 1995).

1.4.2 Th2 polarization

To respond to helminth and parasite infections, immunity has generated a specific arm: the type 2 immunity. The PRRs engaged that lead to a Th2 polarization are mainly CLRs, but specific TLRs have been shown to promote this skewing. Indeed, some ligands of TLR2 trigger ERK in DCs with subsequent phosphorylation of c-Fos, resulting in the inhibition of IL-12p70 and favoring Th2 polarization (Dillon et al. 2018). Furthermore, since helminths and parasites exhibit many thousand-fold greater size than bacteria or viruses, their infiltration into the host provokes cell and tissue damage and release of DAMPs that may trigger DCs (Everts et al. 2010). Additionally, these pathogens release several immunomodulatory molecules to force their migration into the host, like cysteine proteases, inducing Th2 skewing in vivo. Despite this, PRRs capable of sensing these enzymes are still lacking and it seems that the Th2 induction in this context may involve other cell types in addition to DCs (Sokol et al. 2008; H. Tang et al. 2010). Indeed, Th2 polarization is less characterized than Th1, even though they were firstly described together by Mossman and Coffman in 1986 (Mosmann et al. 1986), since the Th2-driving cytokine, IL-4, is poorly produced by DCs, thus corroborating the hypothesis of a cooperation between cell subsets. The most potent source of IL-4 are basophils and they appear to drive Th2 polarization

synergistically with DCs during helminths infections (Giacomin et al. 2012; Siracusa et al. 2011). As emerged, the precise mechanisms underlying innate immune sensing and activation following parasites encounter are still elusive, but efficiently lead to Th2 production of cytokines critical in fighting these pathogens: IL-4, IL-5 and IL-13 (Allen and Maizels 2011). These mediators promote IgE class switching in B cells, smooth muscle contractility, mucus production and recruitment of innate immune cells (Bao and Reinhardt 2015).

1.4.3 Th17 polarization

Th 17-polarized lymphocytes intervene during fungal and bacterial infections and have been reported to concur in the pathogenesis of autoimmune diseases as psoriasis, rheumatoid arthritis, SLE and others (Tesmer et al. 2008). The skewing towards a Th17 profile begins with the recognition of fungal or bacterial components via CLRs and TLRs on DCs. As for Th1 induction, DCs maturation and upregulation of costimulatory molecules and cytokines, through NF-κB activation, is required to drive Th17 differentiation. Differently from Th1 and Th2, the "signal 3" that expand Th17 pool is more heterogeneous and, to some extent, not fully characterized. *In vitro* Th17 differentiation requires a cocktail of distinct cytokines: IL-1β, IL-6, IL-23 and

TGF-β (Luis, Haines, and Cua 2013), while *in vivo* the requirement for IL-1β and IL-6 depends on the site of immunization (Hu et al. 2011). Furthermore, IL-23 acts in synergy with IL-1β, since naïve T cells do not express IL-23 receptor (Sutton et al. 2006). Following their activation, Th17 secrete IL-17A, IL-17F, IL.22, GM-CSF and TNF-α, playing a major role in recruitment and triggering of neutrophils to the site of infection (Kolls 2010; Kumar, Chen, and Kolls 2013).

1.4.4 CTLs activation

Immune responses against intracellular pathogens, either viruses and bacteria, rely on CTLs activity. Indeed, viral infections are sensed via nucleic acids sensors that promote release of type I IFNs, with antiviral properties, and IL-12, leading to the establishment of CTLs. As already mentioned, $CD8^+$ lymphocytes require MHC I antigen presentation, thus CTLs are capable of recognizing virally infected cells via their MHC I, and further mediate their killing. In addition, CTLs are informed about the presence of viruses by specific DCs subsets, specialized in cross presentation. Batf3-dependent $CD103^+$ DCs, residing in peripheral tissues, and Batf3-dependent $CD8\alpha^+$ DCs, localized in lymphoid organs, resulted to be necessary for cross-presentation and amplification of $CD8^+$ T cells in several

contexts, due to their specialized profile in sensing viruses and virally infected cells (Edelson et al. 2010; Radtke et al. 2015; Waithman et al. 2013). Despite their intrinsic talent, the cooperation of multiple DCs subsets results in more potent anti-viral responses, since optimal clonal expansion, differentiation and memory properties of $CD8^+$ CTLs requires signals from elicited $CD4^+$ T cells (Eickhoff et al. 2015). Moreover, the site and route of infection can shape the scenario and the kinetics of the immune response. Indeed, cutaneous viral infections preferentially rely on dermal DCs, whose maturation leads to the elicitation of $CD4^+$ T cell responses (Mount et al. 2008; Seneschal, Jiang, and Kupper 2014). Similarly, distinct skin-resident DCs subsets drive preferential T cell polarization, for instance upon fungal infections (Igyártó et al. 2011; Kashem et al. 2015).

Providing insights into the intrinsic specialization of the multitude of DC subsets and their functional contribution to the polarization of T cells, either into Th or CTLs, will enlighten the immunological relevance of each subtypes in the promotion of specific responses that can be artificially shaped and skew depending on the pathological context. Among the variety of outcome that an external insult can provoke, the common feature of DCs, being professional APCs, is antigen-presentation, TCR engagement and the further signaling cascade.

1.5 TCR Signaling

Priming of T lymphocytes is achieved via the activation of different but synergic signaling pathways in parallel to the engagement of the TCR. Following TCR encounter with its cognate antigen complexed with MHC molecules, a conformational change of the TCR-CD3 complex occurs, generating high accessibility of the CD3 subunits to phosphorylation (Kuhns, Davis, and Garcia 2006). In the meantime, the binding of several TCRs on a single T cell to their cognate antigen-MHC complex on an APC leads to the TCR clustering. This congregation of receptors allows the co-receptors, CD4 or CD8, and their associated Src-family kinase Lck (Kim et al. 2003), together with the membrane associated kinase Fyn, to phosphorylate the immunoreceptor tyrosine-based activation motifs (ITAMs) on the conformationally accessible CDE chains (Qi-jing Li et al. 2004). These phosphorylations on the ζ chains of the TCR generate docking sites for ζ-associated protein of 70 kDa (ZAP-70) that binds these sites through its tandem Src homology 2 (SH2) domains and is further phosphorylated by Lck. ZAP-70 in turn phosphorylates both the membrane-associated linker for T cells activation (LAT) and the SH2 domain-containing leukocyte protein of 76 kDa (SLP-76). SLP-76 is then recruited via the SH2 domain on its constitutively associated adapter protein Grb2-related adapter

downstream of shc (GADS) to phosphorylated LAT. Phosphorylated LAT and SLP-76 create a docking site for the Phospholipase Cγ1 (PLCγ1), activated by the Tec family kinase Itk. PLCγ1 cleaves phosphatidylinositol 4,5-bisphosphate (PIP2) generating diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3), as second messengers. DAG remains localized at the plasma membrane where it diffuses laterally to recruit RasGRP1 and Protein Kinase Cθ (PKC-θ). In parallel, IP3 generation and binding to IP3 receptors (IP3R) in the endoplasmic reticulum (ER) membrane result in a calcium flux from the ER compartment into the cytosol. This ER calcium depletion is sensed by the stromal interaction molecule (STIM) with a mechanism not fully understood, probably mediated by STIM binding of calcium via a helix-loop-helix (EF-hand) motif. Then, it signals and triggers the opening of the calcium release-activated calcium (CRAC) channels in the plasma membrane, rapidly generating an influx of extracellular calcium into the cytoplasm (S Feske 2007). CRAC channels are highly specific for calcium and allow a large influx of this ion. The process of replenishment of calcium from the extracellular space in response to the ER calcium depletion is named store-operated calcium entry (SOCE). The SOCE mechanism leads to calmodulin and calcineurin (Cn) activation and further NFAT translocation to the nucleus, whose pathway will be discussed in detail below.

While IP3 induces NFAT pathway activation, DAG promotes the MAPK cascade, involving ERK, JNK and p38 (Dong, Davis, and Flavell 2002), which phosphorylate Fos and Jun that together comprise the AP-1 TF (Johnson and Lapadat 2002). In addition, LAT recruits other proteins that enhance MAPK signaling via Ras and Rac. It has emerged that AP-1 activation is largely PLCγ1 independent, even though DAG enforces Ras involvement that in turn leads to ERK1/2 signaling transduction. Moreover, Cn could participate in JNK activation (Werlen et al. 1998), but JNK1/2 appear to be dispensable for T cell activation and consequent IL-2 production (Sabapathy et al. 2001).

In addition to NFAT and AP-1, also NF-κB participates in T cell priming. At the steady state, NF-κB heterodimer is associated to the cytosolic inhibitor of κB (IκB) that hides its nuclear localization sequence (Vallabhapurapu and Karin 2009). Both NF-κB and IκB exhibit several family members, but p65:p50:IκBα complex is the more represented in T cells. As already mentioned, upon TCR engagement, DAG promotes the activation of PKC-θ (Isakov and Altman 2002) that, in turn, induces the activation of the trimeric IKK complex via the CARMA:Bcl-10:MALT (CBM) complex. IKK comprises the regulatory subunit IKKγ as well as the catalytic subunits IKKα and IKKβ that phosphorylate IκBα mediating its polyubiquitination and thus proteasomal degradation. The

removal of IκBα allows the translocation of NF-κB into the nucleus.

The complete activation of T lymphocytes requires the involvement of more than one TFs whose cooperation efficiently sustains the required transcriptional program. In addition, the interaction of costimulatory receptors with costimulatory molecules on APCs plays a fundamental role in T cell priming, leading to the formation of the critical immunological synapse.

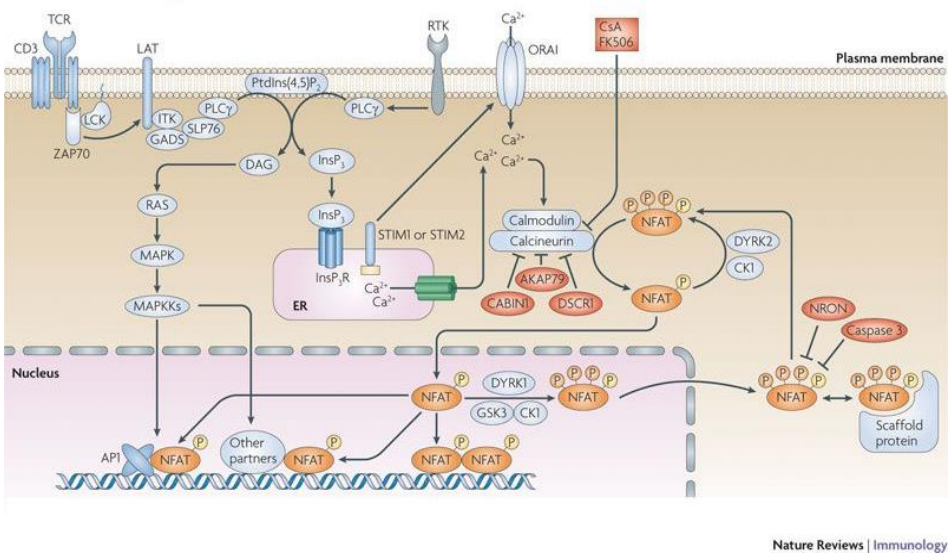

Figure 4: TCR signaling cascade

Upon TCR engagement, the signaling cascade leads to the production of DAG and IP3, which binds IP3R on the ER, resulting in an increase in the intracellular calcium concentration and further calmodulin/calcineurin activation. The pathway culminates with the dephosphorylation of NFAT by calcineurin and promotion of transcription in concert with other TFs. Adapted from Muller and Rao 2010.

1.6 Downstream the TCR: NFAT TFs

NFAT is a TF whose roles are still currently emerging nowadays since its discovery goes back only to 1988 when it was initially identified as an inducible TF able to bind Il-2 promoter in activated T cells (Shaw et al. 1988). Since then, many efforts have been made to molecularly and functionally characterize the TF, which has recently emerged to be expressed also in innate immunity. Nonetheless, its regulatory functions are conducted also in non-immune-related tissues, including the central nervous system, in particular in the outgrowth of axons (Weider et al. 2018), blood vessels, bone, intervening in the differentiation of osteoclasts during bone formation (Grössinger et al. 2017; Winslow et al. 2006), heart, especially in the generation of cardiac septa and valves in embryos, kidney, skeletal muscle and hematopoietic stem cells.

The NFAT family encompasses five members: NFAT1 (also known as NFATp or NFATc2), NFAT2 (also known as NFATc or NFATc1), NFAT3 (also known as NFATc4), NFAT4 (also known as NFATx or NFATc3) and NFAT5 (also known as Tonicity-responsive enhancer-binding protein, TonEBP or OREBP). NFAT1-4 activity relies on calcium signaling, while NFAT5 responds to osmotic stress. The members involved in the regulation of immune functions are mainly NFAT1-4, differently expressed in every cell type. As already mentioned, NFAT-

mediated gene-transcription is strictly dependent on changes in the amplitude and oscillation frequency of intracellular calcium concentration. Indeed, slow oscillations and low sustained intracellular calcium influx more efficiently activate NFAT TFs (Dolmetsch et al. 1997; Dolmetsch, Xu, and Lewis 1998). This notion is further corroborated in severe combined immunodeficiency patients with defects in SOCE mechanism that fail to maintain a sustained augmentation of intracellular calcium concentration and thus, NFAT activation (Stefan Feske et al. 2000).

NFAT TFs activation relies on the serine-threonine protein phosphatase Cn, the calcium signaling transducer par excellence. Calcium entry in the cell and subsequent binding to calmodulin leads to its binding to Cn and Cn activation. It is a heterodimeric protein consisting of both a catalytic (CnA) and a regulatory (CnB) domains (Klee, Ren, and Wang 1998). There are three isoforms of CnA (CnAα, CnAβ and CnAγ) and two isoforms of CnB (CnB1 and CnB2), whose expression is tissue-specific, as for CnAα and CnB1, found in lymphoid tissues (Chan, Wong, and Ohashi 2002). Calcium-bound calmodulin displaces the autoinhibitory domain of CnB, which structurally blocks CnA phosphatase activity, opening the catalytic site and promoting signaling transduction (Ye et al. 2013). NFAT TFs are the main targets of activated Cn and their protein structure allows Cn intervention. Indeed, the calcium-regulated NFAT1-4 display

Cn-binding site(s) in the moderately conserved core region, named NFAT homology region (NHR) that bears most of the other NFAT regulatory domains: transactivation domain, nuclear localization signal(s) and multiple phosphorylation sites. In resting conditions, cytosolic NFAT proteins are phosphorylated on several serine residues in the NHR. Cn-mediated dephosphorylation induces conformational changes that expose nuclear localization signals, promoting NFAT transportation to the nucleus (Heidi Okamura et al. 2000). Here, NFAT cooperates with other TFs to promote transcription of selected genes. AP-1 complex, GATA3, CEBP, or forkhead box protein 3 (Foxp3) are only some of the functional partners of NFAT (Hermann-Kleiter and Baier 2010; Macian, Lopez-Rodriguez, and Rao 2001; Y. Wu et al. 2006; T. T. C. Yang et al. 2006). Indeed, NFAT promiscuity relies on a highly conserved Rel homology region (RHR) at its N-terminus, a structural region that confers DNA-binding specificity but also provides protein-protein interaction sites. It is thought that the NFAT family members are evolutionarily related to the Rel family of TFs because of a recombination event, occurred about 500 million years ago, between a Rel domain and an NFAT precursor, explaining the presence of the latter only in vertebrates (Graef et al. 2000).

1.6.1 Regulatory mechanisms in NFATc pathway

While Cn is capable of dephosphorylating all calcium-regulated NFAT TFs, the kinases that phosphorylate NFAT are member-specific. On the one hand, cytosolic maintenance kinases keep NFAT in a hyperphosphorylated state and, on the other hand, nuclear export kinases phosphorylate NFAT in the nucleus to shuttle it out (Porter, Havens, and Clipstone 2000). Indeed, Casein kinase 1 (CK1) is a serine/threonine kinase that is involved in both cytosol maintenance and nuclear export of NFAT, by targeting the first serine-rich region (SRR-1) motif in the TF (H Okamura et al. 2004). The SRR-1 of NFAT1 and NFAT4 is also target of other kinases, the two mitogen-activated protein kinases (MAPKs) p38 and JNK (Chow et al. 1997; Gomez del Arco et al. 2000). Concerning the nuclear export and NFAT inactivation, Glycogen synthase kinase 3 (GSK3) in the nucleus acts on NFAT1 and NFAT2 (Beals et al. 1997) while the Dual-specificity tyrosine-phosphorylation regulated kinases (DYRK) family members prime NFAT for further phosphorylation by CK1 and GSK3 to negatively regulate the TFs (Arron et al. 2006; Gwack et al. 2006) (Figure 5). The network composing the machinery that controls NFAT activation is further complicated by the presence of other regulatory mechanisms. This is the case for the sumoylation of sites displayed only at the C-terminus of

NFAT1, which promotes nuclear retention and transcriptional activity (Terui et al. 2004). On the contrary, it has emerged how the poly-ADP-ribosylation of NFAT1 and NFAT2 by poly-ADP-ribose polymerase-1 (PARP-1) is implicated in the promotion of the phosphorylation of these TFs, thus ceasing their activation (Valdor et al. 2008). Furthermore, indirect effects on NFAT activity are mediated by long-noncoding RNAs (lnc-RNAs). Indeed, the non-coding repressor of NFAT (NRON) has been identified as a cytoplasmic repressor of the TF by forming an RNA-protein complex together with the calmodulin-binding protein IQGAP1 and the nuclear transport protein KPNB1, a member of the importin-β family. This complex sequesters cytosolic phosphorylated inactive NFAT, preventing its translocation into the nucleus (Willingham et al. 2005). The integrated actions of these mediators lead to a complex regulation of NFAT TFs that is still partially unexplored.

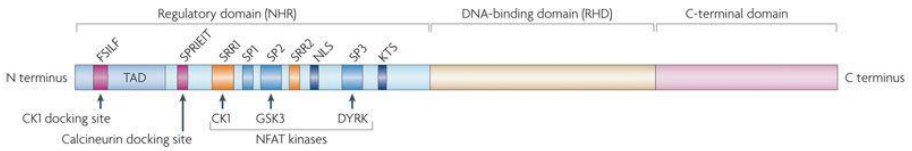

Figure 5: NFAT TF structure

NFAT principally exhibits 3 domains: C-terminal domain, DNA-binding domain and a highly conserved regulatory domain, which displays several sites for kinases and phosphatases that decide for NFAT activation or repression. Adapted from Muller and Rao 2010.

1.7 NFATc signaling pathways in innate immunity

Recent advances have revealed that the activation of NFAT TFs is no longer prerogative of only T lymphocytes, but there is growing evidence of its crucial role also in innate immunity. Indeed, several works have shed light on the activation of NFAT TFs in DCs, neutrophils, basophils, mast cells and, in particular contexts, also in macrophages (Zhihua Liu et al. 2011). So far, multiple PRRs have been revealed to activate NFAT and among these, two major and well-described signaling pathways are: that triggered by Dectin-1 and that by CD14.

1.7.1 Dectin-1 pathway

Dectin-1 (also named CLEC-7A) belongs to the CLRs family, whose main ligand is β-glucan carbohydrates, thus playing a crucial role in the sensing of zymosan and live pathogenic fungi, as *Candida albicans*, *Aspergillus fumigatus*, *Pneumocystis carinii*, in both macrophages and DCs (Brown et al. 2003; Rogers et al. 2005). Intriguingly, it has recently been reported the activation of Dectin-1 pathway in the recognition of mycobacteria that lack β-glucans, thus questioning a promiscuous sensing of this receptor for other ligands yet to be

discovered (H. Lee et al. 2009; Rothfuchs et al. 2007; Shin et al. 2008). Initially, Dectin-1 was thought to be specifically expressed by DCs, but sooner it had appeared to be exhibited also by monocytes, macrophages, neutrophils and a subset of T cells (Taylor et al. 2002). This receptor displays an extracellular C-terminal C-type lectin domain, a stalk region, a transmembrane domain and a short intracellular tail displaying an ITAM-like motif (Ariizumi et al. 2000). Indeed, the conventional ITAM displays two YxxL sequences, while Dectin-1 is provided with one YxxL sequence, since the other presents an additional amino acid residue YxxxL and it is not functional. Therefore, to differentiate this domain from the classical ITAM, it has been defined as hemITAM (LeibundGut-Landmann et al. 2007; Slack et al. 2007). The hemITAM motif is crucial for the signaling cascade, since mutations of this motif abolish the pathway and result in recurrent candidiasis in humans (Ferwerda et al. 2009). Upon engagement, Dectin-1 is indeed phosphorylated by Src family kinases that lead to the recruitment of Syk, a tyrosine kinase, which in turn phosphorylates and induces PLC-γ2 activity. PLC-γ2 mediates the hydrolyzation of PIP2 into DAG and IP3, which is thought to induce the release of ER calcium with further Cn activation and thus NFAT translocation (Tassi et al. 2009) (Figure 6). The activation of this pathway leads to the induction of early growth response (Egr) family TFs Egr2 and Egr3, as well as inflammatory mediators as cyclooxygenase-2

(COX-2), IL-2, IL-10 and IL-12p70 (Goodridge, Simmons, and Underhill 2007), involved in anti-fungi responses. Hence, ChIP-seq for genome-wide mapping of NFAT1 targets in DCs treated with zymosan has highlighted the upregulation of genes related to the Th17 pathway, as IL-12b and IL23a that heterodimerize to form the required cytokine for Th17 differentiation IL-23. Other targets identified are GM-CSF and TGF-β3 growth factors, again involved in the pathological Th17 differentiation process (Jiao et al. 2014; Sharma, Kaveri, and Bayry 2013; H. Yu et al. 2015).

1.7.2 CD14 pathway in DCs

As aforementioned, CD14 constitutes another molecule that leads to NFAT activation in innate immune cells and, specifically, in DCs. Initially, in 2001, Granucci and colleagues identified in DCs, both murine and human, another cellular source of IL-2, in addition to T lymphocytes, thus explaining the professional role of DCs in initiating adaptive responses (F Granucci et al. 2001). They demonstrated that LPS, zymosan and live yeasts, and not pro-inflammatory cytokines, are the mediators of IL-2 production by several tissue-specific DCs (Francesca Granucci et al. 2003). Intriguingly, inhibition of Cn with CsA leads to the abrogation of only IL-2 production and not of DCs maturation. Since IL-2 had been found to be

transcriptionally regulated by NFAT, the pathway that leads from LPS to IL-2 may have required this TF and, therefore, it needed to be characterized. Finally, in 2009 Zanoni and colleagues reported that the receptor that induces NFAT activation in innate immune cells upon LPS stimulation is CD14, the coreceptor of TLR4 (Ivan Zanoni et al. 2009). CD14 is a glycosylphosphatidylinositol (GPI)-anchored protein that lacks an intracellular tail and its TLR4-independent signal transduction is not surprising since other GPI-anchored proteins exhibit signaling capabilities in lipid raft, as CD59. Indeed, also for CD14-signaling, the localization of this molecule in the lipid rafts is indispensable for NFAT activation in response to smooth LPS, since disrupting lipid rafts via cholesterol depletion abolishes calcium influx and further NFAT translocation in the nucleus of LPS-treated DCs. The pathway that leads to NFAT activation via LPS-CD14 is still partially unknown. Zanoni and colleagues have reported the involvement of Src-family kinases that in turn phosphorylate PLC-γ2 probably leading to the generation of DAG and IP3, as already mentioned. IP3 is then involved in the opening of unknown channels on the plasma membrane of DCs to promote calcium influx from the extracellular space, since the use of the calcium chelator ethylene glycol tetraacetic acid (EGTA) sequestering calcium in the medium, abrogates calcium influx and then NFAT activation (Ivan Zanoni et al. 2009). The precise intermediate players of the

pathway, as the identity of the plasma membrane channels, are currently under investigation in Granucci's laboratory (Figure 6). Interestingly, macrophages resulted to be incapable of activating the CD14-NFAT pathway, even though they expressed higher levels of CD14 when compared to DCs. Therefore, the NFAT pathway in DCs represents an intrinsic biological difference that attributes to DCs very specific functions. Indeed, a kinetic microarray analysis identified the genes regulated specifically by NFAT in LPS-treated DCs inhibiting or not the NFAT pathway. It emerged that the pathway is involved in exhaustion and, in particular, in regulating the life cycle of activated DCs, promoting their apoptosis via NFAT1-mediated transcription of *Nur77, Gadd45g, Ddit3 and CHOP-10*. This is in line with the notion that macrophages do not activate the pathway since they exhibit crucial activities also in the later stages of immune responses, by endorsing resolution of inflammation, clearance of apoptotic cells and tissue regeneration. On the contrary, DCs apoptotic death is required to prevent exaggerated adaptive responses that may result in systemic autoimmunity and to establish peripheral tolerance (M. Chen et al. 2006; Stranges et al. 2007). In addition to *Nur77*, another gene emerged to be induced by NFAT activation in DCs: Ptges1, encoding for the microsomal PGE synthase-1 (mPGES-1), crucial in the release of prostaglandin E2 (PGE2) starting from membrane phospholipids, in collaboration with the cytosolic PLA2 (cPLA2)

and the COX-2 enzymes. Indeed, upon inflammation, cPLA2 translocates from the cytosol to the plasma membrane and generates arachidonic acid by hydrolyzing phospholipids. COX2 converts arachidonic acid into PGG2 and then into PGH2. The latter is the substrate of mPGES-1 that forms PGE2 from PGH2 (Park, Pillinger, and Abramson 2006). PGE2 is a multifaceted prostanoid that contributes to edema formation and vasodilation upon local inflammation (Daniel F Legler et al. 2010), phenomena that emerged to be CD14- and NFAT-dependent in an LPS-induced model of edema in mice (Ivan Zanoni, Ostuni, et al. 2012).

NFAT activation in DCs via CD14 represents an evolutionary adaptation to the higher level of complexity introduced with the adaptive immunity. As a matter of fact, NFAT pathway induction participates in both the early and the late events of immune responses: i) it promotes fast IL-2 production for the sustaining of NK cells activity (F Granucci et al. 2004) ii) rapid PGE2 release for the regulation of antigen and further DCs migration through the afferent lymphatic vessels via local edema formation (Kabashima et al. 2003; D. F. Legler et al. 2006) iii) it initiates adaptive responses through late IL-2 production iv) it induces apoptosis in terminally differentiated DCs preventing overt T cell immunity.

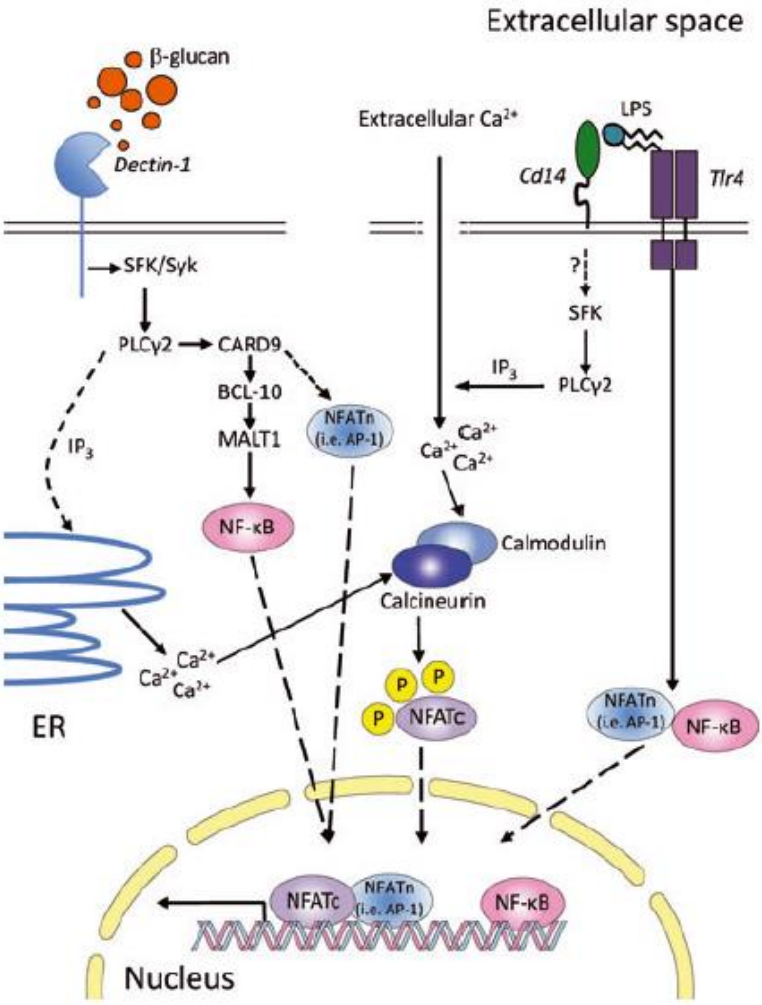

Figure 6: NFATc pathway in innate immune cells

NFATc pathway is activated downstream the engagement of Dectin-1 via β-glucan and CD14 via LPS and though the involvement of SFK and PLCγ2, intracellular calcium increases and triggers calmodulin that in turn elicits calcineurin, promoting NFATc translocation and its transcriptional functions. Adapted from Zanoni and Granucci 2012.

1.7.3 Newly emerged NFAT pathway in innate immunity

In the last years, the role of other PRRs in the induction of NFAT has been explored. Indeed, in mast cells, Pam3CyS binding to the heterodimer of TLR1-TLR2 has been reported to recruit Fc-γ receptor, which contains ITAM domains, for triggering NFAT pathway (Jin et al. 2016). In addition, *Aspergillus fumigatus* signals via a phagosomal TLR9-dependent and Bruton's tyrosine kinase-dependent pathway in macrophages, leading to NFAT activation and heterodimerization with NF-κB for TNF-α production (Herbst et al. 2015). Furthermore, ITAM-mediated recruitment of Syk and subsequent calcium influx and NFAT elicitation have been observed upon engagement of CLEC-2 (Robinson et al. 2011; Séverin et al. 2011) and macrophage-inducible calcium-dependent lectin (Mincle) (Yamasaki et al. 2008). Interestingly, also the truncated form of LPS, the so-called rough LPS, is capable of inducing NFAT activation in a CD14-independent manner (Ivan Zanoni, Bodio, et al. 2012). It has been recently reported that, in addition to microbial molecules, even sterile particulates are capable of inducing IL-2 production in DCs, suggesting an implication of NFAT in these contexts. Indeed, alum, monosodium urate crystals and SiO2, once phagocytosed by DCs, trigger Syk and Src kinases, calcium mobilization and

finally, Cn-mediated NFAT activation, thus promoting IL-2 release (Khameneh et al. 2017) as for the pathogenic ligands already discussed. Therefore, it is presumable that other exogenous or even endogenous molecules may be capable of inducing the activation of NFAT in innate cells, and specifically, in DCs. The identification of those ligands and possibly the pathogenic contexts in which NFAT pathway is active in DCs, will be of utmost importance for the immunoregulation of these APCs, both to promote elicitation of NFAT activation and thus sustaining T cell responses, as for vaccines (Khameneh et al. 2017) and to inhibit an overt adaptive reaction in autoimmunity, preventing IL-2 production (Wuest et al. 2011).

1.8 Calcineurin inhibitors

Due to the crucial relevance of NFAT activation in mediating adaptive responses, the inhibition of this pathway has always acquired much attention for several clinical setting, from transplantation to autoimmunity. Cyclosporin A (CsA) and FK-506 (Tacrolimus) represent the most used immunosuppressive drugs that abrogate NFAT translocation and further transcriptional activity.

CsA is a cyclic endecapeptide that was originally derived from the filamentous fungus *Tolypocladium inflatum* Gams for an

antibiotic screening program. In addition, it was also tested for its immunosuppressive and cytostatic properties, which resulted to be excellent on T cells, with lower myelotoxicity if compared to other immunosuppressants of that time. Similarly, FK-506 is a macrolide antibiotic, which was isolated in 1987, from a soil fungus, *Streptomyces tsukubaensis,* which exhibited effective immunosuppressive activity (Kino et al. 1987). The mechanism of action of these compounds was still elusive when they were firstly applied into the clinics, in 1979 for CsA and 1989 for FK-506. Only in 1991, it appeared clear that Cn was the common target of both CsA and FK-506 (J. Liu et al. 1991). Hence, since they share the same pharmacodynamic properties, these compounds are generally classified as Cn inhibitors (CNIs). The mechanism of action of CNIs relies on their binding to intracellular immunophilins: cyclophilins in the case of CsA and the FK-binding proteins for FK-506. These newly formed complexes bind Cn, resulting in the abrogation of its enzymatic activity thus impeding NFAT TFs activation (Richard et al. 1993). The effectiveness of CsA and FK-506 in inhibiting Cn clearly relies on the binding with their respective immunophilins, leading to the notion that they are not active inhibitors by themselves (Schreiber and Crabtree 1992). Immunophilins belong to the class of peptidyl-prolyl *cis-trans* isomerases (PPIases) and exhibit several cellular functions, as *de novo* protein folding (J. Liu et al. 1991). The binding to CsA or FK-506

abolishes the PPIase activity but confers the complexes the capability to limit the access of proteins substrates to the catalytic core of Cn. Moreover, the interaction between immunosuppressant-immunophilin and Cn masks the docking site for the NFAT LxVP motif at Cn (Rodriguez et al. 2009). Therefore, CsA and FK-506 specifically inhibit Cn, and not other Ser/Thr protein phosphatases as PP1, PP2A or PP2C. Despite this selective specificity, CNIs use to prevent NFAT triggering in T cells, entails several side effects. Indeed, CNIs act upstream NFAT activation and Cn itself modulates other TFs, as NF-κB, AP-1, Elk1 and CREB (Dolmetsch, Xu, and Lewis 1998; Frantz et al. 1994; Oetjen et al. 2005; Sugimoto, Stewart, and Guan 1997). In addition, Cn interferes with other signaling pathways as that triggered by TGF-β or the MAPK cascade (Q. Liu, Busby, and Molkentin 2009; Ninomiya-tsuji et al. 1999), hence the abrogation of Cn activity via CNIs leads to several side effects. The adverse events reported during the administration of CNIs regard neurotoxicity, nephrotoxicity, vascular toxicity and still remain a major challenge. Even though CsA and FK-506 exhibit the same mechanism of action, they have different toxicity profiles. Indeed, FK-506 appears to be less vasoconstrictive and fibrogenic as compared to CsA, but it can be more diabetogenic (Bagnis et al. 1996; Heisel et al. 2004; Jain, Bicknell, and Nicholson 2000). Despite this, after 40 years from their discovery, CNIs remain the

standard of care, at least, in organ transplantation, which is the field most revolutionized with the introduction of these drugs.

1.8.1 Cn inhibitors effects on DCs

CNIs have widely been used to inhibit adaptive responses in autoimmune diseases and to prevent T cell alloreactivity in mismatched transplants without considering the biological effects that these drugs may have on other immune cells. Indeed, DCs treated with FK-506 or CsA exhibit an immature phenotype, with low expression of costimulatory molecules, as CD80, CD86, CD40 but also of MHC I and II, reducing DCs capability of antigen-presentation and T cell activation (Imai et al. 2007; Y. Lee et al. 2005). In parallel, conditioning with CNIs results in reduced production of pro-inflammatory cytokines as TNF-α, IL-6 and IL-12, but leads to the upregulation of IL-10 and TGF-β, known to be mediators of tolerance (Imai et al. 2007; Y. Lee et al. 2005; Ren et al. 2014). Intriguingly, pre-treatment with FK-506 maintains DCs anti-inflammatory profile even when LPS or IFN-γ are used to promote DCs maturation (Ren et al. 2014). This phenotypical evidence revealed to be associated with a reduction in the stimulating capabilities of DCs as APCs. Indeed, allogeneic T cells in the presence of FK-506- or CsA-conditioned DCs reduce their proliferation rate and production of crucial mediators as

IFN-γ, IL-4 and IL-2 while DCs reduced their release of IL-6, IL-12p40 and IL-12p70 (Matsue et al. 2002). Surprisingly, FK-506-treated DCs suppress the proliferation of T cells induced by mature DCs, suggesting a potential tolerogenic activity by Tacrolimus-treated DCs, applicable also to abolish immune responses *in vivo* (Ren et al. 2014). Indeed, in a model of autoimmune arthritis, mice that received injection of FK-506-treated DCs achieve better outcomes, with a more physiologic histology of the joints with few infiltrated cells and absence of damage (Ren et al. 2014). In humans the effects of CsA and FK-506 on DCs have been mainly investigated in monocyte-derived DCs. It is important to note that both the studies with CsA and FK-506 led to same controversies. Treatment with either the CNIs does not impair moDCs differentiation (Woltman et al. 2000) even though reduces their expression of costimulatory molecules as CD80, CD86 and CD83 and DC-lysosomal-associated-membrane protein (LAMP), depending on the stimulus of maturation (Duperrier et al. 2002; Koski et al. 1999). Furthermore, MLR with moDCs treated with a therapy dose of Tacrolimus skew T cells towards a Th2 profile (Shimizu et al. 2000) when they do not induce T cell hyporesponsiveness (Szabo, Gavala, and Mandrekar 2001). Even though there is no clear evidence of the beneficial role of CNIs-treated DCs in autoimmune contexts or in transplantation, the induction of

tolerogenic DCs may be part of the benefits of the therapy with CsA and FK-506.

1.9 Unresponsitivity

As continuously emerging in almost all fields of biology, unresponsiveness is not a passive phenomenon. It is not merely the ceasing of action, but it requires a specific epigenetic and transcriptional program to sustain the unresponsive status. In immunology and, specifically, in T cell biology, a reduced responsive profile is usually named through multifaceted definition: tolerance, anergy in $CD4^+$ T helper cells and exhaustion in $CD8^+$ cytolytic T cells.

Tolerance is a necessary phenomenon that allows organisms not to mount immune responses against self-antigens and thus prevents autoimmunity. The so-called central tolerance is achieved via clonal elimination of self-reactive lymphocytes during development in the thymus, and further, the ones who survive this first line of intervention, are rendered tolerant to self-antigens in the periphery though a process referred as to peripheral tolerance (Kamradt and Mitchison 2001). Peripheral tolerance is achieved via two different mechanisms: anergy induction, defined as a condition in which T cells fail to mount an appropriate response against an antigen, remaining

functionally inactivated but alive for an extended period of time (also known as recessive tolerance), and Tregs intervention (also known as dominant tolerance). In particular, Ron Schwartz, one of the pioneers of anergy research, defines two diverse forms of T cell anergy: clonal anergy and adaptive tolerance (or *in vivo* anergy) (Schwartz 2003). Clonal anergy originates with the incomplete activation of T cell clones, usually previously activated, resulting in growth inhibition and an incomplete block of the effector functions. Conversely, adaptive tolerance arises in naïve T cells when costimulation is insufficient or inhibition is present and leads to growth inhibition and loss of effector activity. Adaptive tolerance may also occur in the presence of persisting antigen exposure. Both these phenomena are clearly related to NFAT TFs.

1.9.1 Recessive tolerance

NFAT does play a role in determining both the active and the unresponsive state of T lymphocytes. As already mentioned, the TCR engagement is necessary but not sufficient to provide fully activation, since costimulation is required as well. Indeed, pre-activation of calcium/Cn signaling alone, in response to a second activatory signal leads to an anergic profile, in terms of reduced proliferation and decreased transcription of effector

cytokines as IL-2, IFN-γ, TNF-α, GM-CSF without affecting IL-10. This status is accompanied by a specific signature related to the increase of mRNAs of negative transcriptional regulators, components of cellular proteolytic pathways and, in general, proteins capable of negatively regulating cell signaling (Heissmeyer et al. 2004). Among these, of note are the E3 ubiquitin ligases Itch, Cbl-b and GRAIL (Anandasabapathy et al. 2003). It seems that the upregulation of these E3 ubiquitin ligases leads to the degradation of T cell activatory molecules as PLC-γ1 and PKC-θ. Finally, these ubiquitin ligases appear to be associated to anergy since mice deficient for Cbl-b or Itch can develop autoimmune syndromes (Bachmaier et al. 2000; D. Fang et al. 2002).

As already mentioned, the anergic profile emerged to be achieved via an increase in the intracellular calcium concentration (Jenkins et al. 1987), Cn activation and it resulted to be dependent on NFATc2 activity in the absence of AP-1, both *in vitro* and *in vivo* (Macian et al. 2002). Indeed, NFATc2$^{-/-}$ animals fail in inducing the anergic profile in T cells. Thus, anergy requires stimulation of TCR and thus induction of NFATc2, but not CD28 engagement, which subsequently would lead to AP-1 activation, resulting in the exclusive triggering of NFATc2. Through the usage of NFATc2(RIP), an engineered version of NFATc2 that can not interact effectively with its effector partner AP-1, it was evidently demonstrated that anergy

relies on the individual NFATc2 transcriptional activity, since NFATc2(RIP) is capable of eliciting the anergic profile in $CD4^+$ and $CD8^+$ T cells (Martinez et al. 2015). Chromatin-immunoprecipitations and DNA-sequencing have revealed a strong binding of the constitutively expressed NFATc2(RIP) to promoter regions of genes similarly upregulated in anergic $CD4^+$ and exhausted $CD8^+$ T cells *in vivo* (Doering et al. 2012; T. Okamura et al. 2009; Wherry et al. 2007). DNA microarray studies of anergic cells indicated that only one-fifth of all the genes observed is either induced or suppressed if compared to fully activated T cells. Moreover, most of these genes are transcriptionally regulated by NFATc2, since their activity was altered in $NFATc2^{-/-}$ T cells. Intriguingly, upon T cell activation, both the activatory and anergic transcriptional programs are simultaneously induced (Macian et al. 2002; Martinez et al. 2015). The discriminatory factor for the dominance of one profile over the other consists in the availability and elicitation of NFAT partner proteins and the stability of NFAT-partner complexes, since NFAT TFs weakly bind to their DNA consensus sequences and their concentration in the nucleus is severely limiting.

1.9.2 Dominant tolerance

To complete the horizon of tolerance, recessive mechanisms are complemented by the dominant tolerance, defined as the suppressive actions promoted by specialized cells: Tregs. They intervene in multiple scenarios, preventing not only autoimmune diseases but also allergies, transplant rejection, graft-versus-host disease and infection-induced organ pathology. The mechanisms underlying these tolerant effects reside in the suppression of effector T lymphocytes and other cells functions (Shimon Sakaguchi 2004).

The concept of tolerance established by cells with suppressive functions appeared in 1969, when Nishizuka and Sakakura provided insights into a thymus-derived population capable of preventing autoimmunity (Nishizuka and Sakakura 1969). Only one year later, the first hypothesis on the existence of Tregs was conceived by Gershon and Kondo, who demonstrated that T cells could also suppress antibody responses (Gershon and Kondo 1970). Only in 1972 the scientific community began to accept the concept of suppressor T cells, in addition to the well-known effector T cells (Gershon et al. 1972). Even though the existence of a regulatory cell population had been demonstrated, we had to wait until 1985 to have the clear evidence of a particular T cell population that promotes self-tolerance preventing a variety of autoimmune diseases. Indeed, Sakaguchi

and colleagues compared the actions of two different T cell population: $CD5^{lo}$ and $CD5^{hi}$. When they transferred $CD5^{lo}$ cells into nude (nu/nu) mice, they induced autoimmunity, while transferring $CD5^{hi}$ did no. Moreover, co-transferring both the cell types prevented the pathology. Subsequently it was demonstrated that the $CD5^{hi}$ tolerant cells were indeed Tregs (S Sakaguchi et al. 1985). Due to the lack of identifying markers, it was difficult to study suppressor T cells, until 1995, when Sakaguchi discovered CD25 as a phenotypic marker for $CD4^+$ suppressor T cells (S Sakaguchi et al. 1995). Even though in 1991, Godfrey, Wilkinson and Russell observed a lymphoproliferative disorder in mice, caused by a mutation in the scurfy (Fork head box P3, Foxp3) gene that may have been associated to an impairment in the Treg compartment, only later this TF was clearly identified as a lineage-marker for this cell subset (Fontenot, Gavin, and Rudensky 2003; Hori, Nomura, and Sakaguchi 2003; Khattri et al. 2003), both in mice and humans (Roncador et al. 2005; Yagi et al. 2004). Since then, functional assays to investigate the capabilities of Tregs *in vitro* and *in vivo* have been performed, including the evaluation of putative roles of Foxp3 in mediating the tolerant effects of Tregs intervention. Foxp3 binds DNA through a winged helix-forkhead DNA binding domain and may act as both transcriptional activator and repressor by recruiting deacetylases as well as histone acetyltransferases (B. Li et al. 2007). As aforementioned,

mutations in this TF lead to a fatal lymphoproliferative disorder with early death of mice 4-5 weeks after birth (Brunkow et al. 2001), while in humans they result in the Immune dysregulation, polyendocrinopathy, entheropathy, X-linked (IPEX) syndrome, due to the conformational changes in the DNA-binding forkhead domain (FKH) and leucin zipper (Ochs, Ziegler, and Torgerson 2005). These clinical manifestations associated to autoimmunity in several organs, evidently indicated a functional role of Foxp3 in inducing tolerant properties in Tregs. Indeed, ectopic expression of Foxp3 confers suppressive activity to $CD4^+$ $CD25^-$ conventional T cells (Fontenot, Gavin, and Rudensky 2003). As a TF, Foxp3 requires partners for efficient transcriptional activity and, among these, it co-operates with NFAT TFs. Indeed, Foxp3 harbors several NFAT-binding sites, as emerged by structural analysis of the TF. Initially, it was thought that Foxp3 competed with NFAT, antagonizing its transcriptional activity. Foxp3 binds to a consensus forkhead binding motif that overlaps with the AP-1 site within the NFAT/AP-1 binding DNA-regulatory elements (Ziegler 2006). In the same years Anjana Rao and Lin Chen demonstrated that the forkhead domain of Foxp2 (and later of Foxp3) and the RHR motif in NFATc2 were found bound to the composed distal NFAT site of the IL-2 promoter, similarly to the NFATc2/AP-1 complex. Therefore, Foxp3 does not compete with NFAT TFs for binding to promoters, as it was suggested (Papers et al. 2001), but actively forms heteromeric

complexes with NFAT TFs that, in the case of IL-2, promote repression of transcription (Y. Wu et al. 2006). To corroborate this hypothesis, mutations in the binding site of Foxp3 to NFAT TFs, and not to DNA, were inserted and resulted in impairment of the suppressive functions of Foxp3 on the NFAT transcriptional activity. Furthermore, Foxp3 mutated in 4 amino acid residues (WWRR Foxp3 mutant, T359W N361W E399R E401R) led to the complete loss of all the suppressive actions of Tregs, thus affecting the repression of transcription mediated by Foxp3-NFAT associated partners. Moreover, the incapability of forming Foxp3-NFAT complexes resulted in suppression of transcription of genes normally upregulated upon stimulation of Tregs, as Ctla4 (CTLA-4), Il2ra (CD25) and Tnfrsf18 (GITR) (Papers et al. 2001; Y. Wu et al. 2006). These genes were also identified as normally upregulated in activated T cells, but they are similarly expressed in $Foxp3^+$ Tregs, as corroborated through transcriptional profiling of Tregs expressing Foxp3 in comparison to naïve or activate conventional T. Therefore, the heterodimer Foxp3-NFAT acts through a dual facet: as suppressor for activatory cytokines and as promoter of Tregs signature molecules. The crucial role of this complex *in vivo* has been demonstrated in mice injected with pancreatic-antigen specific T cells that would generate autoimmune diabetes. When the injected T cells are transfected with the WWRR Foxp3 mutant, they fail to acquire suppressive functions and promote

autoimmunity, hence mice develop diabetes cause by the adoptive co-transfer of pancreatic-antigen specific T cells, which are suppressed when co-transduced with the wild type form of Foxp3 protein (Y. Wu et al. 2006). Therefore, NFAT is strictly necessary to confer full regulatory profile to Tregs via the protein-protein interaction with Foxp3 TF.

Other studies brought to light the issue of the constitutive activation of Foxp3 to promote transcription of markers that specifically confer the regulatory phenotype of Tregs, as CD25 and CTLA-4 as well as the constitutive suppression of IL-2 production (Gavin et al. 2007; Ye Zheng and Rudensky 2007). Therefore, it was expected that Foxp3 would be constantly bound to DNA and that was the case. The TF was found to be constitutively bound to almost 700 target genes in freshly isolating Tregs (Ye Zheng et al. 2007). Consequently, given the necessity of a protein-protein interaction of Foxp3 with NFAT, it was assumed that NFAT could be present directly in the nucleus. Indeed, NFATc2 and NFATc1 have been detected in the nucleus of $CD4^+$ $CD25^+$ murine Tregs and their mobilization from the nucleus by CsA treatment was unaffected (Qiuxia Li et al. 2012). Controversially, the use of Cn inhibitors dramatically reduces Tregs number and their suppressive functions, underlying the role of Cn and, possibly, of NFAT in Tregs biology (Ma et al. 2009; Vaeth et al. 2012).

Thus, the role of calcium/Cn and NFAT activity supporting Foxp3 is still controversial, even though it seems that NFAT may have a role in Tregs biology.

1.9.4 NFATc role in Tregs generation

Tregs originate via two different developmental processes. Natural Tregs (nTregs) arise in the thymus during T cell education as lymphocytes with high-avidity TCR for self-antigens that survive the clonal deletion. Induced Tregs (iTregs) or adaptive Tregs are generated from conventional naïve $CD4^+$ T cells in peripheral tissues.

By evaluating the role of NFATc1, c2 and c3, the most expressed in the T cell compartment, during the generation of Tregs and execution of their suppressive functions, multiple single and combined NFAT KO animals were used. It appeared clear that both nTregs and iTregs activate NFAT TFs during their life-time, even if these cell subtypes express less NFAT TFs when compared with peripheral $CD4^+$ conventional T cells. nTregs development seems to require NFAT, because of the necessity of the calcium signals in this process (Oh-hora and Rao 2010; Shimon Sakaguchi and Sakaguchi 1988). Despite this, a severe reduction in NFAT level does not prevent nTregs generation, but a block or impairment in calcium/Cn signaling does (Oh-hora

and Rao 2010). In addition, some studies revealed that nTregs in mice lacking NFATc2 and c3 were not impaired in their suppressive functions nor they were reduced in numbers (Bopp et al. 2005), hence indicating that NFAT TFs may be dispensable in nTregs functional activities. Indeed, Foxp3 expression in nTregs mainly depends on CNS3, via the recruitment of a c-Rel enhanceosome (Ruan et al. 2010). As a matter of fact, NFATc2$^{-/-}$, NFATc2$^{-/-}$ X NFATc3$^{-/-}$, NFATc1$^{-/-}$ and NFATc2$^{-/-}$ X NFATc1$^{-/-}$ animals exhibit an unaffected thymic nTregs development (Bopp et al. 2005; Vaeth et al. 2012) suggesting that NFAT TFs are not strictly required for Foxp3 expression and thus generation of nTregs or that even a very low expression of only one member of the NFAT family is enough to promote Foxp3 expression in thymocytes. Another hypothesis regards the requirement of calcium flux and further Cn engagement for the activation of other pathways, as the NF-κB and AP-1 signaling cascades (Kiani, Rao, and Aramburu 2000). When compared to NFAT, these TFs seem to necessitate lower calcium oscillation frequencies (Fisher et al. 2006). To corroborate this evidence, Stim1$^{fl/fl}$ X CD4-Cre animals display regular nTregs development while Stim1/2 double KO, showing almost total block in calcium flux, completely abrogate nTregs generation (Oh-hora et al. 2009). It is important to note that development of nTregs requires high affinity/avidity TCR interaction completed by costimulatory signals via CD28 engagement, calcium/Cn

signaling cascade that leads to AP-1 and c-Rel/NF-κB activation. Conversely, NFAT TFs are required for peripheral iTregs generation. Indeed, NFAT TFs bind to the enhancer1/CNS1 sequence of the promoter of Foxp3, the *cis*-regulatory element that encourages the induction of Foxp3 in peripheral iTregs (Y Zheng et al. 2010). Furthermore, iTregs exhibit a generation process paradoxically opposite to the nTregs one, in terms of TCR stimulation. Indeed, iTregs develop after a suboptimal TCR engagement that results in NFAT activation, similarly to anergy induction in peripheral $CD4^+$ conventional T cell (Borde et al. 2006). Despite this, it seems that minimal levels of NFAT activity suffice for suppressive functions in both iTregs and nTregs, thanks to its interaction with Foxp3 and that NFAT role resides in the generation of iTregs.

1.9.5 Beyond NFATc: the role of IL-2 in Tregs homeostasis and function

Several studies have shown the crucial role of IL-2 both during the nTregs generation process in the thymus and for their subsequent maintenance in peripheral tissues. Indeed, the abundant and constitutive expression of CD25 on Tregs, firstly reported by Sakaguchi and colleagues, brought to light the

hypothesis for a fundamental role of IL-2 also for Tregs suppressive functions. This cytokine binds with low affinity to CD25 or to heterodimers of the common γ-chain (γc; CD132) and IL-2Rβ (CD122), but when these three subunits together interact with IL-2, their affinity for the cytokine increases of about 1.000-fold (Waldmann 1989). The signaling activated downstream IL-2R engagement involves JAK kinases and subsequently STAT5, fundamental TF for the expression of Foxp3 and Tregs differentiation in the thymus (M A Burchill et al. 2018). As a matter of fact, IL-2 permits the conversion of thymic $CD25^{+}$ $Foxp3^{-}$ and $CD25^{-}$ $Foxp3^{low}$ Tregs progenitor cells into mature Tregs (Matthew A Burchill et al. 2009; Lio and Hsieh 2009; Vang et al. 2018). In addition, IL-2 and TGF-β synergistically contribute to the differentiation of peripheral iTregs (Wanjun Chen et al. 2003). While it appears clear that IL-2 does have a critical role in promoting nTregs and iTregs generation, the role of IL-2 in sustaining Tregs homeostasis and activity following their differentiation has recently emerged to be fundamental. Indeed, mice lacking either IL-2 or the IL-2R α or β-chains display severe autoimmunity (Sadlack et al. 1995; Suzuki et al. 1995; Wilierford et al. 1995). Moreover, neutralization of IL-2 antibody-mediated in adult mice that have undergone thymectomy results in the reduction of Tregs number and Foxp3 expression (Rubtsov et al. 2014; Setoguchi et al. 2005). In addition, deficiency in STAT5 abrogates Foxp3 expression that, surprisingly, is recovered via

the increase of the anti-apoptotic mediator Bcl2, suggesting that IL-2 may be involved in the survival of Tregs or their precursor (Malin et al. 2010). To corroborate this hypothesis, it has been demonstrated that ablation of the pro-apoptotic molecule Bim results in the rescue of Tregs or their precursors from apoptosis in the absence of IL-2 or IL-2R and recovered the number of Tregs without preventing autoimmunity (Barron et al. 2011). Conversely, forced expression of IL-2Rβ exclusively in thymocytes rescues the fatal autoimmunity developed in Il2rβ$^{-/-}$ mice, suggesting that IL-2R expression may be dispensable in iTregs (Malek et al. 2002; Malek and Bayer 2004). Chinen and colleagues have recently demonstrated that IL-2 pathway activation, via IL-2R and STAT5, in Tregs is fundamental not only for their generation but also for their suppressive functions. Indeed, IL-2R expression on Tregs is indispensable for sequestering IL-2 from CD8^{+} T cells, abrogating their effector responses (Chinen et al. 2016). In addition, an observational cohort study reported that daily subcutaneous administration of low doses of IL-2 resulted to be associated to an expansion of the Tregs compartment and even an amelioration of the clinical manifestations of graft-versus-host disease (Koreth et al. 2011). This emerging role of IL-2 in the development and homeostasis of Tregs must be taken into account for therapeutic strategies in the context of autoimmunity and transplant rejection.

Intriguingly, Tregs are unable to produce IL-2, thus it has been questioned what the cellular source/s of IL-2 is/are. Germain and colleagues demonstrated via intravital imaging the localization of pSTAT5$^+$ Tregs clusters around activated effector T cells producing IL-2 in peripheral lymphoid organs (Z Liu et al. 2015) as confirmed by the work of Setoguchi (Setoguchi et al. 2005). Another scenario, not mutually exclusive, reported a direct dependency of Tregs from DCs-derived IL-2, especially for optimal Tregs differentiation in the thymus (Weist et al. 2015). Recently, Farrar and colleagues reported the strict requirement of T cell-derived IL-2 for Tregs development, since in its absence, they observed a severe defect in Tregs development in the thymus and a reduction in peripheral Tregs in lymphoid organs. They demonstrated that the IL-2 derived from B cells or DCs is neither necessary nor sufficient to supply the absence of the bystander IL-2 production from T cells, which seems to suffice Tregs development in the thymus and their maintenance in spleen and lymph nodes (Owen et al. 2018). Conversely, deficiency in IL-2 production by DCs in mesenteric lymph node leads to a reduction in Tregs abundance. Therefore, it is presumable that diverse subsets of Tregs, located in different tissues or organs, may rely on various IL-2 sources. Moreover, the role of IL-2 and its cellular producers in non-lymphoid organs, such as the skin, has not been explored yet. Recently, an evident and critical role of DCs-derived IL-2 for the gut

homeostasis has emerged. Indeed, intestinal DCs prevent spontaneous inflammation in the gut by balancing immunity and tolerance, via the production of IL-2. In this context, DCs-derived IL-2 skews the balance towards a tolerogenic profile.

1.9.6 The mechanisms of dominant tolerance

As aforementioned, Tregs represent the population of cells in chief for the promotion of tolerance. To address this issue, they exploit several diverse mechanisms, either cell-to-cell contact-dependent and via the release of immunomodulatory agents. This specific subset is specialized in suppressing a variety of other immune cells, among these B cells, NK cells, NKT cells, $CD4^+$ and $CD8^+$ T cells, as well as monocytes and DCs. Therefore, Tregs capability to promote suppression of effector functions in T cells could exploit the intermediation of DCs. Several mechanisms of imposed tolerance have been proposed, acting of distinct aspects of the life cycle of cells. Indeed, one of the molecule exploited by Tregs to modulate immune responses is the already mentioned CTLA-4, which is constitutively expressed by Tregs and located on the plasma membrane upon activation (Dieckmann et al. 2001). The precise mechanisms underlying CTLA-4 functionalities are still not fully understood. It may act as a sequester of APCs via the binding of costimulatory

molecules and thus preventing antigen presentation to conventional T cells and it can even reduce the expression of CD80/CD86 on DCs, probably via CTLA-4 (Onishi et al. 2008). The role of CTLA-4 exhibits some controversies, since its blockade does not necessarily result in overt immune responses, suggesting that Tregs do have compensatory or redundant mechanisms to dampen adaptive immunity or even that CTLA-4 involvement is context-dependent (Levings, Sangregorio, and Roncarolo 2001; Read, Malmström, and Powrie 2000). Indeed, Tregs display a wide repertoire of suppressive molecules, in addition to CTLA-4, and among these, Lymphocyte Activation Gene-3 (LAG-3). It binds to MHC class II, again sequestering APCs (Liang et al. 2008). LAG-3 is upregulated by Tregs in the presence of conventional T cells and its ectopic expression similarly results in suppression of adaptive responses (C. Huang et al. 2004). In addition to cell-to-cell contact mechanisms, Tregs create a suppressive milieu and conditionate surrounding cells via the release of specific cytokines: TGF-β and IL-10. Again, the notions regarding TGF-β as a suppressor factor are controversial. Indeed, mice lacking the most expressed form of TGF- β in immune cells, which is TGF-β1, develop T cell-mediated autoimmunity within several weeks after birth, in terms of enhanced Th1 and Th2 responses and development of colitis (M. O. Li, Sanjabi, and Flavell 2006). Some works reported the reduced suppressive capabilities of Tregs when TGF-β is

abrogated (Levings, Sangregorio, and Roncarolo 2001; Nakamura et al. 2004; Nakamura, Kitani, and Strober 2001) while others did not correlate the cytokine with the suppression of conventional T cells (Godfrey et al. 2005; Oberle et al. 2007). The discrepancy observed among distinct studies may be due to the dual role that TGF-β holds: putative suppressor and Tregs inducer. Indeed, TGF-β is directly involved in the generation of Tregs (W Chen and Konkel 2015; Fu et al. 2004), mediated by DCs (H. Huang et al. 2010; R. M. Steinman, Hawiger, and Nussenzweig 2003). In fact, tolerogenic DCs exhibit the peculiar capability to differentiate naïve T cells into Tregs via different factors and, among these, TGF-β. Other molecules released by tolerogenic DCs as "signal 3" are: IL-10, retinoic acid and indoleamine 2,3-dioxygenase (IDO), which catalyzes the conversion of tryptophan to kynurenine (Gu et al. 2015; Zhong-min Liu et al. 2015; Lu et al. 2014; Xie et al. 2015). Indeed, there is a mutual interplay between Tregs and tolerogenic DCs, since both the subsets may be the induction of the other (Min et al. 2003). Therefore, the exact suppressive functions of Tregs still require investigations to provide a clear role of Tregs in the distinct physiological and pathological contexts and to putatively modulate these functions to generate highly specific cell therapy (Q. Tang and Vincenti 2017).

1.10 Transplantation: when self meets non-self

Solid organ transplantation is a viable therapeutic approach that provides life-extending treatment for patients with failing organs. Despite improvements in short-term post-transplant outcomes, the success of the graft is limited to the development of rejection when antigen mismatch between donor and recipient occurs. Indeed, rejection was initially identified as the result of the host adaptive immune response, T cell-mediated, against donor MHC antigens. Nowadays, the scenario of rejection appears to be more complicated and involves diverse degree of the immune response.

1.10.1 The distinct types of rejection

The rejection process is constituted by a specific sequential cascade of events, starting with the surgery and the generation of tissue damage and injury. Soon after the transplant, several factors, discussed below, promote activation of innate immune cells that promote inflammation. In parallel, APCs maturate, upregulate costimulatory molecules and chemokine receptors and released cytokines. Therefore, donor APCs migrate to the draining secondary lymphoid organs where they encounter alloreactive naïve T cells, differentiating them into T helper cells.

Subsequently, they migrate into the graft and activate macrophages, granulocytes, which have reached and infiltrated the transplant via inflammatory mediators, and residing cells. Therefore, a feed-forward loop has established: alloreactive T cells promote effector cells, which contribute to the lesion formation directly or through the release of pro-inflammatory mediators, and, in turn, these effector cells sustain the adaptive response. Depending on the underlying mechanisms and kinetics progression, rejection can be defined as hyperacute, acute or chronic.

Hyperacute rejection occurs very rapidly, in terms of minutes after the transplantation of vascularized organs only. This fast phenomenon is due to the presence of anti-donor antibodies in the recipient that promote complement activation and concurrent stimulation of endothelial cells, which release Von Willebrand pro-coagulant factor. The result consists in massive platelets adhesion and aggregations, generating intravascular thrombosis with lesion and, finally, graft loss.

Acute rejection exhibits a more variable progression and may occur from 1 week to several month following transplantation. This phenomenon results from the instauration of alloreactive immune responses, in terms of T-cell-mediated acute cellular rejection and B cell-mediated acute humoral rejection. Acute rejection is the more frequent form of rejection, even though the

administration of immunosuppressive drugs, as CNIs, has reduced to less than 15% acute rejection event, both at a clinical and subclinical manifestation (Wehmeier et al. 2017).

Chronic rejection occurs several months and even years after the transplantation. The mechanisms underlying this event are still poorly understood. This process seems to be mediated by humoral or cellular events driven by memory/plasma cells and antibodies. Further, endothelial and immune cells release fibrosis-inducing mediators and pro-inflammatory cytokines that exacerbate the rejection. The main clinical manifestation of this pathological phenomenon is luminal narrowing and occlusion of arteries due to the proliferation of intimal smooth-muscle cells (Waaga et al. 2000).

1.10.2 Alloreactive T cells

The allorecognition of the donor MHC, or in humans to the human leukocyte antigens (HLA), differs from the canonical response to the classical antigens, either of microbial origin or derived from self damaged tissues, since it appears to be extremely strong. The mechanism underlying this phenomenon relies on the size and diversity of the alloreactive repertoire. Indeed, the alloimmune T cell repertoire against an allogeneic MHC haplotype has been estimated to represent 1-10% of the

entire T cell population (Dewolf et al. 2018). The extreme strength of alloresponses resides in the polymorphism of MHC/HLA and their crucial role in presenting antigens engaging the TCR. The infinite combinations of alleles of MHC/HLA in the individual, and at a higher level, in the population provide a high degree of defense via the recognition of several, if not all, pathogens, avoiding species destruction (Felix and Allen 2007). Allorecognition is categorized based on the protagonists of the encounter and it can be: direct, indirect or semidirect (Ali et al. 2013) (Figure 7). The direct alloresponse occurs when T cells react directly to alloantigens presented by donor APCs and usually leads to acute rejection. The indirect allorecognition is mediated by the encounter of T cells with self or recipient APCs presenting antigens derived from donor MHC or polymorphic proteins. This alloreactivity reveals to be more similar to typical immune responses, in particular when compared to the potency of the direct allorecognition. Indirect allorecognition has emerged to be the phenomenon mostly involved in chronic rejection, since donor APCs in the graft are continuously replaced by recipient APCs over time. Finally, the semidirect allorecognition involves the so-called "cross-dressing" of recipient cells with donor MHC-peptide complexes, possibly via microvesicles like exosomes (Herrera et al. 2004) or through the internalization and processing of donor MHC as peptides on recipient MHC (Smyth et al. 2006). The

allorecognition is a process that implicates $CD4^+$ and $CD8^+$ T cells of both the naïve and memory compartments and, of note, memory T cells are the most feared component of the process since they may not require costimulation for activation and further, they persist at higher number in the circulation if compared to naïve T cells. In addition to T cells-mediated rejection, there is growing evidence of a crucial role for innate immunity, and not only in terms of APCs functionalities.

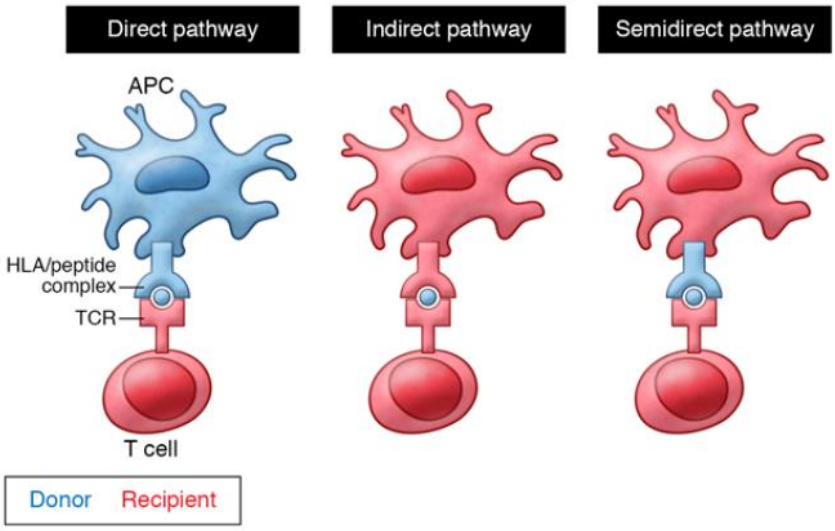

Figure 7: Pathway of activation of alloreactive T cells

Both donor and recipient's DCs concur at the generation of alloreactive T cells. Donor's DCs triggers T lymphocytes via the peptide – MHC complex (Direct pathway), while recipient's DCs can present donor-derived antigens (Indirect pathway) or they can be "cross-dressed" with peptide-MHC derived from donor's APCs (Semidirect pathway). Adapted from Dewolf and Sykes 2017.

1.10.3. Innate immunity in transplantation

1.10.3.1 Inflammation

As reported, adaptive responses play a crucial role in mediating allograft rejection, but early proinflammatory signals originate independently of the adaptive immunity, since they are prerogative of innate immune cells. Indeed, 1 day after a heart transplant, the expression of genes associated to the inflammatory process are comparable in normal mice and in mice deficient for T and B cells (He, Stone, and Perkins 2003). Indeed, inflammatory signals are crucial to create a milieu where the further alloreactive response will take place.

1.10.3.2 Self signals: DAMPs

Transplantation constitutes a complex medical approach for substituting failing organs and, from a molecular point of view, it provides several distinct stimuli, and, among these, DAMPs. Indeed, all grafted organs undergo and sustain some degree of ischemia/riperfusion injury (IRI), basically due to explantation and subsequent transplantation. Compelling evidence shows that IRI generates DAMPs (H Wu et al. 2007). To worsen this phenomenon, alloreactive responses themselves against the grafted tissue generate DAMPs that persist even

when IRI has resolved and exacerbate the rejection process. Therefore, DAMPs increased the cohort of antigens that may be presented by APCs, exaggerating alloreactivity. As aforementioned, DAMPs can bind PRRs, and among these, TLRs. Indeed, blockade of TLRs pathway in donor, recipient or both, via MyD88 deficiency, improves the outcome of the kidney and heart transplant (Goldstein et al. 2003; Tesar et al. 2004; Huiling Wu et al. 2012). Interestingly, patients with a loss-of-function polymorphism in TLR4 receiving lung or kidney transplant exhibit reduced acute rejection and improved long-term outcomes, corroborating the hypothesis of the involvement of TLRs and possibly DAMPs in the rejection process (Kruger et al. 2009; Palmer et al. 2003).

1.10.3.3 Non-self signals: microbiota

Solid organ transplantation involves not only the generation of stress and damage, but also a change in the commensal communities. In the last years, microbiota has risen much interest, and its role in pathological conditions is emerging, resulting in modulation of local or even distal immune responses (Belkaid and Hand 2014). People exhibit diverse bacterial composition, therefore when transplanting the donor organ, also its microbiota is introduced. The contribution of microbiota in this context is still elusive, even though it is already

compelled that organs colonized with bacteria as lungs and intestine have a poorer outcome if compared to organs considered sterile, as heart and kidney. Indeed, it has been suggested that acute rejection of intestine and lung may be in part due to a shift in the microbiota, even though acute rejection itself may have caused the changes in the commensal flora (Charlson et al. 2012; Oh et al. 2012). Interestingly, in a minor antigen-mismatch setting, pre-treating donor and recipients with antibiotics prolongs skin allograft survival, even when mice are in-house-bred littermates, minimizing microbiota differences. Indeed, the same group observed improved graft outcome when donor and recipients are germ-free animals, devoid of live bacteria (Lei et al. 2016). The antibiotics treatment (and so the absence of live microorganisms in germ-free animals) reduces $CD4^+$ IFN-γ-producing T cells, hence dampening alloreactivity. Therefore, it is presumable that a reduction in the microbiota may limit APCs activation and, in turn, decrease alloreactive T cells.

As emerged, transplantation constitutes a biological phenomenon characterized by several variables, provoking the appearance of distinct stimuli, both self and non-self to some extent, that alert the innate immune system concomitantly leading to the instauration of alloreactivity against the graft. The introduction of immunosuppressors has dramatically reduced the frequency of rejection in parallel with an increase of severe

side effects that are exacerbated by the life-long administration of the drugs.

1.11 Scope of the thesis

Innate immune cells represent the highly conserved defense mechanism that acts very rapidly upon insult. Their effector functions limit the dissemination of invading pathogens, provide precise information about the type of offence the host is undergoing, and, above all, elicit the more specific and efficient adaptive responses. Indeed, vertebrates are provided with both the two branches of immunity that confer them a functional superiority in facing exogenous agents coming from the surrounding environment.

Being the more ancient form of protection, shared among species, from plants to human, innate immunity may be considered as an elementary but efficiently functional instrument. Conversely, with the appearance of acquired immunity, the degree of complexity in immune responses required a review process, in evolutionary terms. Therefore, novel signaling pathways have emerged and "old-fashion" factors renewed, acquiring additional properties. This is the case for the Nuclear factor of activated T cells (NFAT) in innate immunity, later after its first appearance concomitantly the development of adaptive immunity.

There is now compelling evidence about the multifaceted soul of NFAT. Its crucial role in T cells survival and expansion was brought to light in 1988, being the transcription factor appointed

for IL-2 production. When the first cues about the NFAT-mediated release of IL-2 by DCs were revealed, the long path for the identification of the stimuli that drive NFAT elicitation and its role in immune cells began to be walked.

In the last years, NFAT has emerged as key player in fungal and bacterial infections, both promoting IL-2 release and regulating the life-cycle of DCs, avoiding overt inflammation and autoimmunity. Furthermore, it has been recently shown that IL-2 production by DCs, *trans*-presented on CD25, is fundamental to induce T cell proliferation that leads to adaptive immunity elicitation (Wuest et al. 2011). Hence the critical role of DCs as APCs relies in particular in the activation of NFAT and, hence in the production of IL-2, necessary both for Th polarization and for Tregs generation.

Therefore, the role of NFAT in DCs in both physiological and pathological contexts is attracting much interest and is currently under investigation. Indeed, we aim at evaluating the effects of the possible NFAT activation in the context of mismatched skin transplant, when DCs regulate the priming of alloreactive T cells. Through the use of NFATc2 Knock Out (KO) mice and of newly generated conditional KO animals, in which $CD11c^+$ DCs express a specific inhibitor of NFAT, we could appreciate the contribute of NFAT triggering in alloreactivity. We hypothesize that the inhibition of NFAT can abrogate the APCs functionalities of DCs

preventing T cell activation and consequent graft loss. In addition to the changes in graft acceptance or rejection, our study aims at revealing NFAT contribution to the entire process of transplantation, from the generated-damage to the early inflammatory phase, from the activation of DCs to their migration to the draining lymph node and, finally, to their priming capabilities towards T cells, evaluating the generation of alloreactive T cells or, putatively, of Tregs and graft tolerance (Figure 1).

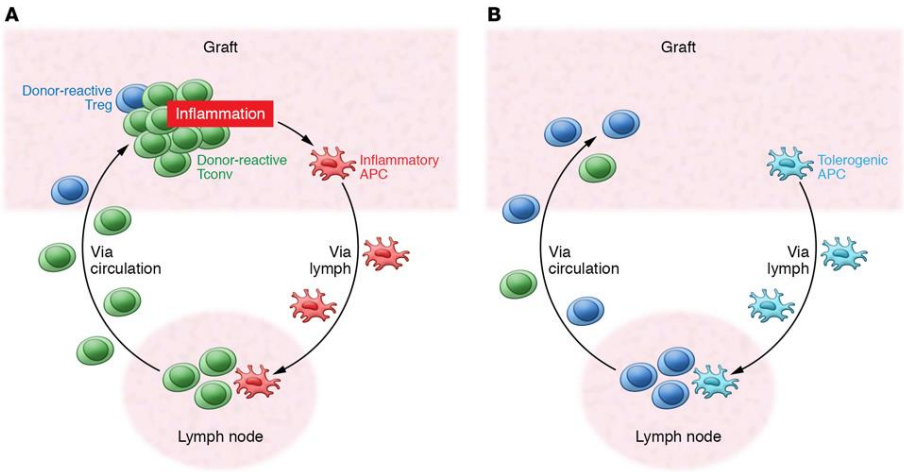

Figure 1: Acceptance vs Rejection

The figure displays the diverse outcomes of organ transplantation, emphasizing the crucial role of innate-based early events and, above all, of DCs that mediate the expansion of alloreactive T cells (A) or of graft-specific Tregs (B) determining graft rejection and acceptance, respectively. Adapted from Tang and Vincenti 2017.

References to Chapter 1

Akira, Shizuo, and Kiyoshi Takeda. 2004. "Toll-like Receptor Signaling." *Nature Reviews Immunology* 4(July): 499–511.

Akira, Shizuo, Satoshi Uematsu, and Osamu Takeuchi. 2006. "Pathogen Recognition and Innate Immunity." *Cell* 124(4): 783–801.

Alcántara-Hernández, Marcela et al. 2017. "High-Dimensional Phenotypic Mapping of Human Dendritic Cells Reveals Interindividual Variation and Tissue Specialization." *Immunity* 47(6): 1037–1050.e6.

Ali, Jason M, Eleanor M Bolton, J Andrew Bradley, and Gavin J Pettigrew. 2013. "Allorecognition Pathways in Transplant Rejection and Tolerance." *Transplantation* 96(8): 681–88.

Allen, Judith E, and Rick M Maizels. 2011. "Diversity and Dialogue in Immunity to Helminths." *Nature Reviews Immunology* 11(6): 375–88. http://dx.doi.org/10.1038/nri2992.

Anandasabapathy, Niroshana et al. 2003. "GRAIL: An E3 Ubiquitin Ligase That Inhibits Cytokine Gene Transcription Is Expressed in Anergic CD4+ T Cells." *Immunity* 18: 535–47.

Ariizumi, Kiyoshi et al. 2000. "Identification of a Novel, Dendritic Cell-Associated Molecule, Dectin-1, by Subtractive CDNA Cloning." *The Journal of Biological Chemistry* 275(26): 20157–67.

Arron, Joseph R et al. 2006. "NFAT Dysregulation by Increased Dosage of DSCR1 and DYRK1A on Chromosome 21." *Nature* 441(June): 595–600.

Bachmaier, Kurt et al. 2000. "Negative Regulation of Lymphocyte Activation and Autoimmunity by the Molecular Adaptor Cbl-B." *Nature* 403: 211–16.

Bagnis, C et al. 1996. "Comparative Acute Nephrotoxicity of FK-506 and Ciclosporin in an Isolated in Situ Autoperfused Rat Kidney Model." *American Journal of Nephrology* 17: 17–24.

Banchereau, J, and R M Steinman. 1998. "Dendritic Cells and the Control of Immunity." *Nature* 392(March): 245–52.

Bao, Katherine, and R Lee Reinhardt. 2015. "The Differential Expression of IL-4 and IL-13 and Its Impact on Type-2 Immunity." *Cytokine* 75(1): 25–37.

Barron, Luke et al. 2011. "Mechanisms of IL-2-Dependent Maintenance of Functional Regulatory T Cells." *The Journal of Immunology*

185(11): 6426–30.
Beals, Chan R et al. 1997. "Nuclear Export of NF-ATc Enhanced by Glycogen Synthase Kinase-3." *Science* 275(5): 1930–34.
Bedoui, Sammy et al. 2009. "Cross-Presentation of Viral and Self Antigens by Skin-Derived CD103+dendritic Cells." *Nature Immunology* 10(5): 488–95.
Belkaid, Yasmine, and Timothy W Hand. 2014. "Role of the Microbiota in Immunity and Inflammation." *Cell* 157(1): 121–41. http://dx.doi.org/10.1016/j.cell.2014.03.011.
Billingham, R E, L Brent, and P B Medawar. 1953. "Actively Acquired Tolerance of Foreign Cells." *Nature* 172: 603–6.
Boehm, Thomas. 2012. "Evolution of Vertebrate Immunity." *Current Biology* 22(17): R722–32. http://dx.doi.org/10.1016/j.cub.2012.07.003.
Bopp, Tobias et al. 2005. "NFATc2 and NFATc3 Transcription Factors Play a Crucial Role in Suppression of CD4+ T Lymphocytes by CD4+ CD25+ Regulatory T Cells." *The Journal of Experimental Medicine* 201(2): 181–87.
Borde, Madhuri et al. 2006. "Transcriptional Basis of Lymphocyte Tolerance." *Immunological Reviews* 210(1): 105–19.
Brown, Gordon D et al. 2003. "Dectin-1 Mediates the Biological Effects of β-Glucans." *The Journal of Experimental Medicine* 197(9): 1119–24.
Brubaker, Sky W, Kevin S Bonham, Ivan Zanoni, and Jonathan C Kagan. 2015. "Innate Immune Pattern Recognition: A Cell Biological Perspective." *Annual Review of Immunology* 33: 257–90.
Brunkow, Mary E et al. 2001. "Disruption of a New Forkhead/Winged-Helix Protein, Scurfin, Results in the Fatal Lymphoproliferative Disorder." *Nature Genetics* 27: 68–73.
Burchill, M A et al. 2018. "IL-2 Receptor b-Dependent STAT5 Activation Is Required for the Development of Foxp3+ Regulatory T Cells." *The Journal of Immunology* 178: 280–90.
Burchill, Matthew A et al. 2009. "Linked T Cell Receptor and Cytokine Signaling Govern the Development of the Regulatory T Cell Repertoire." *Immunity* 28(1): 612–25.
Burnet, Sir M. 1959. "Auto-Immune Disease. II. Pathology of the Immune Response." *British Medical Journal* 2(5154): 720–25.
Burnet, Sir Macfarlane. 1959. "Auto-Immune Disease. I. Modern Immunological Concepts." *British Medical Journal* 2(5153): 645–50.
Celona, Barbara et al. 2011. "Substantial Histone Reduction Modulates Genomewide Nucleosomal Occupancy and Global

Transcriptional Output." *PLoS Biology* 9(6): 1–16.
Chan, Vera S F, Camilla Wong, and Pamela S Ohashi. 2002. "Calcineurin Aα Plays an Exclusive Role in TCR Signaling in Mature but Not in Immature T Cells." *European journal of Allergy and Clinical Immunology* 32: 1223–29.
Chang, John T et al. 2000. "The Costimulatory Effect of IL-18 on the Induction of Antigen-Specific IFN-γ Production by Resting T Cells Is IL-12 Dependent and Is Mediated by up-Regulation of the IL-12 Receptor B2 Subunit." *European Journal of Immunology* 30: 1113–19.
Charlson, Emily S et al. 2012. "Lung-Enriched Organisms and Aberrant Bacterial and Fungal Respiratory Microbiota after Lung Transplant." *Am J Respir Crit Care Med* 186(6): 536–45.
Chen, Min et al. 2006. "Dendritic Cell Apoptosis in the Maintenance of Immune Tolerance." *Nature* 311(February): 1160–64.
Chen, W, and J E Konkel. 2015. "The Development of Thymic Foxp3+ Regulatory T Cells: TGF-β Matters." *European Journal of Immunology* 45(4): 958–65.
Chen, Wanjun et al. 2003. "Conversion of Peripheral CD4+ CD25+ Naive T Cells to CD4+ CD25+ Regulatory T Cells by TGF-b Induction of Transcription Factor Foxp3." *The Journal of Experimental Medicine* 198(12): 1875–86.
Chinen, Takatoshi et al. 2016. "An Essential Role for IL-2 Receptor in Regulatory T Cell Function." *Nature Immunology* 17(11): 1322–33.
Chow, Chi-wing et al. 1997. "Nuclear Accumulation of NFAT4 Opposed by the JNK Signal Transduction Pathway." *Science* 278(November): 2–5.
Cisse, Babacar et al. 2008. "Transcription Factor E2-2 Is an Essential and Specific Regulator of Plasmacytoid Dendritic Cell Development." *Cell* 135(1): 37–48.
Collin, Matthew, and Paul Milne. 2016. "Langerhans Cell Origin and Regulation." *Current Opinion in Hematology* 23(1): 28–35.
Cosenza, Humberto, Lee D Leserman, and Donald A Rowley. 1971. "The Third Cell Type Required for the Immune Response of Spleen Cells in Vitro." *The Journal of Immunology* 107(2): 414–21.
Crozat, K. et al. 2011. "Cutting Edge: Expression of XCR1 Defines Mouse Lymphoid-Tissue Resident and Migratory Dendritic Cells of the CD8 + Type." *The Journal of Immunology* 187(9): 4411–15. http://www.jimmunol.org/cgi/doi/10.4049/jimmunol.1101717
Dewolf, Susan et al. 2018. "Quantifying Size and Diversity of the Human T Cell Alloresponse." *Journal of Clinical Investigation* 3(15).

Dieckmann, By Detlef et al. 2001. "Ex Vivo Isolation and Characterization of CD4+ CD25+ T Cells with Regulatory Properties from Human Blood." *The Journal of Experimental Medicine* 193(11): 1303–10.

Dillon, Stephanie et al. 2018. "A Toll-like Receptor 2 Ligand Stimulates Th2 Responses in Vivo, via Induction of Extracellular Signal-Regulated Kinase Mitogen-Activated Protein Kinase and c-Fos in Dendritic Cells." *The Journal of Immunology* 172(8): 4733–43.

Doering, Travis A et al. 2012. "Network Analysis Reveals Centrally Connected Genes and Pathways Involved in CD8+ T Cell Exhaustion versus Memory." *Immunity* 37(6): 1130–44. http://dx.doi.org/10.1016/j.immuni.2012.08.021.

Dolmetsch, R. E., R. S. Lewis, C. C. Goodnow, and J. I. Healy. 1997. "Differential Activation of Transcription Factors Induced by Ca2+ Response Amplitude and Duration." : 855–58.

Dolmetsch, R. E., K. Xu, and R. S. Lewis. 1998. "Calcium Oscillations Increase the Efficiency and Specificity of Gene Expression." *Nature* 392(April): 933–36.

Dong, Chen, Roger J Davis, and Richard A Flavell. 2002. "MAP Kinases in the Immune Response." *Annual Review of Immunology* 20: 55–72.

Doran, Kelly S, Anirban Banerjee, Olivier Disson, and Marc Lecuit. 2013. "Concepts and Mechanisms: Crossing Host Barriers." *Cold Spring Harbor Perspectives in Medicine* 3: 1–20.

Dorner, Brigitte G. et al. 2009. "Selective Expression of the Chemokine Receptor XCR1 on Cross-Presenting Dendritic Cells Determines Cooperation with CD8+ T Cells." *Immunity* 31(5): 823–33.

Duperrier, K et al. 2002. "Cyclosporin A Inhibits Dendritic Cell Maturation Promoted by TNF-α or LPS but Not by Double-Stranded RNA or CD40L." *Journal of Leukocyte Biology* 72(November): 953–61.

Edelson, Brian T. et al. 2010. "Peripheral CD103 + Dendritic Cells Form a Unified Subset Developmentally Related to CD8α + Conventional Dendritic Cells." *The Journal of Experimental Medicine* 207(4): 823–36. http://www.jem.org/lookup/doi/10.1084/jem.20091627.

Eickhoff, Sarah et al. 2015. "Robust Anti-Viral Immunity Requires Multiple Distinct T Cell-Dendritic Cell Interactions." *Cell* 162(6): 1322–37.

Epaulard, Olivier et al. 2014. "Macrophage- and Neutrophil-Derived TNF-α Instructs Skin Langerhans Cells To Prime Antiviral

Immune Responses." *The Journal of Immunology* 193(5): 2416–26. http://www.jimmunol.org/lookup/doi/10.4049/jimmunol.1303339.

Everts, Bart, Hermelijn H Smits, Cornelis H Hokke, and Maria Yazdanbakhsh. 2010. "Helminths and Dendritic Cells: Sensing and Regulating via Pattern Recognition Receptors, Th2 and Treg Responses." *European Journal of Immunology* 40: 1525–37.

Fang, Deyu et al. 2002. "Dysregulation of T Lymphocyte Function in Itchy Mice: A Role for Itch in Th2 Differentiation." *Nature Immunology* 3(3): 281–87.

Fang, Hongliang et al. 2011. "Toll-like Receptor 4 (TLR4) Is Essential for Hsp70-like Protein 1 (HSP70L1) to Activate Dendritic Cells and Induce Th1 Response." *The journal of Biological Chemistry* 286(35): 30393–400.

Felix, Nathan J, and Paul M Allen. 2007. "Specificity of T-cell Alloreactivity." *Nature Reviews Immunology* 7: 942–53.

Ferwerda, B et al. 2009. "Human Dectin-1 Deficiency and Mucocutaneous Fungal Infections." *The New England Journal of Medicine* 361(18): 1760–67.

Feske, S. 2007. "Calcium Signalling in Lymphocyte Activation and Disease." *Nature Reviews Immunology* 7: 690–702.

Feske, Stefan et al. 2000. "The Duration of Nuclear Residence of NFAT Determines the Pattern of Cytokine Expression in Human SCID T Cells." *Journal of immunology (Baltimore, Md. : 1950)* 165: 297–305.

Fisher, Wayne G F, P Yang, R K Medikonduri, and M S Jafri. 2006. "NFAT and NF-KB Activation in T Lymphocytes: A Model of Differential Activation of Gene Expression." *Annals of Biomedical Engineering* 34(11): 1712–28.

Flacher, V. et al. 2014. "Murine Langerin+ Dermal Dendritic Cells Prime CD8+ T Cells While Langerhans Cells Induce Cross-Tolerance." *EMBO Molecular Medicine* 6(9): 1191–1204. http://embomolmed.embopress.org/cgi/doi/10.15252/emmm.201303283.

Fogg, D K et al. 2006. "A Clonogenic Bone Marrow Progenitor Specific for Macrophages and Dendritic Cells." *Science* 311(5757): 83–87.

Fontenot, Jason D, Marc A Gavin, and Alexander Y Rudensky. 2003. "Foxp3 Programs the Development and Function of CD4+ CD25+ Regulatory T Cells." *Nature Immunology* 4(4): 330–36.

Frantz, Betsy et al. 1994. "Calcineurin Acts in Synergy with PMA to Inactivate IkB/MAD3, an Inhibitor of NF-KB." *The EMBO Journal* 13(4): 861–70.

Fu, Shuang et al. 2004. "TGF-β Induces Foxp3+ T-Regulatory Cells from CD4+ CD25− Precursors." *American Journal of Transplantation* 4(10): 1614–27.

Gavin, Marc A et al. 2007. "Foxp3-Dependent Programme of Regulatory T-Cell Differentiation." *Nature* 445(7129): 771–75.

Gershon, R K, P Cohen, R Hencin, and S A Liebhaber. 1972. "Suppressor T Cells." *The Journal of Immunology* 108(3): 586–90.

Gershon, R K, and K Kondo. 1970. "Cell Interactions in the Induction of Tolerance : The Role of Thymic Lymphocytes." *Immunology* 18: 723–37.

Ghosh, Hiyaa S et al. 2010. "Continuous Expression of the Transcription Factor E2-2 Maintains the Cell Fate of Mature Plasmacytoid Dendritic Cells." *Immunity* 33(6): 905–16. http://dx.doi.org/10.1016/j.immuni.2010.11.023.

Giacomin, Paul R et al. 2012. "Thymic Stromal Lymphopoietin-Dependent Basophils Promote Th2 Cytokine Responses Following Intestinal Helminth Infection." *The Journal of Immunology* 189: 4371–78.

Godfrey, Wayne R et al. 2005. "Cord Blood CD4+ CD25+-Derived T Regulatory Cell Lines Express FoxP3 Protein and Manifest Potent Suppressor Function." *Blood* 105(2): 750–58.

Goldstein, Daniel R, Bethany M Tesar, Shizuo Akira, and Fadi G Lakkis. 2003. "Critical Role of the Toll-like Receptor Signal Adaptor Protein MyD88 in Acute Allograft Rejection." *The Journal of Clinical Investigation* 111(10): 1571–78.

Gomez del Arco, Pablo et al. 2000. "A Role for the P38 MAP Kinase Pathway in the Nuclear Shuttling of NFATp." *The journal of Biological Chemistry* 275(18): 13872–78.

Goodridge, H. S., R. M. Simmons, and D. M. Underhill. 2007. "Dectin-1 Stimulation by Candida Albicans Yeast or Zymosan Triggers NFAT Activation in Macrophages and Dendritic Cells." *The Journal of Immunology* 178(5): 3107–15. http://www.jimmunol.org/cgi/doi/10.4049/jimmunol.178.5.3107.

Gornati, Laura, Ivan Zanoni, and Francesca Granucci. 2018. "Dendritic Cells in the Cross Hair for the Generation of Tailored Vaccines." *Frontiers in Immunology* 9: 1–14.

Graef, Isabella A, Julie M Gastier, Uta Francke, and Gerald R Crabtree. 2000. "Evolutionary Relationships among Rel Domains Indicate Functional Diversification by Recombination." *PNAS* 98(10): 5740–45.

Grakoui, Arash et al. 1999. "The Immunological Synapse: A Molecular Machine Controlling T Cell Activation." *Science* 285: 221–27.

Granucci, F et al. 2001. "Inducible IL-2 Production by Dendritic Cells Revealed by Global Gene Expression Analysis." *Nature Immunology* 2(9): 882–88. papers2://publication/doi/10.1038/ni0901-882.

Granucci, Francesca et al. 2004. "A Contribution of Mouse Dendritic Cell-Derived IL-2 for NK Cell Activation." *The Journal of Experimental Medicine* 200(3): 287–95.

Granucci, Francesca et al. 2003. "The Immune Response Is Initiated by Dendritic Cells via Interaction with Microorganisms and Interleukin-2 Production." *Journal of Infect* 187: 0–4.

Greter, Melanie et al. 2012. "GM-CSF Controls Nonlymphoid Tissue Dendritic Cell Homeostasis but Is Dispensable for the Differentiation of Inflammatory Dendritic Cells." *Immunity* 36(6): 1031–46. http://dx.doi.org/10.1016/j.immuni.2012.03.027.

Grössinger, Eva M et al. 2017. "Ca 2+ -Dependent Regulation of NFATc1 via KCa3.1 in Inflammatory Osteoclastogenesis."

Gu, Jian et al. 2015. "All-Trans Retinoic Acid Favors the Development and Function of Regulatory T Cells from Liver Transplant Patients." *International Immunopharmacology* 28(2): 906–10. http://dx.doi.org/10.1016/j.intimp.2015.03.052.

Guilliams, Martin et al. 2015. "Dendritic Cells, Monocytes and Macrophages: A Unified Nomenclature Based on Ontogeny." *Nature Reviews Immunology* 14(8): 571–78.

Gwack, Yousang et al. 2006. "A Genome-Wide Drosophila RNAi Screen Identifies DYRK-Family Kinases as Regulators of NFAT." *Nature* 441(June): 646–50.

Haniffa, Muzlifah et al. 2012. "Human Tissues Contain CD141hiCross-Presenting Dendritic Cells with Functional Homology to Mouse CD103+Nonlymphoid Dendritic Cells." *Immunity* 37(1): 60–73.

Hartmann, K, R W Dutton, Margaret M Mccarthy, and R I Mishell. 1970. "Cell Components in the Immune Response. Cellular Immunology." *Cellular Immunology* 1: 182–89.

He, Hongzhen, James R Stone, and David L Perkins. 2003. "Analysis of Differential Immune Responses Induced by Innate and Adaptive Immunity Following Transplantation." *Immunology* 109: 185–96.

Heisel, Olaf, Rochelle Heisel, Robert Balshaw, and Paul Keown. 2004. "New Onset Diabetes Mellitus in Patients Receiving Calcineurin Inhibitors: A Systematic Review and Meta-Analysis." *American Journal of Transplantation* 4: 583–95.

Heissmeyer, Vigo et al. 2004. "Calcineurin Imposes T Cell Unresponsiveness through Targeted Proteolysis of Signaling Proteins." *Nature Immunology* 5(3): 255–65.

Henri, Sandrine et al. 2010. "CD207+ CD103+ Dermal Dendritic Cells Cross-Present Keratinocyte-Derived Antigens Irrespective of the Presence of Langerhans Cells." *The Journal of Experimental Medicine* 207(1): 189–206.

Herbst, S et al. 2015. "Phagocytosis-Dependent Activation of a TLR9-BTK-Calcineurin-NFAT Pathway Co-Ordinates Innate Immunity to Aspergillus Fumigatus." *EMBO Molecular Medicine* 7(3): e201404556. http://embomolmed.embopress.org/cgi/doi/10.15252/emmm.201404556%5Cnpapers2://publication/doi/10.15252/emmm.201404556.

Hermann-Kleiter, Natascha, and Gottfried Baier. 2010. "NFAT Pulls the Strings during CD4+ T Helper Cell Effector Functions." *Blood* 115(15): 2989–98.

Herrera, Osquel Barroso et al. 2004. "A Novel Pathway of Alloantigen Presentation by Dendritic Cells." *The Journal of Immunology* 173: 4828–37.

Honda, K et al. 2005. "IRF-7 Is the Master Regulator of Type-I Interferon-Dependent Immune Responses." *Nature* 434: 772–77.

Hori, Shohei, T Nomura, and S Sakaguchi. 2003. "Control of Regulatory T Cell Development by the Transcription Factor Foxp3." *Science* 299(5609): 1057–61.

Hu, Wei, Ty Dale Troutman, Ramakrishna Edukulla, and Chandrashekhar Pasare. 2011. "Priming Microenvironments Dictate Cytokine Requirements for T Helper 17 Cell Lineage Commitment." *Immunity* 35(6): 1010–22. http://dx.doi.org/10.1016/j.immuni.2011.10.013.

Huang, Ching-tai et al. 2004. "Role of LAG-3 in Regulatory T Cells." *Immunity* 21: 503–13.

Huang, Hui et al. 2010. "Tolerogenic Dendritic Cells Induce CD4+ CD25hi Foxp3+ Regulatory T Cell Differentiation from CD4+ CD25−/Lo Foxp3− Effector T Cells." *The Journal of Immunology* 185: 5003–10.

Hunter, Christopher A, and Rob Kastelein. 2012. "Interleukin-27: Balancing Protective and Pathological Immunity." *Immunity* 37(6): 960–69. http://dx.doi.org/10.1016/j.immuni.2012.11.003.

Igyarto, B. Z. et al. 2009. "Langerhans Cells Suppress Contact Hypersensitivity Responses Via Cognate CD4 Interaction and

Langerhans Cell-Derived IL-10." *The Journal of Immunology* 183(8): 5085–93.
http://www.jimmunol.org/cgi/doi/10.4049/jimmunol.0901884

Igyártó, Botond Z. et al. 2011. "Skin-Resident Murine Dendritic Cell Subsets Promote Distinct and Opposing Antigen-Specific T Helper Cell Responses." *Immunity* 35(2): 260–72.

Imai, Akihito et al. 2007. "Inhibition of Endogenous MHC Class II-Restricted Antigen Presentation by Tacrolimus (FK506) via FKBP51." *European Journal of Immunology* 37: 1730–38.

Isakov, N, and A Altman. 2002. "Protein Kinase Cθ in T Cell Activation." *Annual Review of Immunology* 20: 761–94.

Jain, S, G R Bicknell, and M L Nicholson. 2000. "Tacrolimus Has Less Fibrogenic Potential than Cyclosporin A in a Model of Renal Ischaemia-Reperfusion Injury." *British Journal of Surgery* 87: 1563–68.

Janeway, Charles A. 1992. "The Immune System Evolved to Discriminate Infectious Nonself from Noninfectious Self." *Immunology Today* 13(1): 11–16.

Jenkins, Marc K et al. 1987. "Molecular Events in the Induction of a Nonresponsive State in Interleukin 2-Producing Helper T-Lymphocyte Clones." *PNAS* 84(August): 5409–13.

Jiao, Zhijun et al. 2014. "The Closely Related CD103+ Dendritic Cells (DCs) and Lymphoid-Resident CD8+ DCs Differ in Their Inflammatory Functions." *PLoS ONE* 9(3): 1–10.

Jin, M et al. 2016. "Toll-like Receptor 2-Mediated MAPKs and NF-KB Activation Requires the GNAO1-Dependent Pathway in Human Mast Cells." *Integrative Biology* 8(9): 968–75.

Johnson, Gary L, and Razvan Lapadat. 2002. "Mitogen-Activated Protein Kinase Pathways Mediated by ERK, JNK, and P38 Protein Kinases." *Science* 298(5600): 1911–12.

Kabashima, Kenji et al. 2003. "Prostaglandin E2–EP4 Signaling Initiates Skin Immune Responses by Promoting Migration and Maturation of Langerhans Cells." *Nature Medicine* 9(6): 744–49.

Kagan, Jonathan C et al. 2008. "TRAM Couples Endocytosis of Toll-like Receptor 4 to the Induction of Interferon-β." *Nature Immunology* 9: 361–68.

Kagan, Jonathan C, and Ruslan Medzhitov. 2006. "Phosphoinositide-Mediated Adaptor Recruitment Controls Toll-like Receptor Signaling." *Cell* 125(5): 943–55.

Kamradt, T, and N A Mitchison. 2001. "Tolerance and Autoimmunity." *The New England Journal of Medicine* 344(9): 655–64.

Kashem, Sakeen W. et al. 2015. "Candida Albicans Morphology and Dendritic Cell Subsets Determine T Helper Cell Differentiation." *Immunity* 42(2): 356–66.

Kautz-Neu, Kordula et al. 2011. "Langerhans Cells Are Negative Regulators of the Anti- *Leishmania* Response." *The Journal of Experimental Medicine* 208(5): 885–91. http://www.jem.org/lookup/doi/10.1084/jem.20102318.

Kawai, Taro, and Shizuo Akira. 2007. "TLR Signaling." *Seminars in Immunology* 19: 24–32.

Khameneh, Hanif Javanmard et al. 2017. "The Syk–NFAT–IL-2 Pathway in Dendritic Cells Is Required for Optimal Sterile Immunity Elicited by Alum Adjuvants." *The Journal of Immunology* 198(1): 196–204. http://www.jimmunol.org/lookup/doi/10.4049/jimmunol.1600420.

Khattri, Roli, Tom Cox, Sue-ann Yasayko, and Fred Ramsdell. 2003. "An Essential Role for Scurfin in CD4+ CD25+ T Regulatory Cells." *Nature Immunology* 4(4): 337–42.

Kiani, Alexander, Anjana Rao, and Jose Aramburu. 2000. "Manipulating Immune Responses with Immunosuppressive Agents That Target NFAT." *Immunity* 12: 359–72.

Kim, Peter W et al. 2003. "A Zinc Casp Structure Tethers Lck to T Cell Coreceptors CD4 and CD8." *Science* 301(5640): 1725–28.

Kimbrell, Deborah A, and Bruce Beutler. 2001. "The Evolution and Genetics of Innate Immunity." *Nature Reviews Genetics* 2(April): 256–67.

King, Irah L, Mark A Kroenke, and Benjamin M Segal. 2010. "GM-CSF-Dependent, CD103+ Dermal Dendritic Cells Play a Critical Role in Th Effector Cell Differentiation after Subcutaneous Immunization." *The Journal of Experimental Medicine* 207(5): 953–61.

Kingston, Dior et al. 2009. "The Concerted Action of GM-CSF and Flt3-Ligand on in Vivo Dendritic Cell Homeostasis." *Blood* 114: 835–43.

Kino, Toru et al. 1987. "FK-506, a Novel Immunosuppressant Isolated from a Streptomyces. II Immunosuppressive Effect of FK-506 in Vitro." *The Journal of Antibiotics* 40(9): 1256–65.

Kissenpfennig, Adrien et al. 2005. "Dynamics and Function of Langerhans Cells in Vivo: Dermal Dendritic Cells Colonize Lymph Node Areas Distinct from Slower Migrating Langerhans Cells." *Immunity* 22(5): 643–54.

Klee, Claude B, Hao Ren, and Xutong Wang. 1998. "Regulation of the Calmodulin- Stimulated Protein Phosphatase, Calcineurin." *The journal of biological chemistry* 273(22): 13367–70.

Kolls, Jay K. 2010. "Th17 Cells in Mucosal Immunity and Tissue Inflammation." *Semin Immunopathol.* 29(10): 1883–89.

Koreth, J et al. 2011. "Interleukin-2 and Regulatory T Cells in Graft-versus-Host-Disease." *New England Journal of Medicine* 365(22): 2055–66.

Koski, Gary K et al. 1999. "Calcium Mobilization in Human Myeloid Cells Results in Acquisition of Individual Dendritic Cell-like Characteristics through Discrete Signaling Pathways." *The Journal of Immunology* 163: 82–92.

Kroczek, Richard A., and Volker Henn. 2012. "The Role of XCR1 and Its Ligand XCL1 in Antigen Cross-Presentation by Murine and Human Dendritic Cells." *Frontiers in Immunology* 3(FEB): 1–5.

Kruger, Bernd et al. 2009. "Donor Toll-like Receptor 4 Contributes to Ischemia and Reperfusion Injury Following Human Kidney Transplantation." *PNAS* 106(9): 3390–95.

Kuhns, Michael S, Mark M Davis, and K Christopher Garcia. 2006. "Deconstructing the Form and Function of the TCR/CD3 Complex." *Immunity* 24(2): 133–39.

Kumar, Pawan, Kong Chen, and Jay K Kolls. 2013. "Th17 Cell Based Vaccines in Mucosal Immunity." *Current Opinion in Immunology* 25(3): 373–80. http://dx.doi.org/10.1016/j.coi.2013.03.011.

Lafferty, K J, and J Cunningham. 1975. "A New Analysis of Allogeneic Interactions." *Australian Journal of Experimental Biology and Medical Science* 53: 27–42.

Lee, Hye-mi et al. 2009. "Innate Immune Responses to Mycobacterium Ulcerans via Toll-like Receptors and Dectin-1 in Human Keratinocytes." *Cellular Microbiology* 11(January): 678–92.

Lee, Young-ran et al. 2005. "Cyclosporin A and Tacrolimus, but Not Rapamycin, Inhibit MHC-Restricted Antigen Presentation Pathways in Dendritic Cells." *Bloo* 105: 3951–55.

Legler, D. F. et al. 2006. "Prostaglandin E2 Is Generally Required for Human Dendritic Cell Migration and Exerts Its Effect via EP2 and EP4 Receptors." *The Journal of Immunology* 176(2): 966–73. http://www.jimmunol.org/cgi/doi/10.4049/jimmunol.176.2.966.

Legler, Daniel F, Markus Bruckner, Edith Uetz-von Allmen, and Petra Krause. 2010. "Prostaglandin E2 at New Glance: Novel Insights in Functional Diversity Offer Therapeutic Chances." *The*

International Journal of Biochemistry & Cell Biology 42(2): 198–201.
Lei, Yuk Man et al. 2016. "The Composition of the Microbiota Modulates Allograft Rejection." *The Journal of Clinical Investigation* 126(7): 2736–44.
LeibundGut-Landmann, S et al. 2007. "Syk- and CARD9-Dependent Coupling of Innate Immunity to the Induction of T Helper Cells That Produce Interleukin 17." *Nature Immunology* 8(6): 630–38.
Levings, By Megan K, Romina Sangregorio, and Maria-grazia Roncarolo. 2001. "Human CD25+ CD4+ T Regulatory Cells Suppress Naive and Memory T Cell Proliferation and Can Be Expanded in Vitro without Loss of Function." *The Journal of Experimental Medicine* 193(11): 1295–12302.
Li, Bin et al. 2007. "FOXP3 Interactions with Histone Acetyltransferase and Class II Histone Deacetylases Are Required for Repression." *PNAS* 104(11): 4571–76.
Li, Ming O, Shomyseh Sanjabi, and Richard A Flavell. 2006. "Transforming Growth Factor-b Controls Development, Homeostasis, and Tolerance of T Cells by Regulatory T Cell-Dependent and -Independent Mechanisms." *Immunity* 25: 455–71.
Li, Qi-jing et al. 2004. "CD4 Enhances T Cell Sensitivity to Antigen by Coordinating Lck Accumulation at the Immunological Synapse." *Nature Immunology* 5: 791–99.
Li, Qiuxia et al. 2012. "Constitutive Nuclear Localization of NFAT in Foxp3+ Regulatory T Cells Independent of Calcineurin Activity." *PNAS* 188: 4268–77.
Liang, Bitao et al. 2008. "Regulatory T Cells Inhibit Dendritic Cells by Lymphocyte Activation Gene-3 Engagement of MHC Class II." *The Journal of Immunology* 180: 5916–26.
Lio, C J, and C Hsieh. 2009. "A Two-Step Process for Thymic Regulatory T Cell Development." *Immunity* 28(1): 100–111.
Liu, Jun et al. 1991. "Calcineurin Is a Common Target of Cyclophilin-Cyclosporin A and FKBP-FK506 Complexes." *Cell* 66(4): 807–15.
Liu, K et al. 2009. "In Vivo Analysis of Dendritic Cell Development and Homeostasis." *Science* 324: 392–97.
Liu, Qinghang, Jennifer Caldwell Busby, and Jeffery D Molkentin. 2009. "Interaction between TAK1–TAB1–TAB2 and RCAN1–calcineurin Defines a Signalling Nodal Control Point." *Nature Cell Biology* 11(2): 154–61.
Liu, Z et al. 2015. "Immune Homeostasis Enforced by Co-Localized Effector and Regulatory T Cells." *Nature* 528(7581): 225–30.
Liu, Zhihua et al. 2011. "The Kinase LRRK2 Is a Regulator of the

Transcription Factor NFAT That Modulates the Severity of Inflammatory Bowel Disease." *Nature Immunology* 12(11): 1063–71.

Liu, Zhong-min, Kun-peng Wang, Jilin Ma, and Song Guo Zheng. 2015. "The Role of All- Trans Retinoic Acid in the Biology of Foxp3 1 Regulatory T Cells." *Cellular & Molecular Immunology* 12: 553–57.

Lu, Ling et al. 2014. "Critical Role of All - Trans Retinoic Acid in Stabilizing Human Natural Regulatory T Cells under Inflammatory Conditions." *PNAS* 19(111): E3432-3440.

Vander Lugt, Bryan et al. 2014. "Transcriptional Programming of Dendritic Cells for Enhanced MHC Class II Antigen Presentation." *Nature Immunology* 15(2): 161–69.

Luis, A Z, Christopher Haines, and Daniel J Cua. 2013. "Th17 Cell Development: From the Cradle to the Grave." *Immunological Reviews* 252: 78–88.

Ma, Anlun et al. 2009. "Combined Therapy of CD4+CD25+ Regulatory T Cells with Low-Dose Sirolimus, but Not Calcineurin Inhibitors, Preserves Suppressive Function of Regulatory T Cells and Prolongs Allograft Survival in Mice." *International Immunopharmacology* 9(5): 553–63. http://dx.doi.org/10.1016/j.intimp.2009.01.033.

Macian, Fernando et al. 2002. "Transcriptional Mechanisms Underlying Lymphocyte Tolerance." *Cell* 109: 719–31.

Macian, Fernando, Cristina Lopez-Rodriguez, and Anjana Rao. 2001. "Partners in Transcription: NFAT and AP-1." *Oncogene* 20: 2476–89.

Mahon, B et al. 1995. "Poliovirus-Specific CD4+ Th1 Clones with Both Cytotoxic and Helper Activity Mediate Protective Humoral Immunity against a Lethal Poliovirus Infection in Transgenic Mice Expressing the Human Poliovirus Receptor." *The Journal of Experimental Medicine* 181: 1285–12921.

Malek, Thomas R et al. 2002. "CD4 Regulatory T Cells Prevent Lethal Autoimmunity in IL-2Rb-Deficient Mice: Implications for the Nonredundant Function of IL-2." *Immunity* 17: 167–78.

Malek, Thomas R, and Allison L Bayer. 2004. "Tolerance, Not Immunity, Crucially Depends on IL-2." *Nature Reviews Immunology* 4(9): 665–74.

Malin, Stephen et al. 2010. "Role of STAT5 in Controlling Cell Survival and Immunoglobulin Gene Recombination during Pro-B Cell Development." *Nature Immunology* 11(2): 171–79.

Martinez, Gustavo J et al. 2015. "The Transcription Factor NFAT

Promotes Exhaustion of Activated CD8+ T Cells." *Immunty* 42(2): 265–78.

Matsue, Hiroyuki et al. 2002. "Contrasting Impacts of Immunosuppressive Agents (Rapamycin, FK506, Cyclosporin A, and Dexamethasone) on Bidirectional Dendritic Cell-T Cell Interaction during Antigen Presentation." *The Journal of Immunology* 169: 3555–64.

Matzinger, Polly. 1994. "Tolerance, Danger and the Extended Family." *Annual Review of Immunology* 12: 991–1045.

Mckenna, Hilary J et al. 2000. "Mice Lacking Flt3 Ligand Have Deficient Hematopoiesis Affecting Hematopoietic Progenitor Cells, Dendritic Cells, and Natural Killer Cells." *Blood* 95(11): 3489–97.

Merad, M et al. 2013. "The Dendritic Cell Lineage: Ontogeny and Function of Dendritic Cells and Their Subsets in the Steady State and the Inflamed Setting." *Annual Review of Immunology* 31(1): 563–604. http://www.annualreviews.org/doi/10.1146/annurev-immunol-020711-074950.

Merad, Miriam et al. 2002. "Langerhans Cells Renew in the Skin throughout Life under Steady-State Conditions." *Nature Immunology* 3(12): 1135–41.

Miller, Jennifer C et al. 2012. "Deciphering the Transcriptional Network of the Dendritic Cell Lineage." *Nature Immunology* 13(9): 888–99. http://dx.doi.org/10.1038/ni.2370.

Min, Wei-ping et al. 2003. "Inhibitory Feedback Loop between Tolerogenic Dendritic Cells and Regulatory T Cells in Transplant Tolerance." *The Journal of Immunology* 170: 1304–12.

Miyake, Kensuke. 2007. "Innate Immune Sensing of Pathogens and Danger Signals by Cell Surface Toll-like Receptors." *Seminars* 19: 3–10.

Mosmann, T R et al. 1986. "Two Types of Murine Helper T Cell Clone . I . Definition According to Profiles of Lymphokine Activities and Secreted Proteins." *The Journal of Immunology* (136): 2348–57.

Mount, Adele M. et al. 2008. "Multiple Dendritic Cell Populations Activate CD4+ T Cells after Viral Stimulation." *PLoS ONE* 3(2).

Naik, S H et al. 2007. "Development of Plasmacytoid and Conventional Dendritic Cell Subtypes from Single Precursor Cells Derived in Vitro and in Vivo." *Nature Immunology* 8(11): 1217–26.

Naik, Shalin H et al. 2006. "Intrasplenic Steady-State Dendritic Cell Precursors That Are Distinct from Monocytes." *Nature*

Immunology 7(6): 663–71.
Nakamura, Kazuhiko et al. 2004. "TGF-B1 Plays an Important Role in the Mechanism of CD4+ CD25+ Regulatory T Cell Activity in Both Humans and Mice." *The Journal of Immunology* 172: 834–42.
Nakamura, Kazuhiko, Atsushi Kitani, and Warren Strober. 2001. "Cell Contact–dependent Immunosuppression by CD4+ CD25+ Regulatory T Cells Is Mediated by Cell Surface–bound Transforming Growth Factor β." *The Journal of Experimental Medicine* 194(5): 629–44.
Nakano, Hideki, Manabu Yanagita, and Michael Dee Gunn. 2001. "CD11c+ B220+ Gr-1+ Cells in Mouse Lymph Nodes and Spleen Display Characteristics of Plasmacytoid Dendritic Cells." *The Journal of Experimental Medicine* 194(8): 171–78.
Ninomiya-tsuji, Jun et al. 1999. "The Kinase TAK1 Can Activate the NIK-IkB as Well as the MAP Kinase Cascade in the IL-1 Signalling Pathway." *Nature* 398(March): 252–56.
Nishizuka, Y, and T Sakakura. 1969. "Thymus and Reproduction: Sex-Linked Dysgenesia of the Gonad after Neonatal Thymectomy in Mice." *Science* 166(3906): 753–55.
Nussenzweig, MC, and RM Steinman. 1980. "Contribution of Dendritic Cells to Stimulation of the Murine Syngeneic Mixed Leukocyte Reaction." *The Journal of Experimental Medicine* 151(May): 1196–1212.
Oberle, Nina et al. 2007. "Rapid Suppression of Cytokine Transcription in Human CD4+ CD25 T Cell by CD4+Foxp3+ Regulatory T Cells: Independence of IL-2 Consumption, TGF-Beta, and Various Inhibitors of TCR Signaling." *The Journal of Immunology* 179(6): 3578–87.
Ochs, Hans D, Steven F Ziegler, and Troy R Torgerson. 2005. "FOXP3 Acts as a Rheostat of the Immune Response." *Immunological Revi* 203(1): 156–64.
Oetjen, Elke et al. 2005. "The Immunosuppressive Drugs Cyclosporin A and Tacrolimus Inhibit Membrane Depolarization-Induced CREB Transcriptional Activity at the Coactivator Level." *British Journal of Pharmacology* 144: 982–93.
Oh-hora, Masatsugu et al. 2009. "Dual Functions of the Endoplasmic Reticulum Calcium Sensors STIM1 and STIM2 in T Cell Activation and Tolerance." *Nature Immu* 9(4): 432–43.
Oh-hora, Masatsugu, and Anjana Rao. 2010. "The Calcium/NFAT Pathway: Role in Development and Function of Regulatory T Cells." *Microbes and Infection* 11(5): 612–19.

Oh, P L et al. 2012. "Characterization of the Ileal Microbiota in Rejecting and Nonrejecting Recipients of Small Bowel Transplants." *American Journal of Nephrology* 12(3): 753–62.

Ohl, Lars et al. 2004. "CCR7 Governs Skin Dendritic Cell Migration under Inflammatory and Steady-State Conditions." *Immunity* 21: 279–88.

Okamura, H et al. 2004. "A Conserved Docking Motif for CK1 Binding Controls the Nuclear Localization of NFAT1." *Molecular and Cellular Biology* 24(10): 4184–95.

Okamura, Heidi et al. 2000. "Concerted Dephosphorylation of the Transcription Factor NFAT1 Induces a Conformational Switch That Regulates Transcriptional Activity." *Molecular Cell* 6(3): 539–50.

Okamura, Tomohisa et al. 2009. "CD4+ CD25- LAG3+ Regulatory T Cells Controlled by the Transcription Factor Egr-2." *PNAS* 106(33): 13974–79.

Onishi, Yasushi, Zoltan Fehervari, Tomoyuki Yamaguchi, and Shimon Sakaguchi. 2008. "Foxp3+ Natural Regulatory T Cells Preferentially Form Aggregates on Dendritic Cells in Vitro and Actively Inhibit Their Maturation." *PNAS* 105(29): 10113–18.

Owen, David L et al. 2018. "Identification of Cellular Sources of IL-2 Needed for Regulatory T Cell Development and Homeostasis." *The Journal of Immunology* 200(12): 3926–33.

Palmer, Scott M et al. 2003. "The Role of Innate Immunity in Acute Allograft Rejection after Lung Transplantation." *Am J Respir Crit Care Med* 168(6): 628–32.

Pancer, Zeev, and Max D Cooper. 2006. "The Evolution of Adaptive Immunity." *Annual Review of Immunology* 24: 497–518.

Panda, Santosh K, Roland Kolbeck, and Miguel A Sanjuan. 2017. "Plasmacytoid Dendritic Cells in Autoimmunity." *Current Opinion in Immunology* 44: 20–25. http://dx.doi.org/10.1016/j.coi.2016.10.006.

Papers, J B C et al. 2001. "Scurfin (FOXP3) Acts as a Repressor of Transcription and Regulates T Cell Activation." *The Journal of Biological Chemistry* 276(40): 37672–79.

Park, Jean Y, Michael H Pillinger, and Steven B Abramson. 2006. "Prostaglandin E2 Synthesis and Secretion: The Role of PGE2 Synthases." *Clinical Imm* 119(3): 229–40.

Parkin, Jacqueline, and Bryony Cohen. 2001. "An Overview of the Immune System." *Lancet* 357: 1777–89.

Pflanz, Stefan et al. 2002. "IL-27, a Heterodimeric Cytokine Composed

of EBI3 and P28 Protein , Induces Proliferation of Naive CD4+ T Cells." *Immunity* 16: 779–90.

Porter, Cynthia M, Michael A Havens, and Neil A Clipstone. 2000. "Identification of Amino Acid Residues and Protein Kinases Involved in the Regulation of NFATc Subcellular Localization." *The journal of biological chemistry* 275(5): 3543–51.

Radtke, Andrea J. et al. 2015. "Lymph-Node Resident CD8a+ Dendritic Cells Capture Antigens from Migratory Malaria Sporozoites and Induce CD8+ T Cell Responses." *PLoS Pathogens* 11(2): 1–23.

Read, By Simon, Vivianne Malmström, and Fiona Powrie. 2000. "Cytotoxic T Lymphocyte–associated Antigen 4 Plays an Essential Role in the Function of CD25+ CD4+ Regulatory Cells That Control Intestinal Inflammation." *The Journal of Experimental Medicine* 192(2): 295–302.

Reizis, Boris et al. 2011. "Plasmacytoid Dendritic Cells: Recent Progress and Open Questions." *Annual Review of Immunology* 29: 163–83.

Ren, Yana et al. 2014. "Tolerogenic Dendritic Cells Modified by Tacrolimus Suppress CD4+ T-Cell Proliferation and Inhibit Collagen-Induced Arthritis in Mice." *International Immunopharmacology* 21(1): 247–54. http://dx.doi.org/10.1016/j.intimp.2014.05.004.

Richard, J et al. 1993. "Identification of the Immunophilins Capable of Mediating Inhibition of Signal Transduction by Cyclosporin A and FK506: Roles of Calcineurin Binding and Cellular Location." *Molecular and Cellular Biology* 13(8): 4760–69.

Roberts, Edward W. et al. 2016. "Critical Role for CD103+/CD141+Dendritic Cells Bearing CCR7 for Tumor Antigen Trafficking and Priming of T Cell Immunity in Melanoma." *Cancer Cell* 30(2): 324–36. http://dx.doi.org/10.1016/j.ccell.2016.06.003.

Robinson, Matthew J et al. 2011. "CLEC-2 Signaling via Syk in Myeloid Cells Can Regulate Inflammatory Responses." *European Journal of Immunology* 41(10): 3040–53.

Rodriguez, Antonio et al. 2009. "A Conserved Docking Surface on Calcineurin Mediates Interaction with Substrates and Immunosuppressants." *Molecular Cell* 33: 616–26.

Rogers, Neil C et al. 2005. "Syk-Dependent Cytokine Induction by Dectin-1 Reveals a Novel Pattern Recognition Pathway for C Type Lectins." *Immunity* 22: 507–17.

Roncador, Giovanna et al. 2005. "Analysis of FOXP3 Protein Expression in Human CD4+ CD25+ Regulatory T Cells at the

Single-Cell Level." *European Journal of Immunology* 35: 1681–91.
Rothfuchs, Antonio Gigliotti et al. 2007. "Dectin-1 Interaction with Mycobacterium Tuberculosis Leads to Enhanced IL-12p40 Production by Splenic Dendritic Cells." *The Journal of Immunology* 179: 3463–71.
Ruan, Qingguo et al. 2010. "Development of Foxp3+ Regulatory T Cells Is Driven by A C-Rel Enhanceosome." *Immunity* 31(6): 932–40.
Rubtsov, Yuri P et al. 2014. "Stability of the Regulatory T Cell Lineage in Vivo." *Science* 329(5999): 1667–71.
Sabapathy, By Kanaga et al. 2001. "C-Jun NH2-Terminal Kinase (JNK) 1 and JNK2 Have Similar and Stage-Dependent Roles in Regulating T Cell Apoptosis and Proliferation." *The Journal of Experimental Medicine* 193(3): 317.
Sadlack, Benjamin et al. 1995. "Generalized Autoimmune Disease in Interleukin-2-Deficient Mice Is Triggered by an Uncontrolled Activation and Proliferation of CD4+ T Cells." *European Journal of Immunology* 25(11): 3053–59.
Sakaguchi, S et al. 1995. "Immunologic Self-Tolerance Maintained by Activated T Cells Expressing IL-2 Receptor Alpha-Chains (CD25). Breakdown of a Single Mechanism of Self-Tolerance Causes Various Autoimmune Diseases." *J immunol* 155(3): 1151–64.
Sakaguchi, S, K Fukuma, K Kuribayashi, and T Masuda. 1985. "Organ-Specific Autoimmune Diseases Induced in Mice by Elimination of T Cell Subset." *The Journal of Experimental Medicine* 161(January): 72–87.
Sakaguchi, Shimon. 2004. "Naturally Arising CD4+ Regulatory T Cells for Immunologic Self-Tolerance and Negative Control of Immune Responses." *Annual Review of Immunology* 22: 531–62.
Sakaguchi, Shimon, and Noriko Sakaguchi. 1988. "Thymus and Autoimmunity. Transplantation of the Thymus from Cyclosporin A-Treated Mice Causes Organ-Specific Autoimmune Disease in Athymic Nude Mice." *The Journal of Experimental Medicine* 167(4): 1479–85.
Sapoznikov, Anita et al. 2007. "Organ-Dependent in Vivo Priming of Naive CD4+, but Not CD8+, T Cells by Plasmacytoid Dendritic Cells." *The Journal of Experimental Medicine* 204(8): 1923–33.
Schlitzer, Andreas et al. 2013. "IRF4 Transcription Factor-Dependent CD11b+Dendritic Cells in Human and Mouse Control Mucosal IL-17 Cytokine Responses." *Immunity* 38(5): 970–83. http://dx.doi.org/10.1016/j.immuni.2013.04.011.

Schreiber, Stuart L, and Gerald R Crabtree. 1992. "The Mechanism of Action of Cyclosporin A and FK506." *Immunology Today* 13(4): 1433–39.

Schwartz, Ronald H. 2003. "T Cell Anergy." *Annual Review of Immunology* 21: 305–34.

See, Peter et al. 2017. "Mapping the Human DC Lineage through the Integration of High-Dimensional Techniques." *Science* 356(6342).

Seneschal, Julien, Xiaodong Jiang, and Thomas S. Kupper. 2014. "Langerin + Dermal DC, but Not Langerhans Cells, Are Required for Effective CD8-Mediated Immune Responses after Skin Scarification with Vaccinia Virus." *Journal of Investigative Dermatology* 134(3): 686–94. http://dx.doi.org/10.1038/jid.2013.418.

Setoguchi, Ruka, Shohei Hori, Takeshi Takahashi, and Shimon Sakaguchi. 2005. "Homeostatic Maintenance of Natural Foxp3+ CD25+ CD4+ Regulatory T Cells by Interleukin (IL)-2 and Induction of Autoimmune Disease by IL-2 Neutralization." *The Journal of Experimental Medicine* 201(5): 723–35.

Séverin, Sonia et al. 2011. "Syk-Dependent Phosphorylation of CLEC-2." *The Journal of Biological Chemistry* 286(6): 4107–16.

Sharma, Meenu, Srini V Kaveri, and Jagadeesh Bayry. 2013. "Th17 Cells, Pathogenic or Not ? TGF-b 3 Imposes the Embargo." *Cellular & Molecular Immunology* 10: 101–2.

Shaw, Jeng-pyng et al. 1988. "Identification of a Putative Regulator of Early T Cell Activation Genes Actity." *Science* 241(11): 202–5.

Shimizu, Kanako et al. 2000. "Tacrolimus (FK506) Treatment of CD34+ Hematopoietic Progenitor Cells Promote the Development of Dendritic Cells That Drive CD4+ T Cells toward Th2 Responses." *Journal of Leukocyte Biology* 68: 633–40.

Shin, Dong-min et al. 2008. "Mycobacterium Abscessus Activates the Macrophage Innate Immune Response via a Physical and Functional Interaction between TLR2 and Dectin-1." *Cellular Microbiology* 10(April): 1608–21.

Shishido, S N et al. 2012. "Humoral Innate Immune Response and Disease." *Clinical Immunology* 144(2): 142–58.

Siracusa, Mark C et al. 2011. "TSLP Promotes Interleukin-3-Independent Basophil Haematopoiesis and Type 2 Inflammation." *Nature* 477(7363): 229–33. http://dx.doi.org/10.1038/nature10329.

Slack, Emma C et al. 2007. "Syk-Dependent ERK Activation Regulates IL-2 and IL-10 Production by DC Stimulated with Zymosan."

European Journal of Immunology 37: 1600–1612.

Smyth, Lesley Ann et al. 2006. "A Novel Pathway of Antigen Presentation by Dendritic and Endothelial Cells: Implications for Allorecognition and Infectious Diseases." *Transplantation* 82(1): 15–18.

Sokol, Caroline L, Gregory M Barton, Andrew G Farr, and Ruslan Medzhitov. 2008. "A Mechanism for the Initiation of Allergen-Induced T Helper Type 2 Responses." *Nature Immunology* 9(3): 310–18.

Steinman, By Ralph M, Gilla Kaplan, Margaret D Witmer, and Zanvil A Cohn. 1979. "Identification of a Novel Cell Type in Peripheral Lymphoid Organs of Mice V. Purification of Spleen Dendritic Cells, New Surface Markers, and Maintenance in Vitro." *The Journal of Experimental Medicine* 149(January): 1–16.

Steinman, Ralph M., and Zanvil A. Chon. 1973. "Identification of a Novel Cell Type in Peripheral Lymphoid Organs of Mice. I. Morphology, Quantitation, Tissue Distribution." *The Journal of experimental medicine* 137: 1142–62.

Steinman, Ralph M., and Zanvil A. Cohn. 1974. "Identification of a Novel Cell Type in Peripheral Lympoid Organs of Mice. II. Functional Properties in Vitro." *The Journal of experimental medicine* 139: 380–97.

Steinman, Ralph M., Daniel Hawiger, and Michel C. Nussenzweig. 2003. "Tolerogenic Dendritic Cells." *Annual Review of Immunology* 21(1): 685–711. http://www.annualreviews.org/doi/10.1146/annurev.immunol.21.120601.141040.

Steinman, Ralph M, and Margaret D Witmer. 1978. "Lymphoid Dendritic Cells Are Potent Stimulators of the Primary Mixed Leukocyte Reaction in Mice." *PNAS* 75(10): 5132–36.

Stoitzner, Patrizia et al. 2003. "Visualization and Characterization of Migratory Langerhans Cells in Murine Skin and Lymph Nodes by Antibodies against Langerin/CD207." *Journal of Investigative Dermatology* 120(2): 266–74.

Stranges, Peter B. et al. 2007. "Elimination of Antigen-Presenting Cells and Autoreactive T Cells by Fas Contributes to Prevention of Autoimmunity." *Immunity* 26(5): 629–41.

Sugimoto, Toshiro, Scott Stewart, and Kun-Liang Guan. 1997. "The Calcium/Calmodulin- Dependent Protein Phosphatase Calcineurin Is the Major Elk-1 Phosphatase." *The journal of Biological Chemistry* 272(47): 29415–19.

Sutton, Caroline et al. 2006. "A Crucial Role for Interleukin (IL) -1 in the Induction of IL-17–producing T Cells That Mediate Autoimmune Encephalomyelitis." *The Journal of Experimental Medicine* 203(7): 1685–91.

Suzuki, Haruhiko et al. 1995. "Deregulated T Cell Activation and Autoimmunity in Mice Lacking Interleukin-2 Receptor B." *Science* 268(5216): 1472–76.

Szabo, Gyongyi, Cristina Gavala, and Pranoti Mandrekar. 2001. "Tacrolimus and Cyclosporine A Inhibit Allostimulatory Capacity and Cytokine Production of Human Myeloid Dendritic Cells." *Journal of Investigative Medicine* 49(5): 442–50.

Tang, Hua et al. 2010. "The T Helper Type 2 Response to Cysteine Proteases Requires Dendritic Cell–basophil Cooperation via ROS-Mediated Signaling." *Nature Immunology* 11(7): 608–17. http://dx.doi.org/10.1038/ni.1883.

Tang, Qizhi, and Flavio Vincenti. 2017. "Transplant Trials with Tregs: Perils and Promises." *The Journal of Clinical Investigation* 127(7): 2505–12.

Tassi, Ilaria et al. 2009. "Requirement of Phospholipase C-γ 2 (PLCγ2) for Dectin-1- Induced Antigen Presentation and Induction of Th1/Th17 Polarization." *European Journal of Immunology* 39: 1369–78.

Taylor, Philip R et al. 2002. "The β-Glucan Receptor, Dectin-1, Is Predominantly Expressed on the Surface of Cells of the Monocyte/Macrophage and Neutrophil Lineages." *The journal of Immunology* 169: 3876–82.

Terui, Yasuhito et al. 2004. "Dual Role of Sumoylation in the Nuclear Localization and Transcriptional Activation of NFAT1." *The journal of biological chemistry* 279(27): 28257–65.

Tesar, Bethany M, Jiasheng Zhang, Qi Li, and Daniel R Goldstein. 2004. "TH1 Immune Responses to Fully MHC Mismatched Allografts Are Diminished in the Absence of MyD88, a Toll-like Receptor Signal Adaptor Protein." *American Journal of Nephrology* 4: 1429–39.

Tesmer, L A, S K Lundy, S Sarkar, and D A Fox. 2008. "Th17 Cells in Human Disease." *Immunological Reviews* 223: 87–113.

Trinchieri, Giorgio, and Alan Sher. 2007. "Cooperation of Toll-like Receptor Signals in Innate Immune Defence." *Nature Reviews Immunology* 7: 179–90.

Vaeth, Martin et al. 2012. "Dependence on Nuclear Factor of Activated T-Cells (NFAT) Levels Discriminates Conventional T Cells from

Foxp3+ Regulatory T Cells." *PNAS* 109(40): 16258–63.
Valdor, Rut et al. 2008. "Regulation of NFAT by Poly (ADP-Ribose) Polymerase Activity in T Cells." *Molecular Immunology* 45: 1863–71.
Vallabhapurapu, Sivakumar, and Michael Karin. 2009. "Regulation and Function of NF-KB Transcription Factors in the Immune System." *Annual Review of Immunology* 27: 693–733.
Vang, Kieng B et al. 2018. "IL-2, -7, and -15, but Not Thymic Stromal Lymphopoeitin, Redundantly Govern CD4+ Foxp3+ Regulatory T Cell Development." *The Journal of Immunology* 181: 3285–90.
Villani, Alexandra-Chloé et al. 2017. "Single-Cell RNA-Seq Reveals New Types of Human Blood Dendritic Cells, Monocytes, and Progenitors." *Science* 356(6335): eaah4573. http://www.sciencemag.org/lookup/doi/10.1126/science.aah4573.
Waaga, Ana Maria, Martin Gasser, Igor Laskowski, and Nicholas L Tilney. 2000. "Mechanisms of Chronic Rejection." *Current Opinion in Immunology* 12(5): 517–21.
Waithman, Jason et al. 2013. "Resident CD8+ and Migratory CD103+ Dendritic Cells Control CD8 T Cell Immunity during Acute Influenza Infection." *PLoS ONE* 8(6): 1–7.
Waldmann, Thomas A. 1989. "The Multi-Subunit Interleukin-2 Receptor." *Annual Review of Biochemistry* 58(1): 875–905.
Waskow, Claudia et al. 2009. "FMS-like Tyrosine Kinase 3 Is Required for Dendritic Cell Development in Peripheral Lymphoid Tissues." *Nature Immunology* 9(6): 676–83.
Wehmeier, Caroline et al. 2017. "Acute Rejection Phenotypes in the Current Era of Immunosuppression: A Single-Center Analysis." *Transplantation Direct* 3(3): 1–10.
Weider, Matthias et al. 2018. "Nfat/Calcineurin Signaling Promotes Oligodendrocyte Differentiation and Myelination by Transcription Factor Network Tuning." *Nature Communications* 2018(MARCH): 1–16. http://dx.doi.org/10.1038/s41467-018-03336-3.
Weist, Brian M et al. 2015. "Thymic Regulatory T Cell Niche Size Is Dictated by Limiting Interleukin 2 from Antigen-Bearing Dendritic Cells and Feedback Competition." *Nature Immunology* 16(6): 635–41.
Werlen, Guy, Estela Jacinto, Ying Xia, and Michael Karin. 1998. "Calcineurin Preferentially Synergizes with PKC-θ to Activate JNK and IL-2 Promoter in T Lymphocytes." *The EMBO Journal*

17(11): 3101–11.
Wherry, E John et al. 2007. "Molecular Signature of CD8+ T Cell Exhaustion during Chronic Viral Infection." *Immunity* 27: 670–84.
Wilierford, Dennis M et al. 1995. "Interleukin-2 Receptor a Chain Regulates the Size and Content of the Peripheral Lymphoid Compartment." *Immunity* 3(4): 521–30.
Willingham, A T et al. 2005. "A Strategy for Probing the Function of Noncoding RNAs Finds a Repressor of NFAT." *Science* 309: 1570–73.
Winslow, Monte M et al. 2006. "Calcineurin / NFAT Signaling in Osteoblasts Regulates Bone Mass." : 771–82.
Woltman, Andrea M et al. 2000. "The Effect of Calcineurin Inhibitors and Corticosteroids on the Differentiation of Human Dendritic Cells." *European Journal of Immunology* 30: 1807–12.
Wu, H et al. 2007. "TLR4 Activation Mediates Kidney Ischemia/Reperfusion Injury." *J Clin Invest* 117(10): 2847–59.
Wu, Huiling et al. 2012. "Absence of MyD88 Signaling Induces Donor-Specific Kidney Allograft Tolerance." *J Am Soc Nephrol* 23(10): 1701–16.
Wu, Yongqing et al. 2006. "FOXP3 Controls Regulatory T Cell Function through Cooperation with NFAT." *Cell* 126: 375–87.
Wuest, Simone C. et al. 2011. "A Role for Interleukin-2 Trans-Presentation in Dendritic Cell-Mediated T Cell Activation in Humans, as Revealed by Daclizumab Therapy." *Nature Medicine* 17(5): 604–9. http://dx.doi.org/10.1038/nm.2365.
Xie, Fang Tao et al. 2015. "IDO Expressing Dendritic Cells Suppress Allograft Rejection of Small Bowel Transplantation in Mice by Expansion of Foxp3+ Regulatory T Cells." *Transplant Immunology* 33(2): 69–77. http://dx.doi.org/10.1016/j.trim.2015.05.003.
Yagi, Haruhiko et al. 2004. "Crucial Role of FOXP3 in the Development and Function of Human CD25+ CD4+ Regulatory T Cells." *International Immunology* 16(11): 1643–56.
Yamasaki, Sho et al. 2008. "Mincle Is an ITAM-Coupled Activating Receptor That Senses Damaged Cells." *Nature Immunology* 9(10): 1179–88.
Yang, Huan et al. 2010. "A Critical Cysteine Is Required for HMGB1 Binding to Toll-like Receptor 4 and Activation of Macrophage Cytokine Release." *PNAS* 107(26): 11942–47.
Yang, Teddy T C et al. 2006. "Role of Transcription Factor NFAT in Glucose and Insulin Homeostasis." *Molecular and Cellular Biology* 26(20): 7372–87.

Ye, Qilu et al. 2013. "Structural Basis of Calcineurin Activation by Calmodulin." *Cellular Signalling* 25(12): 2661–67. http://dx.doi.org/10.1016/j.cellsig.2013.08.033.

Yokosuka, Tadashi et al. 2010. "Spatiotemporal Basis of CTLA-4 Negative Regulation of T Cell Activation." *Immunity* 33(3): 326–39. http://dx.doi.org/10.1016/j.immuni.2010.09.006.

Yu, Hong-bing et al. 2015. "NFATc2 Mediates Epigenetic Modification of Dendritic Cell Cytokine and Chemokine Responses to Dectin-1 Stimulation." *Nucleic Acids Research* 43(2): 836–47.

Yu, Li, Liantang Wang, and Shangwu Chen. 2010. "Endogenous Toll-like Receptor Ligands and Their Biological Significance." *Journal of Cellular and Molecular Medicine* 14(11): 2592–2603.

Zanoni, I et al. 2011. "CD14 Controls the LPS-Induced Endocytosis of Toll-like Receptor 4." *Cell* 147: 868–80. http://dx.doi.org/10.1016/j.cell.2011.09.051.

Zanoni, Ivan et al. 2009. "CD14 Regulates the Dendritic Cell Life Cycle after LPS Exposure through NFAT Activation." *Nature* 460(7252): 264–68. http://dx.doi.org/10.1038/nature08118.

Zanoni, Ivan, Renato Ostuni, et al. 2012. "CD14 and NFAT Mediate Lipopolysaccharide- Induced Skin Edema Formation in Mice." *The Journal of Clinical Investigation* 122(5): 1747–57.

Zanoni, Ivan, Caterina Bodio, et al. 2012. "Similarities and Differences of Innate Immune Responses Elicited by Smooth and Rough LPS." *Immunology Letters* 142(1–2): 41–47. http://dx.doi.org/10.1016/j.imlet.2011.12.002.

Zheng, Y et al. 2010. "Role of Conserved Non-Coding DNA Elements in the Foxp3 Gene in Regulatory T-Cell Fate." *Nature* 463(7282): 808–12.

Zheng, Ye et al. 2007. "Genome-Wide Analysis of Foxp3 Target Genes in Developing and Mature Regulatory T Cells." *Nature* 445(7130): 936–40.

Zheng, Ye, and Alexander Y Rudensky. 2007. "Foxp3 in Control of the Regulatory T Cell Lineage." *Nature Immunology* 8(5): 457–62.

Ziegler, Steven F. 2006. "FOXP3: Of Mice and Men." *Annual Review of Immunology* 24(1): 209–26.

CHAPTER 2. LONG-TERM GRAFT TOLERANCE INDUCTION BY NFATC PATHWAY INHIBITION IN INNATE IMMUNE CELLS

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Transplantation constitutes a surgical process that triggers diverse concomitant phenomena as well as pathways in the more disparate cells leading to a complex scenario still unraveled to some extent. Indeed, the pathways that are elicited in this context are still partially unexplored. Furthermore, in a mismatched setting, the non-identical MHC or HLA antigens leads to the instauration of alloreactive T cells that mediate the rejection of the graft (Felix and Allen 2007). T cell activation relies on different signals provided by professional APCs, as DCs. If DCs fail in supporting adaptive responses with the already mentioned signal 1, signal 2 and signal 3, T cells may undergo anergy or differentiation into Tregs (Schwartz 2003). Therefore, DCs maturation is a crucial event in the subsequent elicitation of T cell effector functions. NFATc TFs are emerging as peculiar TFs involved in pathogen and sterile adjuvant sensing, providing DCs activation and maturation (Khameneh et al. 2017; Zanoni et al. 2009; Zanoni and Granucci 2012). Indeed, one of the crucial signals for adaptive responses is DCs-mediated IL-2 production that relies on NFATc activation (Granucci et al. 2001; Khameneh et al. 2017). Thus, we wondered whether NFATc TFs in innate immunity may be involved in the complex scenario of transplant rejection.

2.1 Rejection kinetics is delayed upon mismatched NFATc2 KO-derived skin transplant

To provide insights about the role of NFATc TFs in the context of mismatched transplantation, we took advantage of a mouse model lacking NFATc2, which is the member of the NFAT family most expressed by DCs. Thus, we performed a well-known model of skin graft rejection based on the minor antigens mismatched, since mice are syngeneic and exhibit the same MHC molecules (Fernandes et al. 2011; Kwun et al. 2011). Donor skin samples were obtained from the skin tail and transplanted onto the dorsum of recipient animals. While female-derived skin graft is completely accepted by female recipients, male-derived skin graft is rejected within 10-14 days. In addition to these experimental groups, we transplanted male NFATc2 KO-derived skin into female and monitored the outcome. As shown in Figure 1A, when transplanting male-derived NFATc2 deficient skin, the kinetics of graft rejection is delayed if compared to the WT counterpart. Indeed, by the day 15 post-transplant, timepoint within which acute rejection should occur, 90% of the recipients of NFATc2 KO skin exhibited acceptance of the graft. The initiation of the rejection process in the recipients of NFATc2 KO skin appears to be shifted of two weeks and reaches the 100% of graft loss only 80 days post

transplantation, while the control animals of rejection had already lost the graft by day 30. To exclude the possibility that DCs in NFATc2 KO skin graft were reduced in number and thus were not capable of mediating rejection as rapidly as observed in WT male-derived skin transplants, we analyzed skin from WT and NFATc2 KO animals by flow cytometry. As reported in Figure 1B, no differences in the DCs quantity were observed between the two mouse models. Therefore, the absence of NFATc2 and consequently of its transcriptional activities prevents the fast and severe outcome of mismatched transplants, suggesting that NFATc2 pathway may be active in donor cells and contributes to the instauration of alloreactivity.

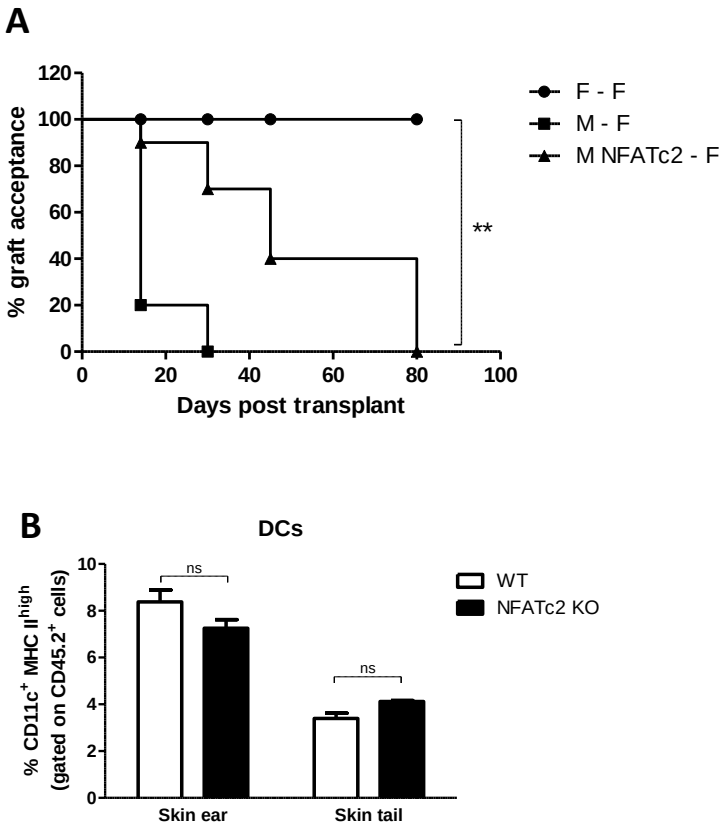


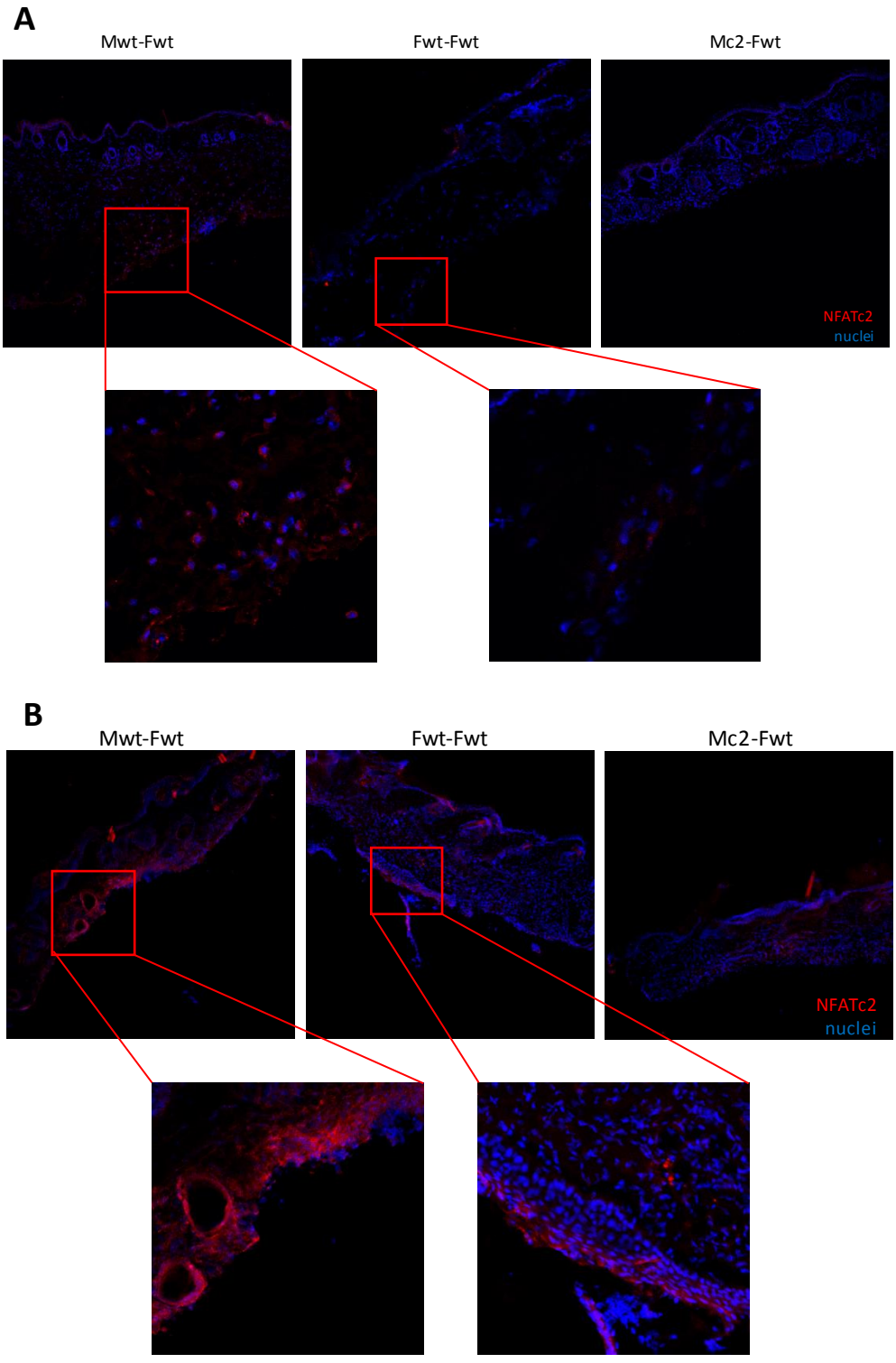
Figure 1: male NFATc2 KO-derived skin transplant

A) 6 weeks-old C57BL/6 WT female animals have been transplanted with C57BL/6 WT female- or C57BL/6 WT male- or male NFATc2 KO-derived skin. Grafts and eventual rejection were monitored at the indicated timepoints until day 80 post-transplant. Log-rank (Mantel-Cox) statistical analysis was performed. B) DCs percentage in skin samples collected by C57BL/6 WT and NFATc2 KO animals, analyzed by flow cytometry.

2.2 NFATc2 TF activation occurs rapidly after transplantation in donor skin cells

To evaluate the putative activation of NFATc2 into the donor cells in the graft, we performed immunofluorescent staining of frozen sections of grafted skin, collected soon after transplantation, in the indicated timepoints, to assess NFATc2 translocation in the nuclei. As reported in Figure 2, male-derived WT skin graft displays NFATc2 activation after 2 and 3 days post-transplant, indicating a fast NFATc2 pathway triggering in donor cells, mediating crucial events for the initiation of the following rejection. Conversely, by evaluating male-derived NFATc2 KO skin graft, it is possible to note the absence of NFATc2 translocation or upregulation at any of the timepoints observed, similarly to the acceptance control, constituted by female-derived WT skin grafting into female recipients. Furthermore, the lack of NFATc2 positive signal in NFATc2 KO skin graft may indicate that even recipient-derived cells are uncapable to infiltrate the transplant within three days and that probably this delayed infiltration reflects the shifted kinetics of skin rejection in this experimental group (see Figure 1).

Taken together these results highlight the critical role of NFATc2 in contributing to the initiation of the rejection process in mismatched grafts.


A
Mwt-Fwt
Fwt-Fwt
Mc2-Fwt
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nuclei
B
Mwt-Fwt
Fwt-Fwt
Mc2-Fwt
NFATc2
nuclei

Figure 2: NFATc2 activations occurs early after transplantation

Female C57BL/6 WT recipients were transplanted with WT female or WT male or NFATc2 KO male-derived skin. Skin grafts were collected at day 2 (A) and 3 (B) post-transplantation and frozen. Immunofluorescent staining was then performed on cryo-sections of graft samples.
NFATc2: red; Nuclei: blue

2.3 The lack of NFATc2 in skin transplants reduces graft infiltration by recipients' cells and IFN-γ production

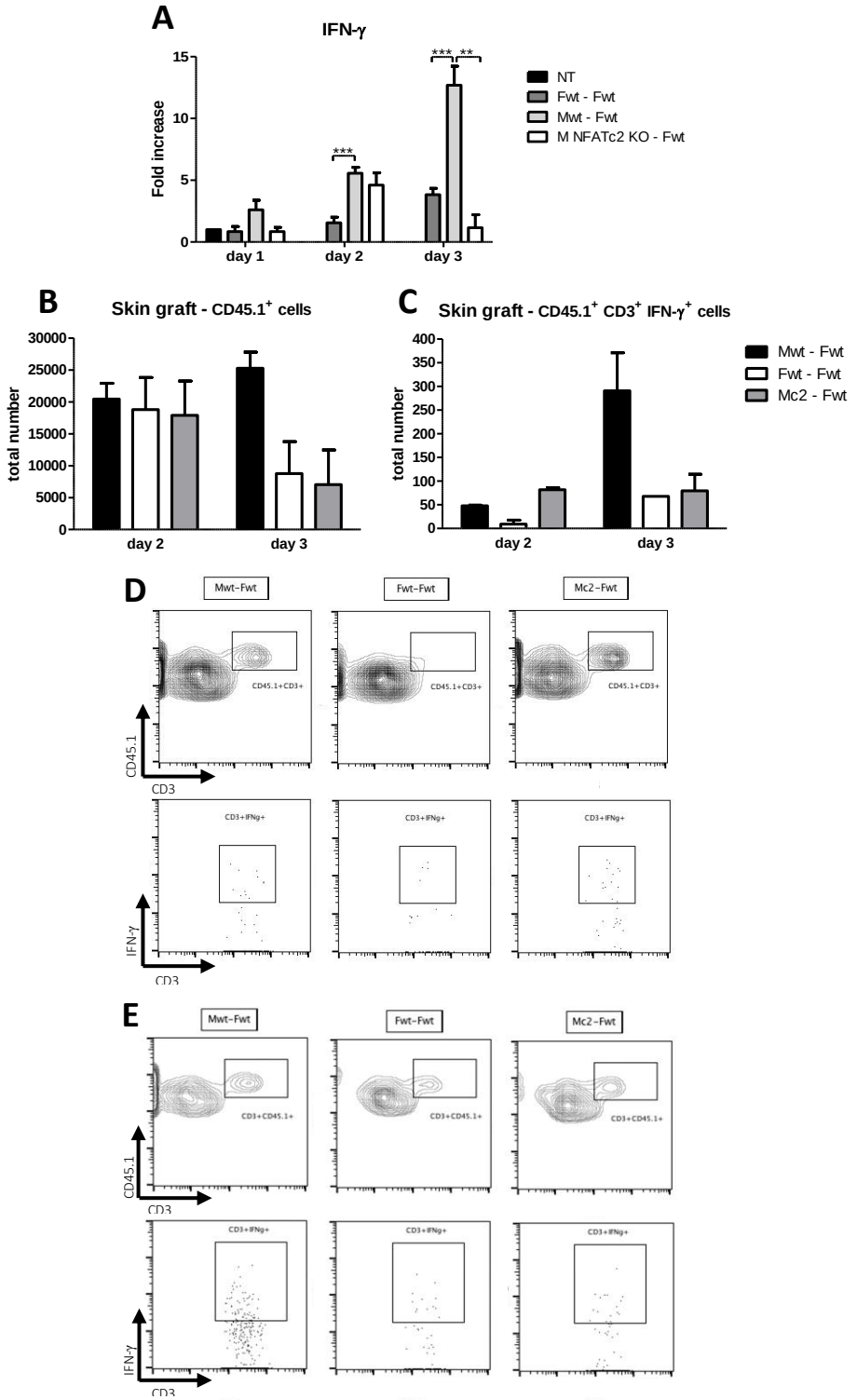
Graft rejection is a phenomenon that resembles tissue damage. For instance, rejected skin appears encrusted, eroded and easily removable by the lipodermal layer below, indicating poor attachment and acceptance. Conversely, matched grafts exhibited complete adhesion and conjunction with the edges of the recipients' skin, displaying elastic behavior upon stretching. The mediators acting upstream rejection are several and, among these, IFN-γ suggests infiltration of the graft by alloreactive T cells, poor tolerance against the skin, cell death and tissue damage (Slavcev et al. 2015). Indeed, IFN-γ release in the skin acts on diverse immune and non-immune cells, promoting T cell infiltration even in sterile conditions (Issekutz, Stoltz, and Van der Meide 1988; Kaplan et al. 1987). Furthermore, IFN-γ has

reported to be involved in improving mobility and migration of T cells into the skin and inducing T cell-mediated keratinocytes killing. In addition, OVA transgenic skin transplanted in IFN-γ$^{-/-}$ animals is not rejected (Bhat et al. 2017). Thus, we wonder whether the delayed rejection observed in recipients of male-derived NFATc2 KO skin may be due to a reduction in IFN-γ production. To evaluate IFN-γ in the graft, we collected skin grafts at the indicated timepoints to perform qRT-PCR. As shown in Figure 3A, the amount of IFN-γ transcript in the grafts derived from male WT animals is elevated when compared to the level exhibited by the skin collected from female WT mice and transplanted into female WT recipients, corroborating the hypothesis on the fundamental role of IFN-γ in mediating skin rejection. In addition, NFATc2 KO skin grafted in female WT recipients displays an IFN-γ transcription comparable to the one of the accepted transplants, suggesting that the absence of NFATc2 in the graft directly or indirectly reduces the production of one of the fundamental mediators of rejection and further, delays the process of rejection itself.

As already described, male NFATc2 KO skin graft exhibited less IFN-γ induction. One of the major sources of IFN-γ are T lymphocytes, already activated and differentiated. In the transplant setting, alloreactive T cells infiltrating the skin constitute evidence for the rejection process. To evaluate whether the less IFN-γ detected in male NFATc2 KO skin graft

could be indicator of a reduced infiltration of alloreactive T cells, we collected the transplants at the indicated timepoints to perform flow cytometric analysis and evaluate both cellular composition infiltrating the grafts and the source of IFN-γ. In order to discriminate between donor- and recipient-derived cells, we transplanted either male NFATc2 KO, male WT or female WT skin, expressing CD45.2, into CD45.1 female recipients. As reported in Figure 2B, the recipient-derived immune infiltrate of male WT skin graft seems to increase over time when compared to the female WT counterpart that, conversely, displays a reduction in the $CD45.1^+$ population. Similarly to the latter, male NFATc2 KO graft exhibits limited infiltrate. Among these $CD45.1^+$ cells, the $CD3^+$ IFN-γ^+ subset is highly represented in the skin transplants derived from male WT, confirming the increase in the IFN-γ transcript in this experimental group observed by qRT-PCR. Conversely, male NFATc2 KO skin graft exhibits lower number of $CD3^+$ IFN-γ^+ lymphocytes, comparable to the amount detected in female WT grafts. In addition, we performed an MLR with splenic DCs isolated from WT or NFATc2 KO animals and $CD4^+$ T cells purified from Balb/c mice to assess the priming capabilities of NFATc2 KO DCs *in vitro*. We could not observe any difference in the proliferation and expansion of T cells but the level of IFN-γ was reduced when $CD4^+$ cells were co-cultured with NFATc2 KO splenic DCs. The cytokine that mostly counteract IFN-γ

effects is TGF-β. Therefore, we added TGF-β to the MLR and observed a clear reduction in the IFN-γ production, as expected. Furthermore, when blocking the TGF-β pathway in the culture we partially restored the IFN-γ level in the WT setting while we assisted even in a significant increase in the NFATc2 KO setting, confirming the action of TGF-β in the stimulatory capacities of NFATc2 KO splenic DCs. These results, in accordance with the previous reported, suggest that in the first events preceding graft rejection, the absence of NFATc2 in the transplants results in a response similar to the one of female WT skin graft, in terms of IFN-γ producing cells, probably via TGF-β action mediated by NFATc2 KO DCs.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day 2
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400
350
300
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200
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100
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Mc2-Fwt
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CD3+IFNg+
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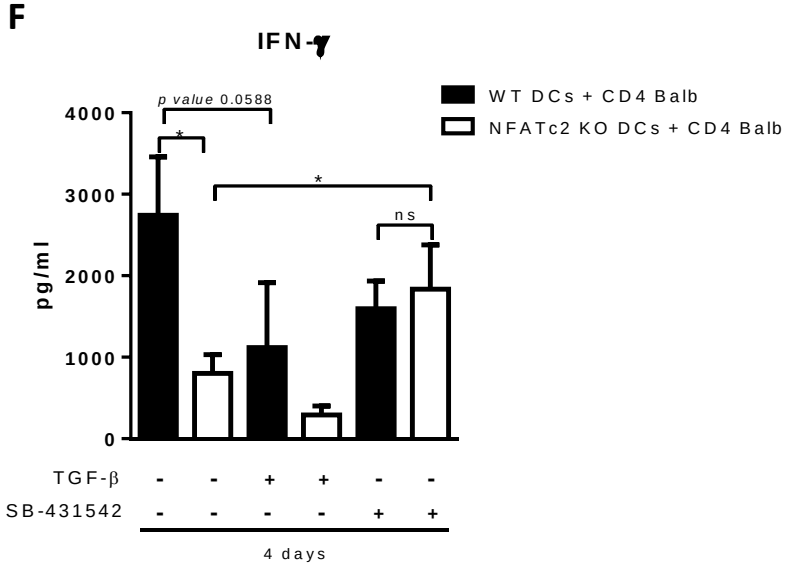


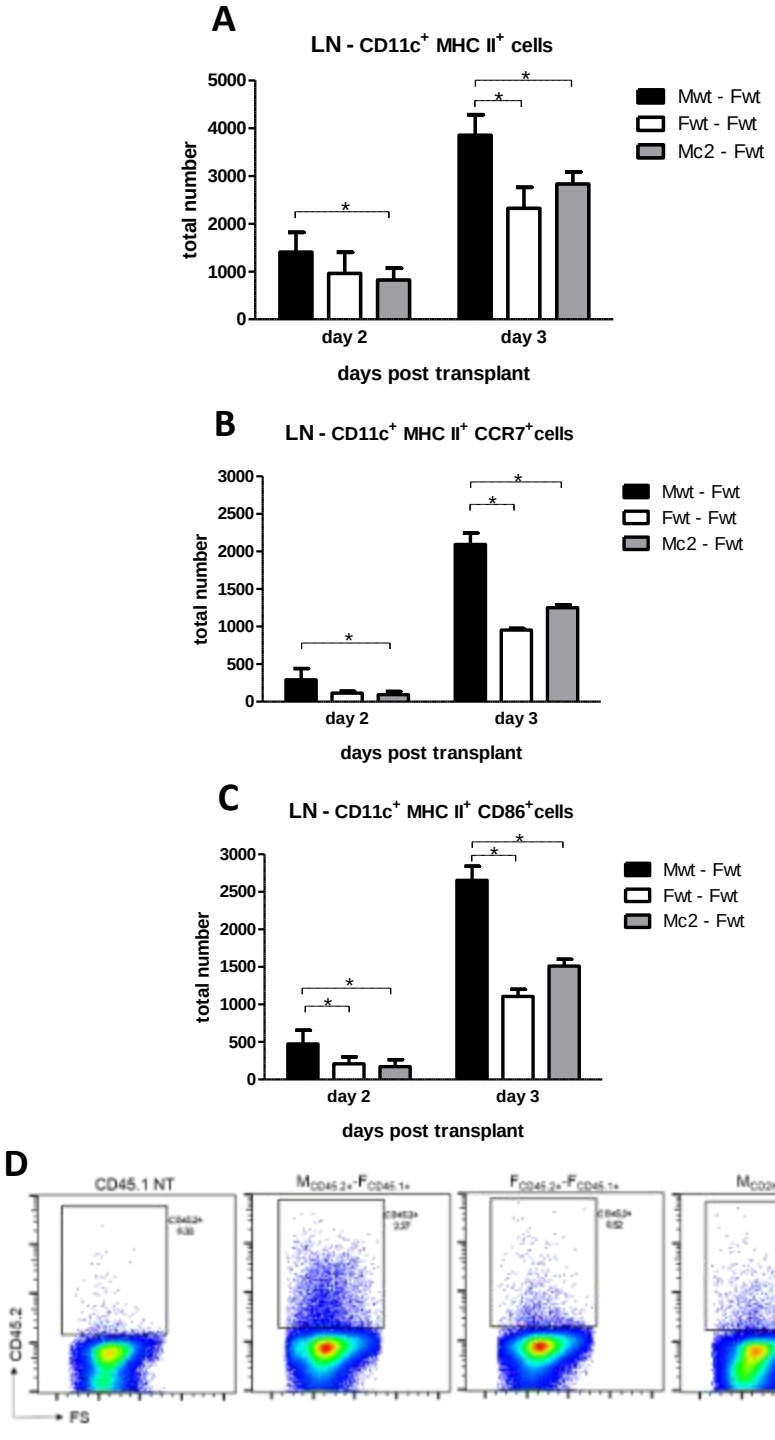
Figure 3: NFATc2 KO male-derived skin graft exhibits reduced infiltrates and less IFN-γ⁺ T cells

C57BL/6 CD45.1 female recipients were transplanted with WT female or WT male or NFATc2 KO male-derived skin. Skin graft were collected after 2 or 3 days post-transplant and RNA was extracted to perform qRT-PCR (A). Skin graft were digested to obtain cellular suspensions for flow cytometric analyses. B) recipients' immune infiltrate. C) recipients' number of $CD3^+$ IFN-γ^+ cells in the graft. Representative dot plots of recipients' $CD3^+$ IFN-γ^+ cells in the graft at day 2 (D) and 3 (E). F) IFN- γ production in MLR with the addition of TGF-β or the inhibitor of its pathway SB-431542.

2.4 DCs in the graft-draining lymph node of NFATc2 KO-skin transplanted mice exhibit a reduced maturation phenotype

Since the efficiency and the skewing of adaptive responses are orchestrated by DCs, the reduced T cell-mediated IFN-γ response observed when transplanting male NFATc2 KO into female WT may be due to an inefficient antigen presentation. In the transplant setting, donor DCs constitute the major player in promoting alloreactivity. Shortly after transplantation, donor DCs migrate to the draining lymph nodes where they present donor antigens via the direct pathway and activate alloreactive T cells (Ali et al. 2013). Thus, the functional profile of donor DCs appears to be fundamental in the first phases of the rejection process. To evaluate the status of the donor NFATc2 KO DCs, we collected skin graft-draining lymph nodes to perform flow cytometric analysis. As shown in Figure 4, recipients of male WT skin display a higher absolute number of DCs, identified as $CD11c^+$ MHC II^+ cells, when compared to the female WT counterpart and even to the female animals transplanted with the male NFATc2 KO skin. The impaired migration of skin DCs to the draining lymph nodes may be due to impaired maturation in the periphery and reduced expression of the crucial chemokine receptor that allows emigration through lymphatic vessels, like CCR7. Indeed, as shown in Figure 4B, $CCR7^+$ DCs in

the lymph node of animals transplanted with female WT or NFATc2 KO skin, are inferior in number if compared to the DCs pool of male WT skin recipients. In addition to CCR7, even the $CD86^+$ DCs population in female WT or NFATc2 KO skin recipients is not as well represented as in the lymph nodes of animals grafted with male WT skin, as reported in Figure 4C. Taken together, these data highlight how the impaired maturation and further improper migration of NFATc2 KO DCs may contribute to the delayed rejection of the graft.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days post transplant
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Fwt - Fwt
Mc2 - Fwt
B
LN - CD11c+ MHC II+ CCR7+cells
3000
2500
2000
1500
1000
500
0
C
LN - CD11c+ MHC II+ CD86+cells
D
CD45.1 NT
CD45.2
FS

Figure 4: DCs of NFATc2 KO male-skin grafted mice exhibit a reduced maturation profile and less migratory capabilities

C57BL/6 CD45.1 female recipients were transplanted with WT female or WT male or NFATc2 KO male-derived skin. Graft-draining lymph nodes were collected after 2 or 3 days post-transplant and analyzed by flowcytometry. Donor's DCs in graft-draining lymph nodes were evaluated in terms of number (A), expression of the chemokine receptor CCR7 (B) and the costimulatory molecule CD86 (C). D) Representative dot plot of flow cytometric analyses of donors' cells in the graft-draining lymph nodes.

2.5 NFATc2 KO skin transplant exhibits reduced lymphatic vessels activation in terms of CCL21 production

To efficiently reach the skin-draining lymph node, fully mature DCs have to up-regulate the chemokine receptor CCR7, which binds CCL21, a chemokine released by lymphatic vessels in inflammatory conditions. Since NFATc2 has emerged to be fundamental in promoting inflammatory processes as edema formation (Zanoni et al. 2012), the absence of this TF in the transplant may have impaired DCs migration, not only reducing their maturation profile but also affecting the inflammatory status of the graft and, thus, lymphatic vessels activation. Therefore, we assessed the production of CCL21 by the lymphatics after the transplantation. To address this issue, whole

mounting immunofluorescent staining on grafted skin was performed. As shown in Figure 5, female-derived skin transplanted in female recipients displays undetectable signal of CCL21, while male-derived grafted skin exhibits up-regulation of the chemokine in close proximity to Lyve-1^+ endothelial cells. Similarly to the female-derived transplant, the NFATc2-deficient skin graft displays no activation of the afferent lymphatic vessels, in terms of CCL21 production. Thus, it is presumable that NFATc2 acts on multiple levels and on distinct cell subsets to orchestrate the proper inflammatory response, hence promoting DCs maturation and migration through the afferent lymphatic vessels to the draining-lymph node.

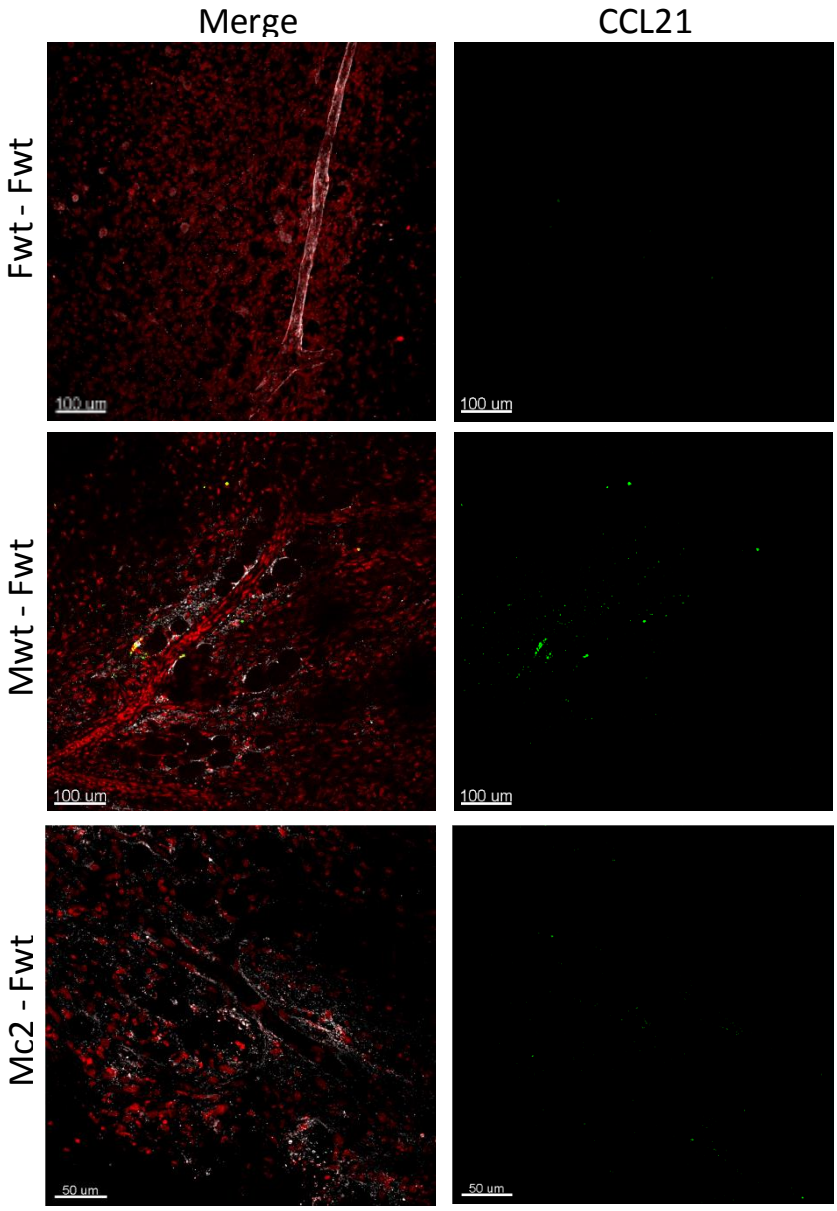


Figure 5: Male NFATc2 KO-derived skin graft exhibits reduced lymphatic vessels activation

Skin graft were collected at day 3 post-transplant and processed as described in Materials and methods. Briefly, whole mounting staining was performed with anti-Lyve-1 (white), anti-CCL21 (green) and DRAQ5 for nuclei staining (red).

2.6 A novel tool to inhibit NFATc pathway in innate immune cells

Gold standard therapies for grafted patients consist in long-life administration of immunosuppressants that aim at inhibiting NFATc-mediated responses by alloreactive T cells, but concomitantly impede the putative generation of tolerance against the graft. If the absence of NFATc2 in the transplant has resulted to be sufficient in delaying the rejection process, hypothetically via DCs impaired maturation, the inhibition of all the NFATc members in APCs may even improve the outcome. To assess this issue, we took advantage of an NFATc specific inhibitor: the VIVIT peptide. The mechanism exploited by the peptide is to impede the protein-protein interaction of Cn and NFATc, since Cn exhibits several docking sites for NFATc, required for proper dephosphorylation and further activation. Therefore, blocking Cn docking onto NFATc will result in NFATc inactivation, though maintaining Cn general phosphatase activity. Research in conceiving putative peptides that would abrogate Cn docking was focused on the main docking site on the N-terminus of Cn, containing the PxIxIT consensus motif (where x is any amino acid). Finally, the discovery of VIVIT (MAGPHPVIVITGPHEE) allowed having a selective and potent inhibitor of Cn (Aramburu et al. 1998, 1999). Therefore, we used VIVIT to specifically inhibit NFATc TFs.

Since our aim is to evaluate the role of NFATc in innate immunity and, specifically, in DCs we required a tool to vehicle VIVIT to these cells. Thus, in collaboration with the Nanobiolab of the University of Milano – Bicocca, we designed polymer-based nanoparticles (NPs) that deliver the VIVIT peptide, avoiding its degradation and promoting the targeting of phagocytes (patent n°: PCT / IB2013/055943).

2.7 MYTS nanoparticles synthesis

The synthesis begins with iron oxide NPs coated with hydrophobic long-chain surfactants with sizes ranging from 3 to 50nm, coated with (PMA) an amphiphilic polymer, dissolved in chloroform (Lin et al. 2008). To obtain a clean NPs dispersion, sodium borate buffer was added and reacted with 2,2-(ethylenedioxy) bis (ethylamine) (EDBE). After washing, NPs were shaken for 4 hours with N-succinimidyl-3-[2-pyridyldithiol]-propionate (SPDP), concentrated and washed again (ref 15). Following, VIVIT peptide and (PEG-SH) (500Da) were added and further shaken for 2 hours. The obtained NPs suspension (MYTS) was concentrated and the concentration determined by UV measurement (Figure 6).

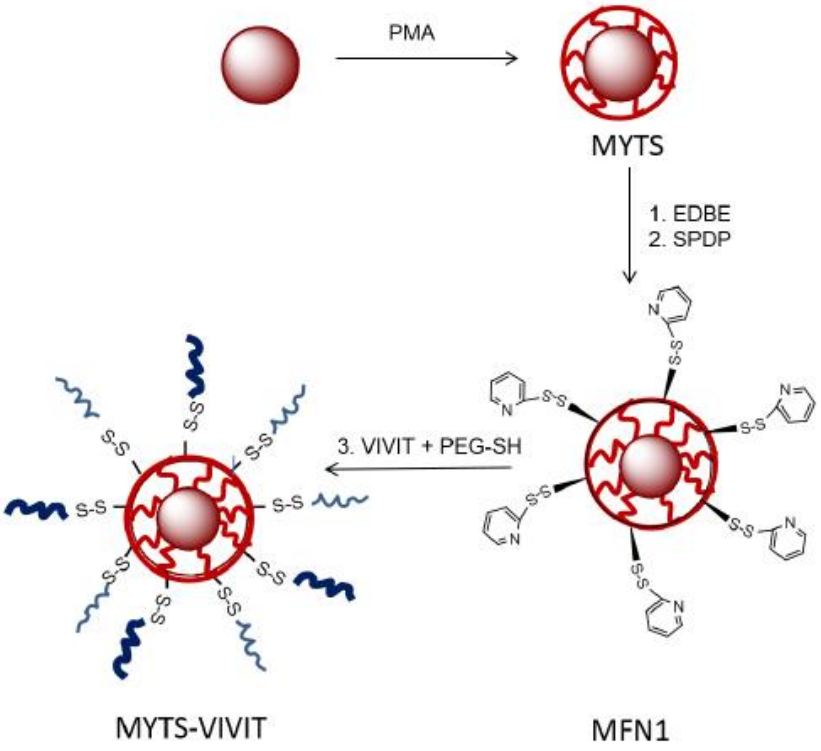


Figure 6: MYTS nanoparticles synthesis

Commercially available iron oxide has been coated with PMA, an amphiphilic polymer. Following, VIVIT peptide and PEG-SH (500 Da) have been added via a disulfide bond. Finally, nanoparticles concentration has been measured.

2.8 MYTS nanoparticles are internalized by phagocytes both *in vitro* and *in vivo*

To evaluate the internalization of MYTS NPs by DCs, we performed both in vitro and in vivo assays. Firstly, we generated bone marrow-derived DCs (BMDCs) from WT mice and incubate BMDCs with MYTS NPs for the indicated timepoints at 37 °C (Figure 7A). To assess the up-take of MYTS NPs, we used MYTS-PEG NPs conjugated with fluorescein isothiocyanate (FITC) and MYTS-PEG NPs as negative control. At the indicated timepoints, BMDCs were harvested and analyzed by flow cytometry. As shown in Figure 8A, BMDCs internalize MYTS-PEG FITC NPs already after 10 minutes of incubation, but the uptake increases over time. We performed the same experiment at 4 °C to verify that the fluorescent signal was not due to non-specific binding of the MYTS-PEG FITC to the BMDCs and we observed a kinetics similar to the experiment conducted at 37 °C (Figure 7B). After assuring the capability of DCs to internalize MYTS NPs, we evaluated the uptake of MYTS-PEG FITC in vivo in WT animals. To address this issue, we administered intraperitoneally (i.p.) 100 µg/mouse of MYTS-PEG FITC and MYTS-PEG as negative control, every other day. After two weeks of treatment, animals were sacrificed and spleen, lymph nodes and skin collected for flow cytometric analyses. Despite the competition with other cell types, MYTS NPs administered *in*

vivo are mainly internalized by phagocytes if compared to the adaptive arm (Figure 7C-E). Indeed, $CD11c^+$ DCs, $CD11b^+$ monocytes/macrophages and $Ly6G^+$ neutrophils were capable of internalizing MYTS NPs while $CD4^+$ T cells did not up-take the NPs, in all the tissues analyzed (Figure 8). Therefore, these internalization and distribution experiments bring to light the intrinsic capability of phagocytes to engulf external particulates thus guaranteeing also a putative vehiculation of drugs and, in our case, of the VIVIT peptide to DCs, residing even in the skin.

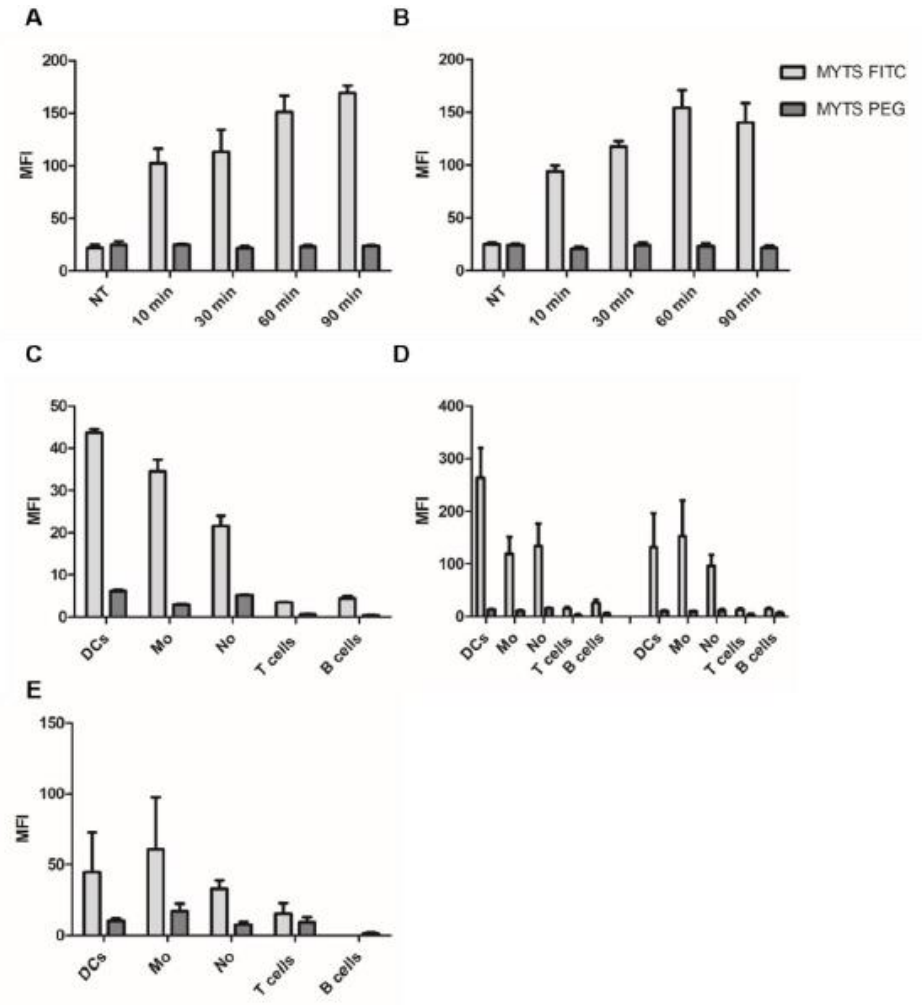


Figure 7: MYTS NPs uptake *in vitro* and *in vivo*

C57BL/6-derived BMDCs were incubated with MYTS-PEG FITC (500 µg/ml) or MYTS-PEG NPs (500 µg/ml) as control, for the indicated timepoints. A) NPs uptake by BMDCs at 37 °C and B) at 4 °C. C57BL/6 mice were administered i.p. with 100ug of MYTS-PEG FITC or MYTS-PEG. Lymphoid or non-lymphoid organs were then collected for flow cytometric analyses. C) NPs uptake by the indicated cell subsets in vivo after 90 minutes post-administration. After two weeks of treatment every other day, spleen and lymph nodes (D) and skin (E)

were collected to evaluate NPs uptake. Data are shown as mean ± SD (n = 2).

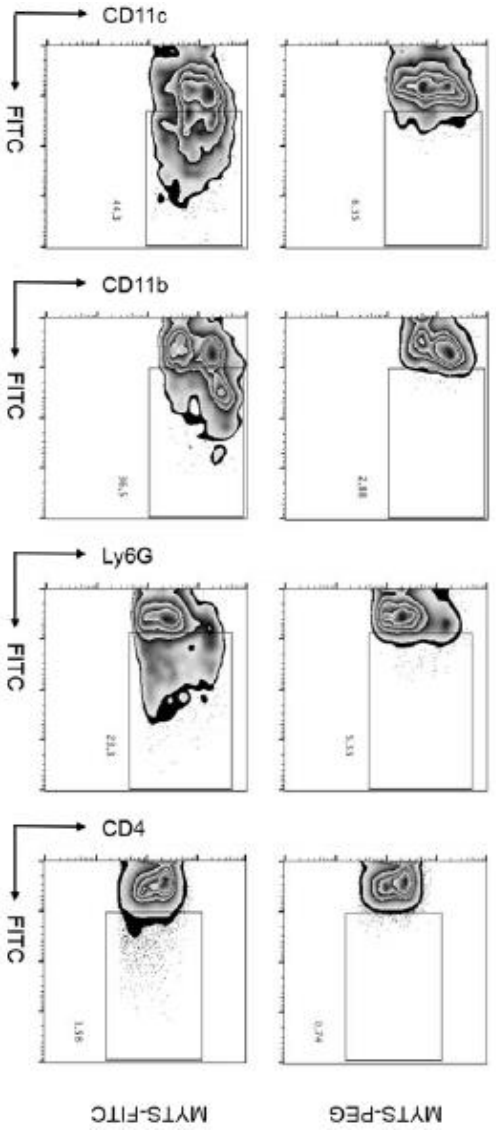


Figure 8: NPs uptake by specific cell subsets

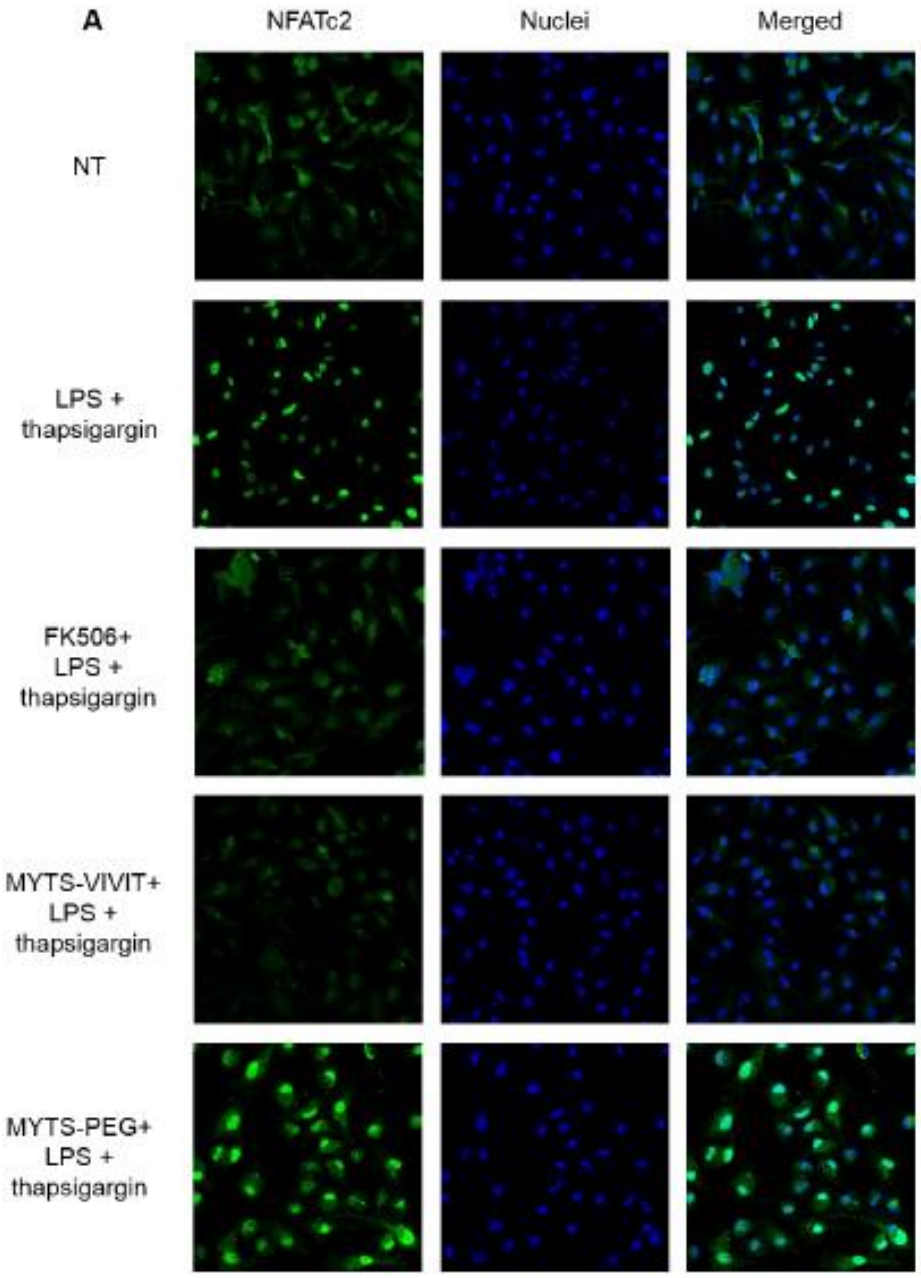
C57BL/6 mice were injected i.p. with 100 µg of MYTS-PEG FITC or MYTS-PEG. After 90 minutes, spleens were collected, and cell

suspensions analyzed by flow cytometry to assess the NPs internalization. DCs: $CD11c^+$; monocytes/macrophages: $CD11b^+$. Neutrophils: $Ly6G^+$. T lymphocytes: $CD4^+$.

2.8 MYTS-VIVIT NPs specifically abrogate NFATc activation without affecting NF-κB pathway

After assessing the targeting and active internalization of the MYTS NPs, we needed to test their efficacy in selectively inhibiting NFATc pathway. The activation of these TFs may be assessed by immunofluorescence staining to detect NFATc translocation to the nucleus but also by measuring the products of the transcriptional activity of these TFs, as IL-2. Indeed, it has been demonstrated that LPS stimulation of DCs leads to NFATc elicitation and consequent IL-2 production, but also NF-kB activation and further TNF-α release. Therefore, BMDCs from WT animals and a mouse DC-line, D1 (Winzler et al. 1997), were pre-treated with MYTS-VIVIT or MYTS-PEG NPs or tacrolimus as control at the concentrations reported in Figure 10 for 2 hours, and then stimulated with thapsigargin and LPS for 40 minutes to induce NFATc activation and thus translocation. Cell were then stained for NFATc2 or NF-kB to evaluate their translocation via confocal microscopy. Images analyses of MYTS-VIVIT NPs-

treated DCs revealed their capability in abrogating LPS and thapsigargin-induced NFATc translocation in the nucleus, similarly to FK-506-treated samples. As expected, MYTS-PEG NPs allowed NFATc activation comparably to the positive control (Figure 9A). Conversely, the pre-treatment either with MYTS-VIVIT or MYTS-PEG did not compromise NF-kB nuclear translocation, as observed also in FK-506-treated cells (Figure 9B).


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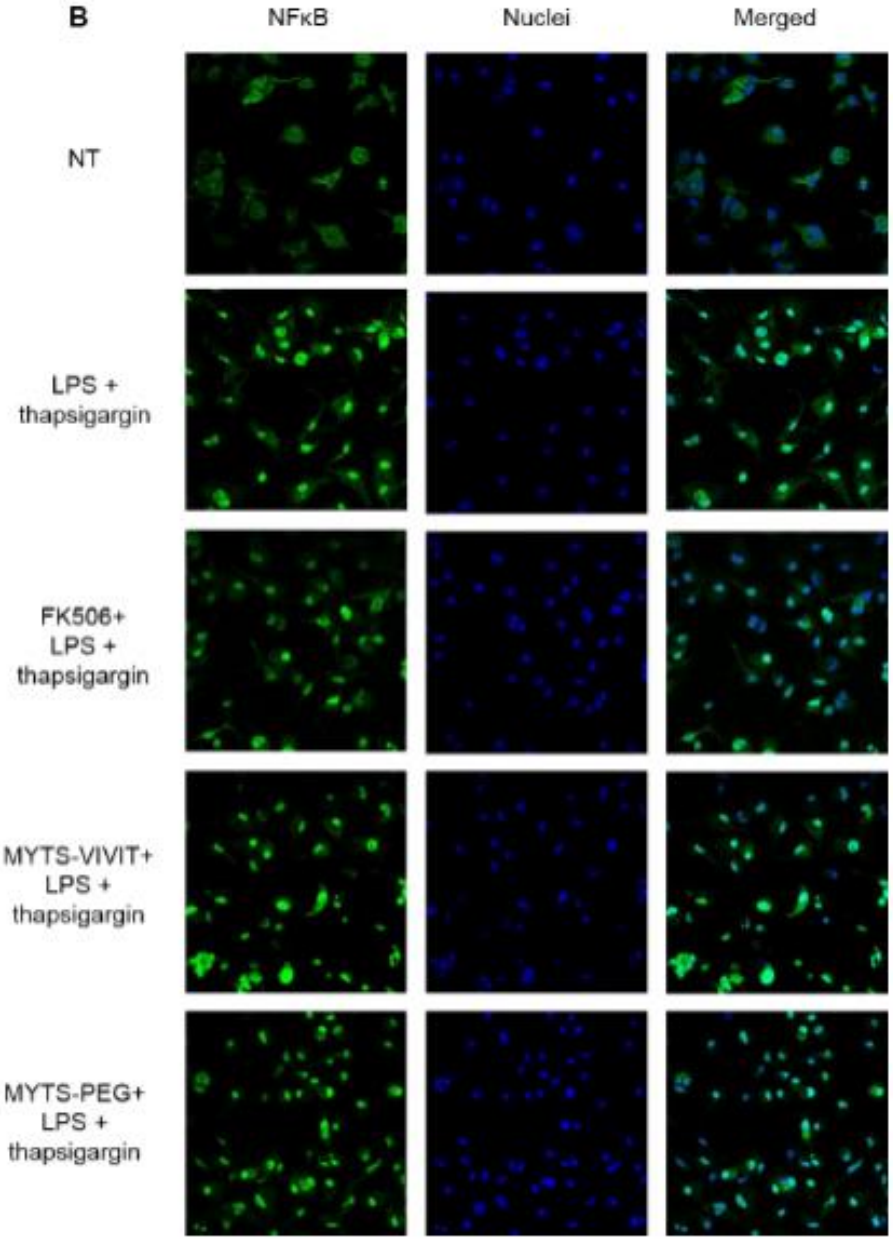
B
NFκB
Nuclei
Merged
NT
LPS +
thapsigargin
FK506+
LPS +
thapsigargin
MYTS-VIVIT+
LPS +
thapsigargin
MYTS-PEG+
LPS +
thapsigargin

Figure 9: MYTS-VIVIT NPs specifically abrogate NFATc2 translocation without affecting NF-κB activation upon LPS and thapsigargin stimulation

100.000 BMDCs were pre-treated for 2 hours with MYTS-VIVIT NPs, MYTS-PEG NPs or FK-506 as control. After the incubation, cells were stimulated with LPS (1 µg/ml) and thapsigargin (50 nM) for 40 minutes. Nuclear translocation of NFATc2 (A) and NF-κB (B) were evaluated via immunofluorescent staining and further confocal microscopy.

Green: NFATc2 (A) and NF-κB (B); Blue: nuclei (DRAQ5)

To assure that the impaired NFATc translocation after MYTS-VIVIT NPs treatment results in functional abrogation of the NFATc-mediated responses, we measured IL-2 production by both BMDCs and D1, after MYTS-VIVIT NPs pre-treatment and LPS stimulation. To verify that the NF-kB pathway is not affected by MYTS-VIVIT NPs, confirming the translocation data observed in Figure 9B, we quantified the NK-kB-dependent cytokine TNF-a in the supernatants of cell cultures. As expected, pre-treatment with MYTS-VIVIT NPs did not impair TNF-a production upon LPS stimulation, both in BMDCs and D1 samples, at all the concentrations tested (Figure 10A and 10C). Conversely, IL-2 production decreased when DCs are pre-treated with MYTS-VIVIT NPs, in a dose-dependent manner (Figure 10B and 10D).

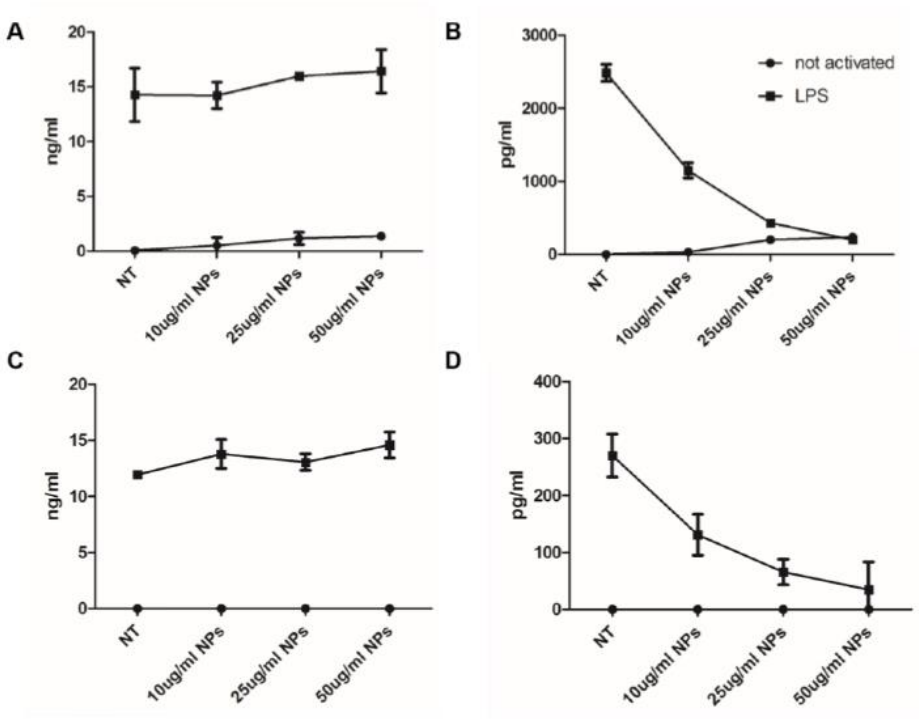

Figure 10: MYTS-VIVIT NPs specifically abrogate IL-2 production in a dose-dependent manner upon LPS stimulation

D1 cells (A-B) or BMDCs (C-D) were pre-treated for 2 hours with MYTS-VIVIT NPs. After the pre-stimulation, LPS (1 µg/ml) was added to the culture for 18 hours, when supernatants were collected to assess IL-2 and TNF-α production by ELISA assay. Data are shown as mean ± SD (n = 3).

2.9 MYTS-VIVIT NPs *in vivo* treatment does not abolish T cell activation

Finally, our aim in the use of MYTS NPs was to inhibit NFATc TFs activation in innate immune cells without affecting NFATc-mediated putative adaptive responses. To assure that T cells were unaffected by NPs treatment, we administered OT II mice with MYTS-VIVIT or MYTS-PEG NPs or FK-506 for 2 weeks, every other day. In parallel, we generated BMDCs that were pulsed with LPS and ovalbumine (OVA). Subsequently, we injected the different OT II treated animals with OVA-pulsed DCs in order to analyze the T cell compartment in lymph nodes, 72 hours post-injection. As shown in Figure 12, mice who received FK-506 did not mount an adaptive response, both in terms of T cell proliferation and expansion (A) nor in the percentage of IL-2^+ $CD4^+$ T lymphocytes (B). Conversely, MYTS-VIVIT NPs treatment did not alter the T cell capacity to activate, displaying a slight decrease in T cell numbers but equal percentage of IL-2-producing cells when compared to not treated OT II mice, injected with OVA-pulsed DCs, and MYTS-PEG-treated animals (Figure 11).

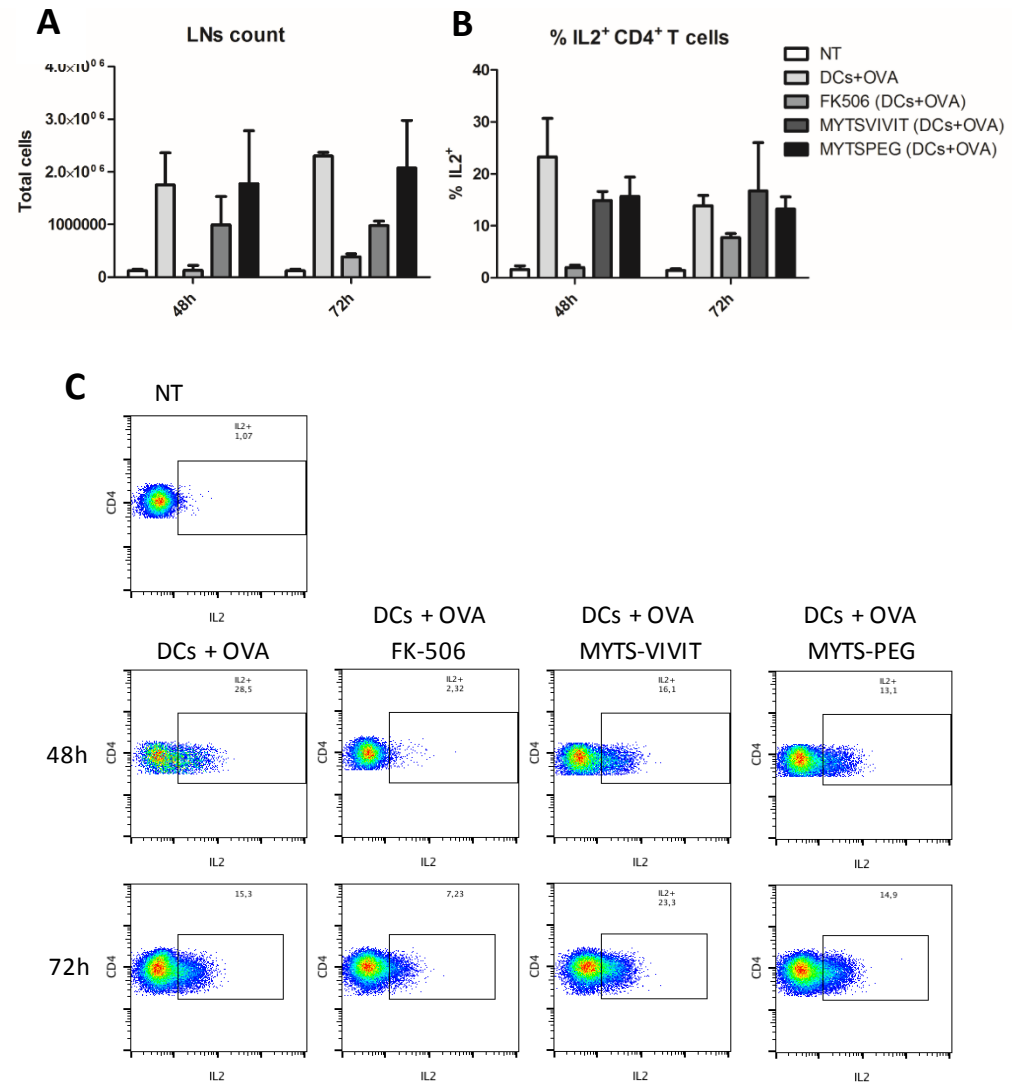

Figure 11: MYTS-VIVIT NPs treatment *in vivo* do not impair T cell activation

OT II mice were treated with 100 MYTS-VIVIT NPs or MYTS-PEG NPs or FK-506 for two weeks every other day. In parallel, C57BL/6-derived BMDCs were pulsed with OVA and LPS (1 g/ml). Then, BMDCs were injected in the treated OT II mice and lymph nodes were collected after 72 hours to assess T lymphocytes count and IL-2 production by T cells

via flow cytometry. A) Absolute number and B) percentage of $CD4^+$ $IL2^+$ T cells. C) Representative dot plot of FACS analyses showing $CD4^+$ $IL2^+$ T cells. Data are shown as mean ± SD.

Taken together, these data highlight the potential of MYTS-VIVIT NPs as a novel tool to selectively inhibit NFATc TFs, preferentially in phagocytes without affecting T cell compartment, still capable to mount immune responses and differentiate into the required effector cells. Therefore, MYTS NPs result to be suitable to study the role of NFATc TFs in innate immunity in a variety of distinct experimental settings for both physiological and pathological conditions.

2.10 MYTS-VIVIT NPs treatment confers protection upon mismatched skin transplant

As already mentioned, DCs are the primary professional APCs that decide for T cell faith and skew adaptive polarization (Kim et al. 2014; Kosten et al. 2017). The multitude of signals derived by DCs synergistically promote T cell responses and recently NFATc TFs have emerged to be crucial for proper adaptive elicitation (Wuest et al. 2011). Indeed, consistently with previous data, the absence of NFATc2 in donor skin revealed to

be sufficient to delay the alloreactive response against the graft in a mismatched setting. Therefore, it is presumable that the complete inhibition of all the NFATc TFs may lead to a better outcome, despite the mismatched graft setting.

We set up the experimental model of transplant previously described, and we add three other groups, in addition to the acceptance group (female-derived skin into female recipients) and the rejection group (male-derived skin into female recipients). We grafted female recipients with male-derived skin and treated some animals with MYTS-VIVIT NPs or MYTS-PEG NPs or FK-506 every other day until the end of the experiment. As reported in Figure 12A, already at day 14 post-transplant, all the animals who received male skin had rejected the graft, exhibiting encrusted and detached transplants. Similarly, already 80% of MYTS-PEG-treated mice rejected the male-derived skin within 14 days while 75% of the MYTS-VIVIT-treated animals accepted the graft for the entire time window observed (day 50 post-transplant). FK-506-treated mice exhibited a slight but constant increase in the percentage of rejecting-animals, reaching almost the 50% at day 50 post-transplant. Thus, it seems that the specific inhibition of NFATc TFs in phagocytes confers protection upon mismatched transplant.

To evaluate whether the protective outcome observed via the MYTS-VIVIT treatment are due to a continuous effect that

acts on phagocytes or the NPs role is crucial in relatively early events that in turn allow to accept the graft even in the absence of NFATc inhibition, we decided to interrupt the treatment and evaluate graft rejection. To address this issue, we repeated the experimental setting just described and interrupt the treatment at day 50. Mice were monitored for other 20 days, until day 70 post-transplant. Surprisingly, MYTS-VIVIT-treated animals did not reject the grafts even in the absence of the treatment while FK-506-treated animals immediately upon treatment interruption, rapidly rejected the skin transplant (almost 100% of the animals at day 70 post-transplant) (Figure 12B).

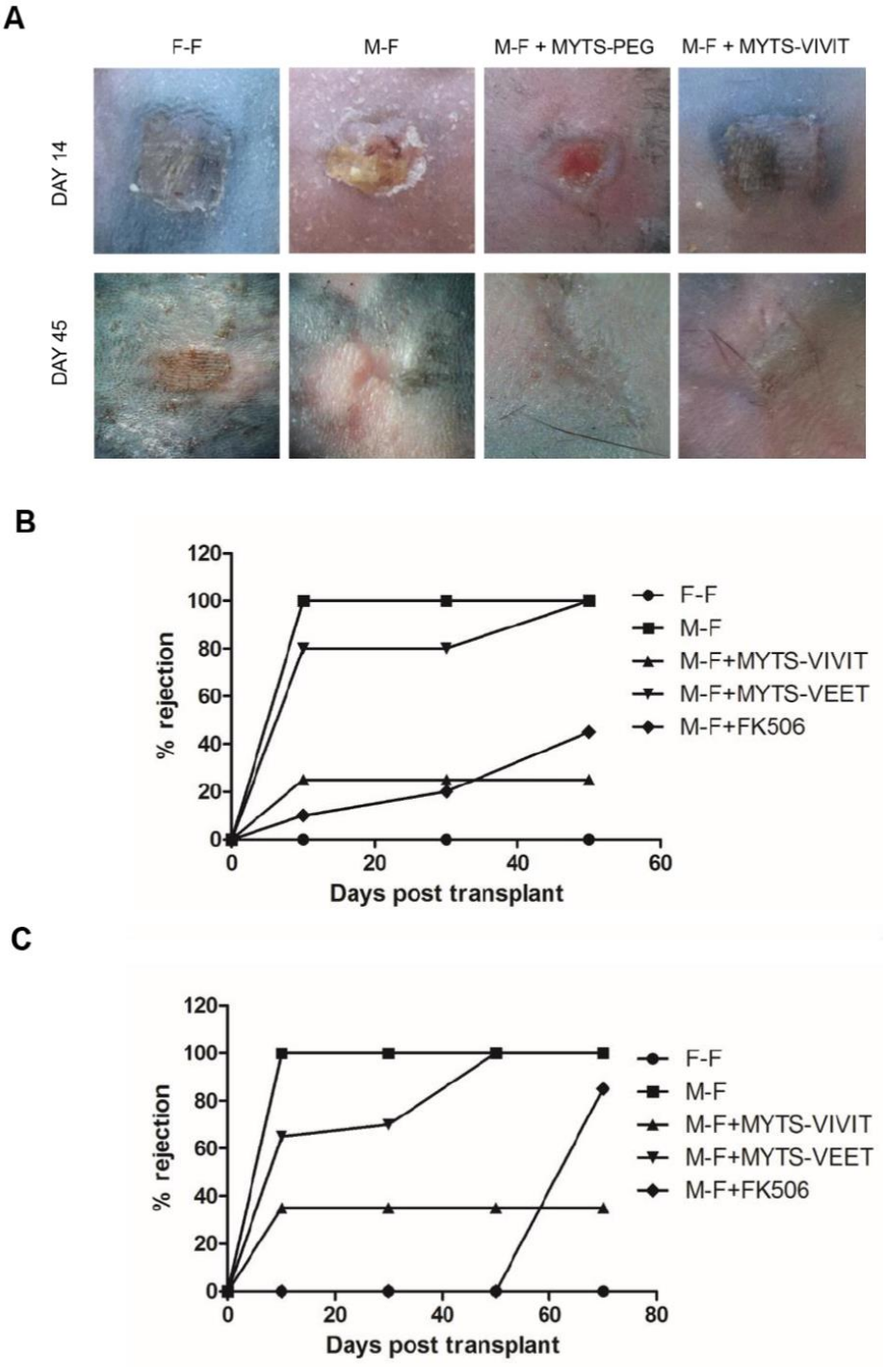
A
F-F
M-F
M-F + MYTS-PEG
M-F + MYTS-VIVIT
DAY 14
DAY 45
B
% rejection
Days post transplant
F-F
M-F
M-F+MYTS-VIVIT
M-F+MYTS-VEET
M-F+FK506
C
% rejection
Days post transplant
F-F
M-F
M-F+MYTS-VIVIT
M-F+MYTS-VEET
M-F+FK506

Figure 12: MYTS-VIVIT NPs treatment mediates acceptance of the mismatched graft

C57BL/6 female mice were transplanted with the tail skin of a donor C57BL/6 male or female mouse. Recipients of the male donor skin were treated i.p. (100 µg) every other day with MYTS-VIVIT NPs, MYTS-VEET NPs or FK-506. Recipients were monitored at the indicated timepoints until day 70 post-transplant. A) graft acceptance or rejection images representative of the four experimental groups. B) % of rejection observed during the timewindow analyzed. C) % of rejection observed with the interruption of the treatment on day 50.

2.11 Temporal treatment with MYTS-VIVIT NPs induces long-term graft acceptance

In order to characterize the immune response occurring in MYTS-VIVIT- and MYTS-PEG-treated animals, we exploited a transgenic animal model known as K5-mOVA, which expresses a specific membrane-bound form of ovalbumin (mOVA) under the promoter of keratin 5 (K5) in the epidermal and hair follicular keratinocytes residing in the skin. By combining k5-mOVA-derived skin with OT II T cells, infused in recipient animals, it is possible to evaluate antigen-specific responses against the skin and, in our case, against the graft. Furthermore, to avoid biases due to the presence of OT II Tregs in the $CD4^+$ T cell

compartment and to simultaneously distinguish OT II from recipient-derived cells, we took advantage of another transgenic animal model: Dereg OT II CD45.1 mouse. Dereg OT II CD45.1 mice are inserted with the Dereg cassette (Lahl et al. 2007) exhibiting expression of the simian diphtheria toxin receptor (10^5-fold more sensitive to diphtheria toxin if compared to its murine counterpart) under the promoter of Foxp3, providing a tool to deplete Foxp3$^+$ Tregs. Therefore, we treated these animals with diphtheria toxin and collected CD4$^+$ T cells, devoid of the Tregs compartment, and infuse them into female recipient mice on the day of the K5-mOVA-derived skin transplantation. Recipients were then treated with either MYTS-VIVIT or MYTS-PEG NPs and monitored for eventual rejection. As expected, even in this potent antigen-specific setting, mice administered with MYTS-VIVIT NPs did not reject male-derived skin graft, while MYTS-PEG NPs-treated animals did (Figure 13A).

To confirm the previous data about the efficacy of a short treatment with MYTS-VIVIT to impede rejection and thus to abolish graft-specific alloreactive responses, we transplanted female K5-mOVA-derived skin into female infused with CD4$^+$ T cells isolated from female Dereg OT II CD45.1, previously treated with diphtheria toxin and thus devoid of Tregs. We shortened the time window of the treatment, reducing it to 21 days post-transplant. Consistently with the previous data, mice receiving MYTS-VIVIT NPs did not reject the grafts for other 30 days after

treatment interruption (day 50 post-transplant) (Figure 13B). This outcome suggests that MYTS-VIVIT treatment may induce long-term tolerance specific for the graft.

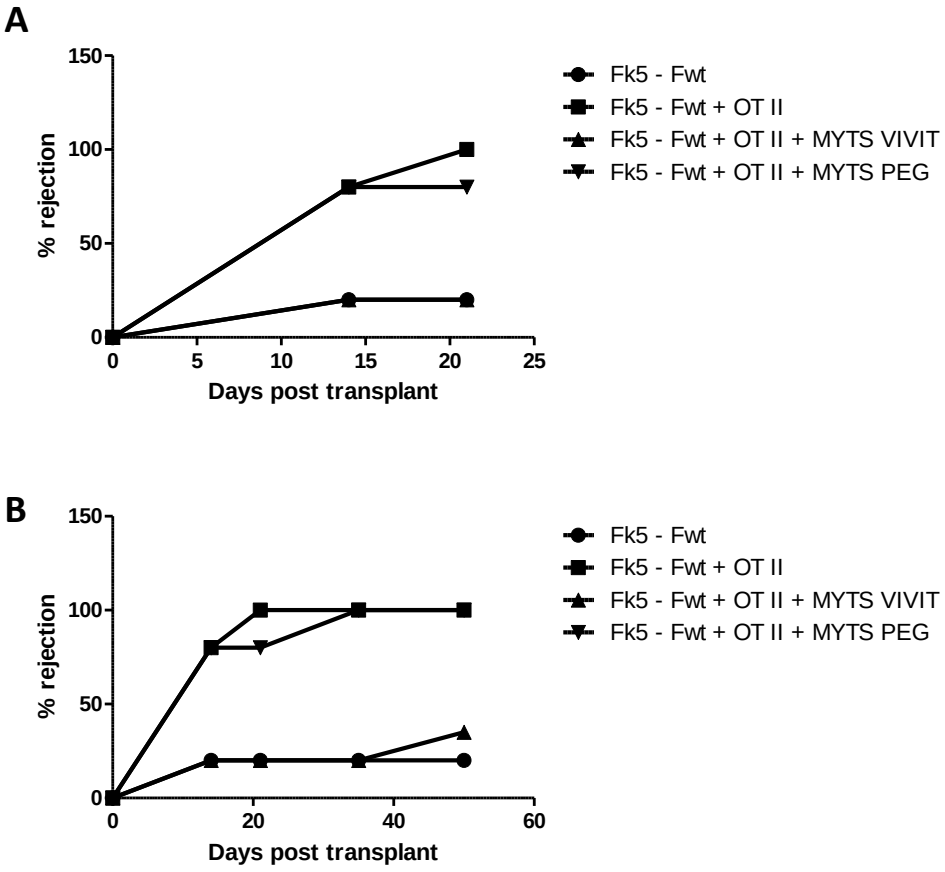

Figure 13: Temporal MYTS-VIVIT NPs treatment induces long-term graft acceptance

Dereg OT II CD45.1 mice were injected with diphtheria toxin for 3 days and then euthanized for spleen collection. CD4+ T (Foxp3-) cells were

isolated and infused into C57BL/6 female recipients. On the same day, the recipients were transplanted with K5-mOVA female-derived tail skin. Some recipients received MYTS-VIVIT NPs or MYTS-PEG NPs every other day until day 21, and rejection was monitored (A). B) The same experimental setting of A was performed but treatment was interrupted on day 21 post-transplant. Grafts were monitored until day 50 post-transplant.

2.12 MYTS-VIVIT NPs treatment results in the expansion of graft-specific Tregs

To dissect the immunological phenomena underlying the hypothesized long-term tolerance mediating K5-mOVA graft acceptance, mice were euthanized at the indicated timepoints and transplant-draining lymph nodes collected for cytofluorimetric analysis. As deeply discussed in the introduction, the protagonists of tolerance are Tregs, specialized suppressors of aberrant responses and self-directed exaggerated reactions. Therefore, analyses were focused on $CD45.1^+$ $CD4^+$ $CD25^+$ $Foxp3^+$ lymphocytes, presumably expanded from the $CD4^+$ T cells derived from Dereg OT II $CD45.1^+$. As shown in Figure 14A, lymph nodes collected from transplanted animals that received MYTS-VIVIT NPs exhibit a higher proportion of

Tregs among $CD4^+$ T cells, at day 7 post-transplant, while at day 14 the increase in the Tregs compartment is even more appreciable, especially if compared to the small percentage of $CD45.1^+$ $CD4^+$ $CD25^+$ $Foxp3^+$ lymphocytes detected in female grafted with female K5-mOVA-derived skin and infused with $CD4^+$ OT II lymphocytes. Conversely, most of the $CD45.1^+$ activated T cells, thus expressing CD25, in these mice do not display Foxp3 expression (Figure 14B). Together, these data suggest that MYTS-VIVIT NPs do induce the expansion of graft-specific Tregs that confers protection upon mismatched transplantation.

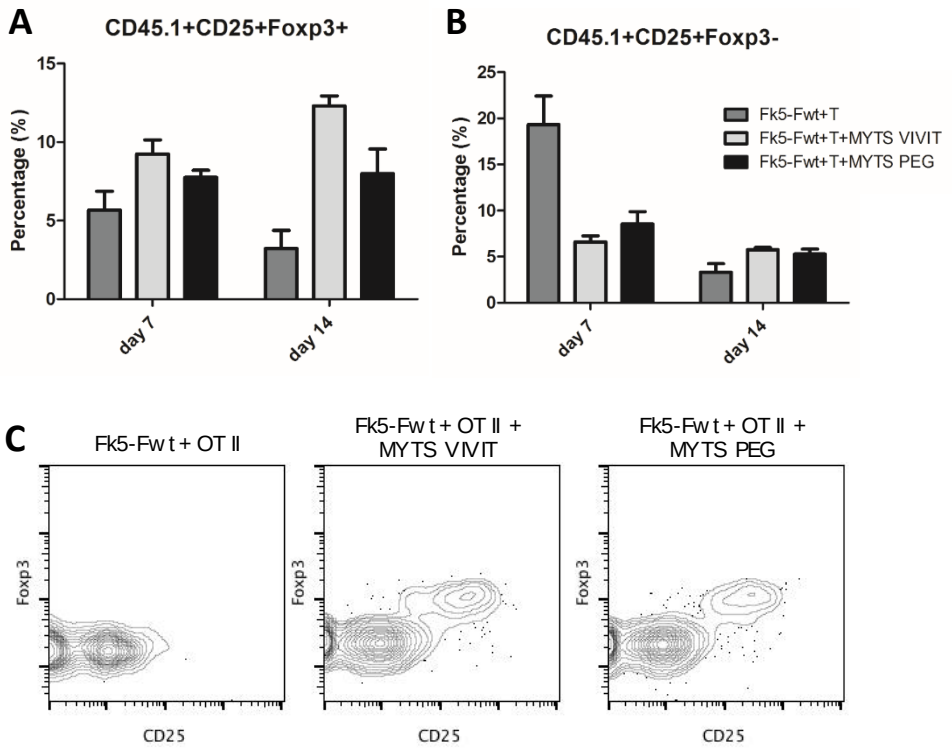

Figure 14: MYTS-VIVIT treatment induces the expansion of CD4⁺ CD25⁺ Foxp3⁺ Tregs

C57BL/6 WT female animals were transplanted with K5-mOVA-derived skin and infused with OT II cells. Some of the recipients received MYTS-VIVIT or MYTS-PEG NPs. At the indicated timepoint graft-draining lymph nodes were collected and analyzed by flow cytometry. A) displays the Foxp3⁺ population, compared to the Foxp3⁻ (B). C) shows a representative dot plot of flow cytometry.

2.13 Graft acceptance depends on Tregs expanded during temporal MYTS-VIVIT NPs treatment

Further, to evaluate the role of the expanded proportion of Tregs in MYTS-VIVIT NPs treated recipients, Dereg OT II mice were grafted with female K5-mOVA-derived skin. Some animals received MYTS-VIVIT NPs treatment until day 14 post-transplant and, subsequently, at day 21 both the groups (MYTS-VIVIT-treated and -untreated) received diphtheria toxin to deplete putative graft-specific expanded Tregs. As shown in Figure 15, 100% of Dereg OT II untreated animals reject the graft within 14 days post-transplant, while the MYTS-VIVIT NPs treated counterpart completely accept the graft. As soon as treated mice are administered diphtheria toxin, they rapidly reject the graft, reaching the 100% of rejection on day 30 post-transplant. These data clearly suggest that long-term graft tolerance is mediated by graft-specific Tregs, since their depletion results in rapid rejection.

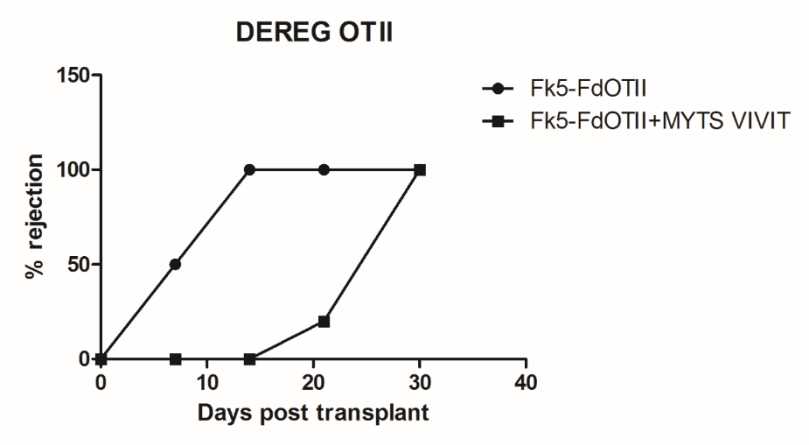

Figure 15: Graft acceptance depends on Tregs expansion during MYTS-VIVIT NPs treatment

Dereg OT II mice were transplanted with female K5-mOVA. Half of them was treated with MYTS-VIVIT NPs until day 14. On day 21 all the mice were administered with diphtheria toxin. Grafts were monitored until all the animals rejected.

2.14 A novel transgenic model to study NFATc TFs specifically in DCs

Since the expansion of Tregs is dependent on MYTS-VIVIT NPs treatment, which acts mainly on phagocytes, we have developed a transgenic conditional KO animal, in which all the calcium-dependent NFATc TFs are inhibited in DCs.

Rosa26 is a 9kb, 3 exons locus located on mouse chromosome 6, still orphan of a specific function. It is widely used for the generation of transgenic animals due to the high efficiency of its targeting. In addition, it drives expression of inserted transgenes among a wide range of cell types, since it is ubiquitous and constitutively expressed in a variety of cells, both of embryonic and adult origins. Furthermore, the insertion of transgenes in the Rosa26 locus has not resulted in any side effects on mouse health and cell viability. Therefore, as reported in Figure 15, we inserted our cassette in the Rosa26 locus, between exon 1 and 2. We inserted a construct that allows us to have stable expression of the VIVIT peptide and a reporter gene (Td tomato). The pCAG promoter has been inserted anti-sense and it is located between two loxP sequences (Figure 16). Therefore, until a Cre recombinase intervenes to flip the pCAG both the VIVIT peptide and the Td tomato are not transcribed and encoded. Thus, we bred these inducible KI animals with mice expressing the Cre under the promoter of CD11c, a lineage-specific marker for DCs. Hence, we will obtain KI animals expressing the NFATc specific inhibitor mainly in DCs and we could further explore the role of these TFs in the professional APCs, promoter of tolerance.

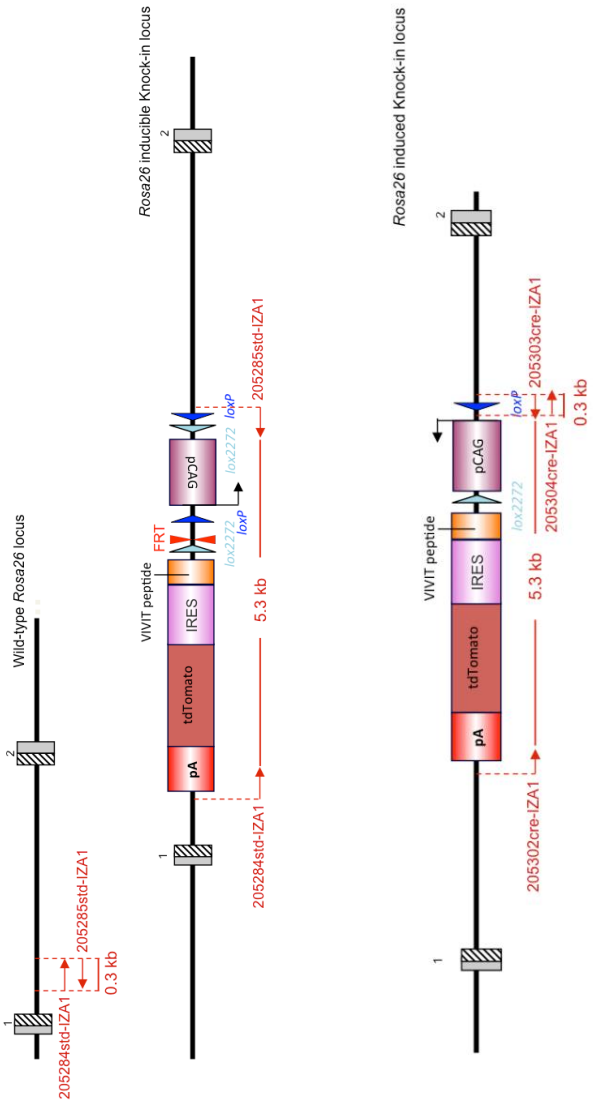

Figure 16: Novel transgenic KI mouse model to study NFATc in DCs

A) Representation of the inserted gene in the Rosa26 locus and inducible KI allele. B) After breeding with Cre-expressing animals, the allele is induced and VIVIT peptide transcribed as well as Td tomato reporter gene.

2.13 Materials and methods

BMDCs and D1 cell line. BMDCs were generated from bone marrow precursors of C57BL/6 WT, flushed from femurs, in Iscove's modified Dulbecco's medium (IMDM) (Euroclone) containing 10% heat-inactivated fetal bovine serum (Euroclone), 100 IU of penicillin, streptomycin (100 µg/ml), 2 mM L-glutamine (Euroclone), and granulocyte-macrophage colony-stimulating factor (GM-CSF) (10 to 20 ng/ml) for 8 days and D1 cells were cultured as previously described (Granucci et al. 2001).

Mice. All mice had been on a B6 background for at least 12 generations and used at 6 to 12 weeks of age. C57BL/6 WT CD45.2 and CD45.1 mice, K5-mOVA and OT II transgenic mice were purchased from Harlan-Italy. E. Serfling (Institute of Virology and Immunobiology, Wurzburg, Germany) provided NFATc2-/- mice. DEREG mice were obtained from T. Sparwasser (Twincore, Hannover, Germany). In DEREG mice a $Foxp3^+$ Tregs ablation can be induced by diphtheria toxin injection (Lahl et al. 2007). 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-kB were purchased from Invitrogen. Antibody against Lyve-1 and CCL21 were purchased from Abcam. TLR4-grade smooth LPS (E. coli, O55:B5) was purchased from Enzo Life Sciences. FK-506, thapsigargin, diphtheria toxin and SB-431542 were purchased from Sigma-Aldrich.

Nanoparticles synthesis. MYTS nanoparticles were kindly provided by D. Prosperi (NanoBioLab, University of Milano-Bicocca, Milano, Italy). In brief, MNPs were synthesized by solvothermal decomposition in octadecene from iron oleate precursors, as described previously (Lin et al. 2008). MNPs (10 mg) suspended in chloroform (5 mg/ml) 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). 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 hours. Next, nanoparticle dispersion was concentrated and washed twice with water. The resulting PMA-coated nanoparticles (PMNPs) were dispersible in aqueous media. For PMNPs suspension, PMNPs 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 (500 Da) 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 YV measurement.

In vitro and in vivo nanoparticles uptake. BMDCs were incubated with MYTS-FITC or MYTS-PEG at 37 °C or 4 °C for 10, 30, 60 and 90 minutes. Cells where then washed with PBS and flow cytometric analyses were performed. For the distribution and uptake of NPs in vivo, mice were injected i.p. for one or two weeks, every other day, with MYTS-FITC or MYTS-PEG NPs (100 µg/mouse). After euthanizing the animals, spleen, lymph nodes and skin were collected and analyzed by flow cytometry.

ELISA assays. Concentration of IL-2, TNF-a and IFN-γ in cell culture supernatants were assessed by ELISA kits purchased from R&D Systems, Invitrogen and eBiosciences, respectively.

In vivo treatment with NPs or FK-506. For in vivo administration, FK-506 was resuspended in 40% w/v HCO-

60/ethanol at the dose of 40 µg/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 the indicated timepoints.

Skin grafting. Section 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 monitored daily until rejection or the end of the experiment. Rejection was determined when the graft became erosive or scale-encrusted.

Foxp3$^+$ Tregs depletion. Diphtheria toxin (40ng/g) was daily administered to Dereg mice through an i.v. injection for 2 consecutive days. Control mice were administered with PBS. Effective Foxp3$^+$ Tregs or DCs depletion was assessed by flow cytometric analyses.

Immunofluorescent staining. 100.000 BMDCs were plated on glass coverslips. Cells were then incubated with MYTS-VIVIT, MYTS-PEG NPs (25 µg/ml) or FK-506 (10 ng/ml) for 90 minutes and then stimulated with LPS (1 µg/ml) plus thapsigargin (50 nM) for 40 minutes. After the stimulation, BMDCs were fixed in paraformaldehyde 4% and permeabilized with 0,2% BSA 0,1%

TritonX-100 in PBS. Successively, cells were kept in blocking solution (BSA 2% in PBS) for 30 minutes. Rabbit anti-mouse NFATc2 or rabbit anti-mouse NF-κB were used. Both primary and secondary antibodies (anti-rabbit AlexaFluor 488 or 555) were diluted in blocking solution and incubated RT. For ex vivo samples, cryostat sections were fixed at room temperature for 15 seconds, air dried, and permeabilized with methanol for 3 minutes. Blocking solution (PBS-BSA 0,1%) was added for 10 minutes. Rabbit anti-mouse NFATc2 was diluted in blocking solution and incubated over/night at 4 °C. Anti-rabbit AlexaFluor 555 diluted in blocking solution was added and incubated RT for 1 hour. All 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.

Whole mounting. Skin transplant were fixed with paraformaldehyde 4% for 2 hours at RT. Blocking solution (PBS BSA 12% + Fc block diluted 1:1.000) was added for 2 hours. Antibody rabbit anti-mouse Lyve-1 and goat anti-mouse CCL21 were added in blocking solution and incubated over/night at 4 °C. Secondary antibodies, anti-rabbit AlexaFluor 555 and anti-goat AlexaFluor 488, were diluted in PBS BSA 12% and incubated 4 hours at 4 °C. DRAQ5 was then added for 20 minutes

RT and finally samples were fixed with paraformaldehyde 4% for 10 minutes RT. Mounting was performed with glycerol and samples acquired at confocal microscopy.

Quantitative real-time PCR ex vivo. Grafted skins were collected, gently washed in cold PBS, lysed in TRIzol solution and mechanically disrupted using a TissueLyser (20 shakes/second for 10 minutes). Total mRNA was then extracted via a RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions. A nanodrop spectrophotometer (Thermo Scientific) was used to quantify mRNA and to assess its purity. 700 ng of mRNA were retrotranscribed to cDNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystem). Then, 10 ng of cDNA were amplified using the TaqMan Gene expression Master Mix (Applied Biosystems) with the TaqMan probe (Ifng, Mm01168134_m1; Gapdh, Mm99999915_g1) in a 7500 Fast Real-Time PCR System (Applied Biosystems), and finally relative mRNA expression was calculated using the ΔC_t method, with *Gapdh* as a reference gene.

Flow cytometry. Single-cell suspensions of spleen, lymph nodes and skin were centrifuged and resuspended with the appropriate amount of antibody in 200 ul of PBS and incubated for 20 minutes on ice in the dark. Cells were washed with 1 ml of

PBS. Intracellular staining for cytokines or transcription factor were performed following manufacturer's instructions. For FACS analyses, the following anti-mouse antibodies were used: anti-CD11c (APC, Pacific Blue), anti-CD86 (Pacific blue), anti-CCR7 (PE), anti-CD11b (FITC), anti-CD3 (FITC), anti-CD4 (APC-Cy7), anti-CD8 (PE), anti-CD45.1 (PE, APC-Cy7), anti-CD45.2 (PE-Cy7), anti-CD25 (PE), anti-Foxp3 (FITC), anti-IFN-γ (APC). Data were acquired using a Beckman-Coulter FACS Gallios and analyzed with FlowJo (TreeStar) software.

Statistical analysis. Means were compared by paired t test. Data are expressed and plotted as mean ± SD. Sample sizes for each experimental setting are provided in the figures and the respective legends.

Reference to Chapter 2

Ali, Jason M, Eleanor M Bolton, J Andrew Bradley, and Gavin J Pettigrew. 2013. "Allorecognition Pathways in Transplant Rejection and Tolerance." *Transplantation* 96(8): 681–88.

Aramburu, J et al. 1998. "Selective Inhibition of NFAT Activation by a Peptide Spanning the Calcineurin Targeting Site of NFAT." *Molecular Cell* 1(5): 627–37.

Aramburu, J et al. 1999. "Affinity-Driven Peptide Selection of an NFAT Inhibitor More Selective than Cyclosporin A." *Science* 285(5436): 2129–33.

Bhat, Purnima, Graham Leggatt, Nigel Waterhouse, and Ian H Frazer. 2017. "Interferon-γ Derived from Cytotoxic Lymphocytes Directly Enhances Their Motility and Cytotoxicity." *Cell Death and Disease* 8: 1–11. http://dx.doi.org/10.1038/cddis.2017.67.

Felix, Nathan J, and Paul M Allen. 2007. "Specificity of T-cell Alloreactivity." *Nature Reviews Immunology* 7: 942–53.

Fernandes, Eduardo et al. 2011. "The Role of Direct Presentation by Donor Dendritic Cells in Rejection of Minor Histocompatibility Antigen-Mismatched Skin and Hematopoietic Cell Grafts." *Transplantation* 91(2): 154–60.

Granucci, F et al. 2001. "Inducible IL-2 Production by Dendritic Cells Revealed by Global Gene Expression Analysis." *Nature Immunology* 2(9): 882–88. papers2://publication/doi/10.1038/ni0901-882.

Issekutz, T B, Jeanette M Stoltz, and P Van der Meide. 1988. "The Recruitment of Lymphocytes into the Skin by T Cell Lymphokines: The Role of γ-Interferon." *Clinical and Experimental Immunology* 73: 70–75.

Kaplan, By Gilla, Andrew D Luster, Gerald Hancock, and Zanvil A Cohn. 1987. "The Expression of a γ-Interferon-Induced Protein (IP-10) in Delayed Immune Rsponses in Human Skin." *The Journal of Experimental Medicine* 166(4): 1098–1108.

Khameneh, Hanif Javanmard et al. 2017. "The Syk–NFAT–IL-2 Pathway in Dendritic Cells Is Required for Optimal Sterile Immunity Elicited by Alum Adjuvants." *The Journal of Immunology* 198(1): 196–204. http://www.jimmunol.org/lookup/doi/10.4049/jimmunol.1600420.

Kim, Taeg S. et al. 2014. "Distinct Dendritic Cell Subsets Dictate the

Fate Decision between Effector and Memory CD8+T Cell Differentiation by a CD24-Dependent Mechanism." *Immunity* 40(3): 400–413. http://dx.doi.org/10.1016/j.immuni.2014.02.004.

Kosten, I.J. et al. 2017. "Comparative Phenotypic and Functional Analysis of Migratory Dendritic Cell Subsets from Human Oral Mucosa and Skin." *PLoS ONE* 12(7): 1–15.

Kwun, Jean, Subramaniam Malarkannan, J William, and Stuart J Knechtle. 2011. "Primary Vascularization of the Graft Determines the Immunodominance of Murine Minor H Antigens during Organ Transplantation." *The Journal of Immunology* 187: 3997–4006.

Lahl, Katharina et al. 2007. "Selective Depletion of Foxp3+ Regulatory T Cells Induces a Scurfy-like Disease." *The Journal of Experimental Medicine* 204(1): 57–63.

Lin, C J et al. 2008. "Design of an Amphiphilic Polymer for Nanoparticle Coating and Functionalization." *Small* 4(3): 334–41.

Schwartz, Ronald H. 2003. "T Cell Anergy." *Annual Review of Immunology* 21: 305–34.

Slavcev, Antonij et al. 2015. "Pre-Transplant Donor-Specific Interferon-Gamma-Producing Cells and Acute Rejection of the Kidney Allograft." *Transplant Immunology* 33(2): 63–68. http://dx.doi.org/10.1016/j.trim.2015.07.007.

Winzler, By Claudia et al. 1997. "Maturation Stages of Mouse Dendritic Cells in Growth Factor–dependent Long-Term Cultures." *The Journal of Experimental Medicine* 185(2): 317–28.

Wuest, Simone C. et al. 2011. "A Role for Interleukin-2 Trans-Presentation in Dendritic Cell-Mediated T Cell Activation in Humans, as Revealed by Daclizumab Therapy." *Nature Medicine* 17(5): 604–9. http://dx.doi.org/10.1038/nm.2365.

Zanoni, Ivan et al. 2009. "CD14 Regulates the Dendritic Cell Life Cycle after LPS Exposure through NFAT Activation." *Nature* 460(7252): 264–68. http://dx.doi.org/10.1038/nature08118.

Zanoni, Ivan et al. 2012. "CD14 and NFAT Mediate Lipopolysaccharide- Induced Skin Edema Formation in Mice." *The Journal of Clinical Investigation* 122(5): 1747–57.

Zanoni, Ivan, and Francesca Granucci. 2012. "Regulation and Dysregulation of Innate Immunity by NFAT Signaling Downstream of Pattern Recognition Receptors (PRRs)." *European Journal of Immunology* 42(8): 1924–31.

CHAPTER 3. FINAL CONSIDERATIONS

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3.1 Summary

The Nuclear Factor of Activated T cells (NFAT) is a TF that initially had been discovered in T lymphocytes (Shaw et al. 1988). It emerged as located on the promoter of IL-2, the cytokine that promotes and sustains T cell clonal expansion, proliferation and survival par excellence. In the last 20 years the solid paradigm of NFAT being egoistically solely associated to the adaptive immunity has been confuted (Goodridge, Simmons, and Underhill 2007; LeibundGut-Landmann et al. 2007). Indeed, studies that elucidate the role of NFATc in innate immunity are increasing (Bendickova, Tidu, and Fric 2017; Zanoni and Granucci 2012). NFATc appearance, in evolutionary terms, corresponds to the development of vertebrates and thus of adaptive immunity (Boehm 2012). Intriguingly, NFAT develops in innate immunity only later, putatively suggesting the necessity to increase the level of complexity of the innate arm to properly sustain the newly originated adaptive branch. Therefore, the functional effects of NFATc activation in the distinct subsets of innate immunity may provide insights on the regulation and orchestration of immune responses in the more disparate contexts (Santus et al. 2017; Zanoni and Granucci 2012).

An additional though fundamental cue was provided when DCs were found to be capable of producing IL-2 (Granucci et al. 2001). DCs are the primary professional APCs, thus their

functionalities aim at promoting elicitation of cognate T cell clones. Hence, the release of IL-2 by DCs complement their intrinsic capability in sustaining adaptive immunity. Moreover, it emerged that DC-derived IL-2 is fundamental for the priming of T cells, therefore NFATc activation in DCs may represent a sort of check point to prevent exaggerated or self-directed responses. Indeed, Zanoni and colleagues reported that NFATc is triggered downstream LPS-mediated CD14 engagement not only to produce IL-2, but also to induce DC apoptosis via Nur77 to avoid overt immune elicitations (Zanoni et al. 2009). In addition, LPS stimulated DCs activate NFATc pathway to up-regulate mPges-1 and release PGE_2, a key inflammatory factor that promotes edema formation (Zanoni et al. 2012). Therefore, NFATc role in DCs mirrors their bridging effector functions: on the one hand, it sustains early events, as local inflammation, antigens and DCs migration through lymphatic vessels and NK activation via early IL-2 production; on the other hand, it guarantees a proper and regulated T cell trigger, via the late production of IL-2 and the induction of DCs apoptosis once they have exploited their functions. Furthermore, in addition to pathogenic contexts, it is emerging how NFATc pathway activation in DCs occurs even in sterile inflammation, as reported by Khameneh (Khameneh et al. 2017), candidating NFATc as a potential novel target to improve T cells responses in vaccination strategy.

Therefore, it appears clear that NFATc TFs act on different levels and in distinct contexts, dependently on the stimulus that activates the pathway, either exogenous and endogenous. Among the diverse settings in which NFATc has been studied, mismatched transplantation is one of the most unexplored, probably because of the complex network of events that concur in the process, from the grafting to the rejection (Ali et al. 2013). The surgery itself generates tissue damage with the dispersal of several DAMPs that have been reported to signal via PRRs engagement on DCs, mimicking pathogenic circumstances (Matzinger 1994; Miyake 2007; Yang et al. 2010). In addition, NFATc TF has proven to be necessary for the development of edema, which is a crucial event even for DC migration to skin-draining lymph nodes (Legler Krause Singer Prostaglandin 2006; Kabashima Sakata Prostaglandin E2-EP4 2003). Indeed, NFATc activation in DCs may be the crucial event that sustains alloreactive T cells proliferation and further rejection of the graft. Hence, we questioned whether NFATc TF might be involved in the process of transplantation, from the early events of local damage and inflammation to the later phases that contribute to the instauration of alloresponses.

3.2 Discussion and future perspectives

The NFAT family encompasses 5 members: NFATc1, c2, c3 and c4 dependent on calcium and Cn signaling, and NFAT5 triggered by osmotic stress (Rao, Luo, and Hogan 1997). Distinct immune subsets express different levels or even diverse members of the family, hence mice deficient of one of the members exhibit disparate phenotypes. Among the distinct members, NFATc2 is the most expressed in DCs, hence we focused out attention on this member (Santus et al. 2017; Zanoni et al. 2009, 2012). Initially, we set up an experimental model of minor histocompatibility antigen-based mismatched (miHAg), exploiting inbred animals of distinct gender. Indeed, male-derived skin grafted onto the dorsum of female recipients, though syngeneic, results in acute rejection mediated by the instauration of alloreactive T cells against male-specific HY antigens. Conversely, female-derived skin transplanted onto female recipients leads to acceptance of the graft. Therefore, these two experimental groups were considered the control of rejection and the control of acceptance, respectively. Since our aim was to elucidate the role of NFATc TFs in innate immunity in the context of transplantation, we firstly evaluate the graft of C57BL/6 NFATc2 KO male-derived skin into C57BL/6 WT female recipients and compared the outcome to the controls of rejection and acceptance already described. Intriguingly, NFATc2 KO male-derived skin graft resulted in delayed

rejection. Indeed, all the recipient animals rejected by the day 50, though displaying a slowed kinetic. Therefore, NFATc2 does play a role in mediating rejection, since its absence solely in the transplant protects from fast acute rejection.

Since alloreactive responses are mediated by the elicitation recipient's T lymphocytes that are triggered by the recognition of donor's antigens, by grafting NFATc2 KO male-derived skin, recipient's adaptive immunity is capable of mounting an alloreactive response. Thus, the delayed kinetic of rejection observed may be due to an alteration of the early events, predominantly driven by innate immunity. Furthermore, despite the three distinct pathways of activation of alloreactivity, donor DCs stand out as the primary APCs involved in eliciting recipient T cells. Then we assessed the status of DCs in the skin of WT versus NFATc2 KO animals, and did not observe any difference in their number, thus suggesting an alteration in their functional capabilities. Indeed, in MLR with Balb/c-derived $CD4^+$ T cells, NFATc2 KO DCs affect the production of IFN-γ by T lymphocytes. Therefore, even though the exact ligands/receptors involved in the process of transplantation are currently missing, it leads to the activation of the NFATc pathway in DCs providing the exact weapons to induce alloreactivity: maturation and migration to the draining lymph nodes to provide antigen presentation. Indeed, consistent with previous works, NFATc acts both on the local inflammatory

environment and on the innate cells protagonists of such scenario (Zanoni et al. 2009, 2012). Intriguingly the temporal abrogation of all the members of the NFATc family, selectively in phagocytes, completely abolishes the alloreactive response and promote acceptance. Indeed, NFATc activation occurs early after transplantation and probably defines the later phases of the whole process, since the blockade of these TFs in phagocytes for a relatively short time window leads to the acceptance of the graft, even when the treatment is interrupted. When grafting male NFATc2 KO-derived skin we could not observe the complete acceptance of the transplants both because skin graft cells could express other NFATc members and because we could not exclude the contribution of the recipients' DCs. Conversely, when abrogating all the members, we assist to the expansion of graft-specific regulatory T cells. Therefore, it appears clear that the temporal inhibition of NFATc pathway in innate immunity promote the instauration of long-term tolerance by modifying the functional properties of DCs.

Presumably, NFATc abrogation upon stimulation of the pathway in DCs induces a sort of tolerogenic profile that skew the response towards tolerance (Imai et al. 2007; Lee et al. 2005; Ren et al. 2014; Szabo, Gavala, and Mandrekar 2001). Indeed, recipients' T lymphocytes are unaffected by the NPs treatment, hence they are capable of activating and skewing their response, dependently on DCs status. Since we could observe the

expansion of Tregs in our setting, grafted patients treated with CNIs do not exhibit similar responses. Actually, the administration of FK-506 or CsA completely paralyzes the immune system, both the innate and the adaptive branches. Thus, while the inhibition of NFATc in innate immunity via CNIs could be beneficial, it could not generate tolerance because these drugs do not select their target cells. Hence, MYTS-NPs constitute a novel tool not only to specifically investigate the role of the NFATc family in innate immunity in pre-clinical models, but also, they would allow naïve T cells to be converted into Tregs, shaping the scenario of the long-life administration of CNIs in transplantation.

The role of NFATc in innate immunity as promoter of T cells responses is already validated by several works that elucidate its potential even as an adjuvant in vaccination strategies (Gornati, Zanoni, and Granucci 2018; Khameneh et al. 2017; Wuest et al. 2011). Thus, the abrogation of NFATc selectively in phagocytes acquires interest in contexts in which the adaptive responses should be dampened or, as we demonstrated, skewed towards tolerance. As already mentioned, NFATc seems to be implicated not only in T cell elicitation, in terms of Th or CTLs polarization, but also in the transcriptional program of anergy and tolerance. Therefore, it appears clear that the administration of CNIs would definitely impede also the expansion of Tregs whenever required. The

introduction of MYTS-VIVIT NPs as a novel therapeutic tool for grafted patients would possibly improve transplants outcome, reduce the severe side effects caused by long-life CNIs treatment and, once the graft-specific Tregs have expanded, the treatment could be interrupted.

To conclude, the novel transgenic KI mouse model that we are currently generating will provide insights on the role of NFATc in the professional APCs. Indeed, further investigations are required to deepen the precise role of NFATc TFs in innate immunity, for instance in the transplantation setting, aiming at identifying the ligands and receptors that provoke the downstream effects in the recipients, resulting in allorejection and provide novel therapeutic approaches for grafted patients.

Reference to Chapter 3

Ali, Jason M, Eleanor M Bolton, J Andrew Bradley, and Gavin J Pettigrew. 2013. "Allorecognition Pathways in Transplant Rejection and Tolerance." *Transplantation* 96(8): 681–88.

Bendickova, Kamila, Federico Tidu, and Jan Fric. 2017. "Calcineurin – NFAT Signalling in Myeloid Leucocytes : New Prospects and Pitfalls in Immunosuppressive Therapy." : 1–10.

Boehm, Thomas. 2012. "Evolution of Vertebrate Immunity." *Current Biology* 22(17): R722–32. http://dx.doi.org/10.1016/j.cub.2012.07.003.

Goodridge, H. S., R. M. Simmons, and D. M. Underhill. 2007. "Dectin-1 Stimulation by Candida Albicans Yeast or Zymosan Triggers NFAT Activation in Macrophages and Dendritic Cells." *The Journal of Immunology* 178(5): 3107–15. http://www.jimmunol.org/cgi/doi/10.4049/jimmunol.178.5.3107.

Gornati, Laura, Ivan Zanoni, and Francesca Granucci. 2018. "Dendritic Cells in the Cross Hair for the Generation of Tailored Vaccines." *Frontiers in Immunology* 9: 1–14.

Granucci, F et al. 2001. "Inducible IL-2 Production by Dendritic Cells Revealed by Global Gene Expression Analysis." *Nature Immunology* 2(9): 882–88. papers2://publication/doi/10.1038/ni0901-882.

Imai, Akihito et al. 2007. "Inhibition of Endogenous MHC Class II-Restricted Antigen Presentation by Tacrolimus (FK506) via FKBP51." *European Journal of Immunology* 37: 1730–38.

Khameneh, Hanif Javanmard et al. 2017. "The Syk–NFAT–IL-2 Pathway in Dendritic Cells Is Required for Optimal Sterile Immunity Elicited by Alum Adjuvants." *The Journal of Immunology* 198(1): 196–204. http://www.jimmunol.org/lookup/doi/10.4049/jimmunol.1600420.

Lee, Young-ran et al. 2005. "Cyclosporin A and Tacrolimus, but Not Rapamycin, Inhibit MHC-Restricted Antigen Presentation Pathways in Dendritic Cells." *Bloo* 105: 3951–55.

LeibundGut-Landmann, S et al. 2007. "Syk- and CARD9-Dependent Coupling of Innate Immunity to the Induction of T Helper Cells That Produce Interleukin 17." *Nature Immunology* 8(6): 630–38.

Matzinger, Polly. 1994. "Tolerance, Danger and the Extended Family."

Annual Review of Immunology 12: 991–1045.

Miyake, Kensuke. 2007. "Innate Immune Sensing of Pathogens and Danger Signals by Cell Surface Toll-like Receptors." *Seminars* 19: 3–10.

Rao, Anjana, Chun Luo, and Patrick G Hogan. 1997. "Transcription Factors of the NFAT Family: Regulation and Function." *Annual Review of Immunology* 15: 707–47.

Ren, Yana et al. 2014. "Tolerogenic Dendritic Cells Modified by Tacrolimus Suppress CD4+ T-Cell Proliferation and Inhibit Collagen-Induced Arthritis in Mice." *International Immunopharmacology* 21(1): 247–54. http://dx.doi.org/10.1016/j.intimp.2014.05.004.

Santus, William et al. 2017. "Skin Infections Are Eliminated by Cooperation of the Fibrinolytic and Innate Immune Systems." *Science Immunology* 2(15): eaan2725. http://immunology.sciencemag.org/lookup/doi/10.1126/sciimmunol.aan2725.

Shaw, Jeng-pyng et al. 1988. "Identification of a Putative Regulator of Early T Cell Activation Genes Actity." *Science* 241(11): 202–5.

Szabo, Gyongyi, Cristina Gavala, and Pranoti Mandrekar. 2001. "Tacrolimus and Cyclosporine A Inhibit Allostimulatory Capacity and Cytokine Production of Human Myeloid Dendritic Cells." *Journal of Investigative Medicine* 49(5): 442–50.

Wuest, Simone C. et al. 2011. "A Role for Interleukin-2 Trans-Presentation in Dendritic Cell-Mediated T Cell Activation in Humans, as Revealed by Daclizumab Therapy." *Nature Medicine* 17(5): 604–9. http://dx.doi.org/10.1038/nm.2365.

Yang, Huan et al. 2010. "A Critical Cysteine Is Required for HMGB1 Binding to Toll-like Receptor 4 and Activation of Macrophage Cytokine Release." *PNAS* 107(26): 11942–47.

Zanoni, Ivan et al. 2009. "CD14 Regulates the Dendritic Cell Life Cycle after LPS Exposure through NFAT Activation." *Nature* 460(7252): 264–68. http://dx.doi.org/10.1038/nature08118.

Zanoni, Ivan et al. 2012. "CD14 and NFAT Mediate Lipopolysaccharide- Induced Skin Edema Formation in Mice." *The Journal of Clinical Investigation* 122(5): 1747–57.

Zanoni, Ivan, and Francesca Granucci. 2012. "Regulation and Dysregulation of Innate Immunity by NFAT Signaling Downstream of Pattern Recognition Receptors (PRRs)." *European Journal of Immunology* 42(8): 1924–31.