Tolerogenic dendritic cell-based immunotherapy in Crohn's disease

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Thesis to obtain the PhD degree in Biomedicine

of the Universitat de Barcelona (UB)

2015

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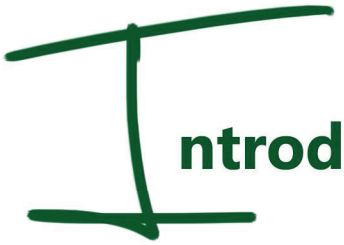
Abbreviations

Abbreviations

AC	Apoptotic cell
Ag	Antigen
AMPs	Antimicrobial peptides
APC	Antigen presenting cell
ASCA	Anti-*Saccharomyces cerevisiae* antibodies
ATG16L1	Autophagy-related protein 16-1
BDCA	Blood-derived dendritic cell antigen
CA	Carbonic anhydrase
CCL	Chemokine (C-C motif) ligand
CCR	Chemokine receptor
cDNA	Complementary DNA
CLEC	C-type lectin domain
CLR	C-type lectin receptor
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
DC	Dendritic cell
DCIR	Dendritic cell immunoreceptor
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
DEC-205	Dendritic and epithelial cells, 205 kDa protein
Dex	Dexamethasone
DNA	Deoxyribonucleic acid
DNGR	Dendritic cell NK lectin group receptor
E.Coli	*Escherichia coli*
e.g.	**"**For example"
EGF	Epidermal growth factor
ER	Endoplasmic reticulum
ERK	Extracellular-signal-regulated kinase
FasL	Fas ligand
FNIII	Fibronectin type III
Foxp3	Forkhead box p3
GATA	Trans-acting T-cell-specific transcription factor
GAS6	Growth arrest specific 6
GC	Glucocorticoids
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GMP	Good manufacturing practice
GWAS	Genome-wide association study
HBD	Human beta-defensins
HD	Human alpha-defensins
HSCT	Hematopoietic stem cells transplantation
IBD	Inflammatory bowel disease
ICAM	Intercellular adhesion molecule
iDC	Immature dendritic cell
IDO	Indoleamine 2,3-dioxygenase
IFNAR	Type I interferon receptor
IFNs	Interferons
IFN-γ	Interferon gamma
IL	Interleukin

ILC	Innate lymphoid cell
IL-12B	Interleukin 12 subunit beta
IL-6R	Interleukin 6 receptor
IL-23R	Interleukin 23 receptor
IRGM	Immunity-related GTPase family M protein
JAK	Janus kinase
LFA-1	Lymphocyte function-associated antigen 1
LG	Laminin G domain
LPS	Lipopolysaccharides
LXR	Liver X receptor
mDC	Mature dendritic cell
Mer-Fc	Recombinant human MERTK-Fc
MHC	Major histocompatibility complex
MIICs	MHCII compartments
MLR	Mixed lymphocyte reaction
MMP	Matrix metalloproteinase
moDC	Monocyte-derived dendritic cell
MPA	Mycophenolic acid
mRNA	Messenger ribonucleic acid
MS	Multiple sclerosis
MSC	Mesenchymal stem cell
MUC	Mucin
myDC	Myeloid dendritic cell
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B-cells
NK cell	Natural killer cell
NLR	NOD-like receptor
NOD2	Nucleotide-binding oligomerization domain containing protein 2
NSAIDs	Non-steroidal anti-inflammatory drugs
OmPC	*E.Coli* Outer membrane porine-C
PAMPs	Pathogen associated molecular patterns
pANCA	Perinuclear anti-neutrophil cytoplasmic
PBMCs	Peripheral blood mononuclear cells
pDC	Plasmacytoid dendritic cell
PD-1	Programmed cell death protein 1
PDL-1	Programmed death-ligand 1
PGE_2	Prostaglandin E_2
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PROS1	Protein S
PRR	Pattern recognition receptor
PS	Phosphatidylserine
QC	Quality control
RA	Rheumatoid arthritis
RIG-like	Retinoic acid-inducible genes
RORγt	RAR-related orphan receptor gamma t
RTK	Receptor tyrosine kinase
SHBG	Sex hormone binding globulin-like domain
SLE	Systemic lupus erythematosus
SMAD7	Mothers against decapentaplegic homolog 7
SNP	Single nucleotide polymorphism

SOCS	Suppressor of cytokine signaling
STAT	Signal transducer and activator of transcription
TAA	Tumor associated antigens
TAM	Tyro3, Axl, Mer
T-bet	T-box transcription factor TBX21
TGF-β	Transforming growth factor beta
Th cell	T helper cell
TKO	Triple knock-out
TLRs	Toll-like receptors
TM	*Trans*-membrane
TNBS	Trinitrobenzenesulfonic acid
TNF-α	Tumor necrosis factor alpha
TNFR2	Tumor necrosis factor receptor 2
Tol-DC	Tolerogenic dendritic cell
TRAF	TNF receptor associated factor
TRAIL	TNF-related apoptosis-inducing ligand
T reg	Regulatory T lymphocytes
Tr1	Type 1 regulatory T-cells
TSR	Thrombin sensitive region
UPR	Unfolded protein response
VIP	Vasoactive intestinal peptide
XBP1	X-box binding protein 1



Introduction

Introduction

Chapter I | Crohn's Disease

Crohn's disease is a lifelong and chronic relapsing inflammatory bowel disease (IBD) that can affect any portion of the gastrointestinal tract from the mouth to the anus. Crohn's disease is characterized by a transmural and discontinuous inflammation that most commonly affects the ileum and colon, leading to a stricturing or even fistulising phenotype in up to 80% of patients. It causes a wide variety of symptoms including abdominal pain, diarrhea, weight loss, anorexia, growth impairment, delayed sexual maturation and fever, resulting in a marked deterioration in the quality of life. Individuals with this condition often experience periods of symptomatic relapse and remission, and after 10 years of diagnosis, more than 40 % of the patients must undergo at least one surgical procedure [1].

Epidemiology

Industrialized countries, such as Western Europe and North America have a higher Crohn's disease incidence than other countries. The highest annual incidence of Crohn's disease has been determined at 12.7 per 100.000 person-years in Europe, and 20.2 per 100.000 person-years in North America [2]. In both cases, the highest prevalence is around 20 times the incidence rate.

The age of onset of Crohn's disease has a bimodal distribution. The first peak occurs between the second and third decade, and the second peak strikes individuals after age 50. But disease onset can occur at any age to all ethnic groups, races and genders.

Etiology

While the underlying etiology of IBD remains unknown, several immunological, genetic and environmental factors may contribute to the disease. It has been proposed that these disorders can result from an over-reactive mucosal immune

response to the gut flora in genetically predisposed individuals [3]. A growing body of evidence suggests that a deregulation in both innate and adaptive immune responses contributes to the aberrant intestinal inflammatory response.

Phenotypes

Crohn's disease can be categorized by the age of onset, the specific region of the gastrointestinal tract affected (namely ileocolitis, ileitis, gastroduodenal Crohn's disease, jejunoileitis and Crohn's colitis) and by the behavior of the disease, which can be purely inflammatory, or develop complications including strictures and penetrating lesions (fistulas and abscess). In addition, presence of perianal disease is considered as a modifying factor in the phenotypic classification systems both in adults [4] and children [5].

Various organs and systems can be affected as extra-intestinal manifestations of the disease, for example, joints, skin, liver, eye and blood coagulation.

Causes

Innate immunity

Immune cells of the innate system, such as dendritic cells (DCs) and macrophages, but also intestinal epithelial cells and myofibroblasts, can sense the intestinal microbiota and initiate rapid and effective inflammatory responses against microbial invasion. This recognition is mediated by receptors that sense conserved structural motifs on microorganisms, known as pathogen-associated molecular patterns (PAMPs). Defective microbial sensing, intestinal barrier disturbances and defects in the process of autophagy are thought to play a crucial role in the pathogenesis of IBD. Both genetic and environmental factors can induce impaired barrier function in the intestinal mucosa facilitating the translocation of microbial products from the gut lumen into the bowel wall. Altogether it triggers the activation of immune cells and cytokine production; however, if acute mucosal inflammation cannot be resolved by anti-inflammatory mechanisms, chronic intestinal inflammation develops (**Figure 1**).

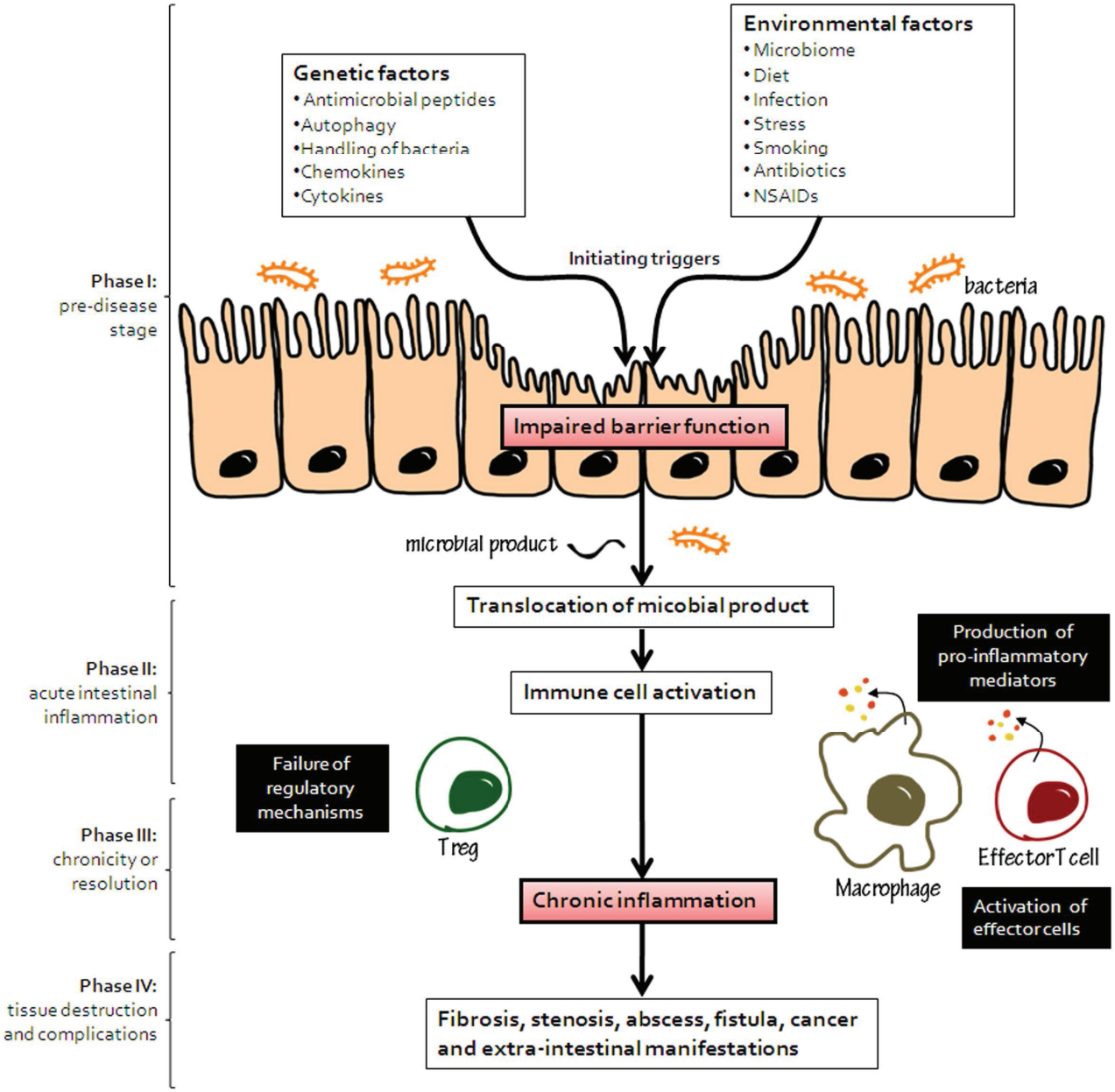


Figure 1. Conceptual framework for the pathogenesis of IBD. NSAIDs: non-steroidal anti-inflammatory drugs; T-reg: regulatory T-cell. *Adapted from [6], Nature Reviews Immunology 2014.*

Intestinal barrier

The **mucus** layer covers the intestinal epithelium and is the first physical barrier that intestinal bacteria and food antigens encounter. It is organized by

polymerization of gel-forming mucins, glycoproteins secreted by specialized epithelial cells, named Goblet cells. When mucosal barrier function is disrupted, permeability is compromised; therefore the risk of bacterial break-through and intestinal inflammation is higher. Indeed, a study showed that *MUC1* mRNA expression in humans was reduced in the inflamed ileum of Crohn's disease patients compared to controls [7]. Several studies have been performed using *MUC2*$^{-/-}$ mice, which develops spontaneous colitis, in order to better understand the involvement of these glycoproteins in IBD pathogenesis.

The second line of defense against bacterial invasion is the **intestinal epithelium**, which provides a selective permeable barrier. Enterocytes and specialized epithelial cells (such as Goblet cells and Paneth cells) maintain the integrity of the epithelial barrier by tight junctions, adherens junctions and desmosomes. Defective epithelial barrier and increased intestinal permeability have long been observed in patients with Crohn's disease [8]. Besides its structural function, epithelial cells can also secrete **antimicrobial peptides** (AMPs) including α- and β-defensins, which exhibit a broad spectrum of bactericidal activity. It is known that, patients with Crohn's disease have a reduced induction of β-defensins HBD2, HBD3 and HBD4 in inflamed colon [9]. Additionally, patients with ileal Crohn's disease present decreased expression of the Paneth cell-derived α-defensins (HD5 and HD6) [10].

Bacterial encounter

The accurate **sensing** of microbial antigens (Ags) is crucial for the initiation of an effective innate response against pathogens in the gut. DCs, macrophages, epithelial cells and myofibroblasts recognize bacterial PAMPs through pattern recognition receptors (PRRs) and are responsible of the induction of adaptive immunity. NLRs are intracytoplasmic PPRs, also known as NOD-like receptors, that recognize components of bacterial peptidoglycan. In 2001, *NOD2* was the first risk gene to be identified to confer increased susceptibility to develop Crohn's disease [11, 12]. Mutations in this receptor directly influence NF-κB activation signaling pathway. The consequences of the impaired function of *NOD2* remain still unclear.

Efficient recycling of cellular components and degradation of invading bacteria requires a highly conserved cellular process known as **autophagy**. A defect in this process has been linked to the pathogenesis of Crohn's disease as mutations in *ATG16L1* and *IRGM* genes have been described in patients. Remarkably, some studies have related NOD2 with autophagy induction and have demonstrated the existence of a mechanistic link between two of the most important Crohn's disease susceptibility genes, *NOD2* and *ATG16L1* [13].

Closely related to autophagy and innate immunity, unresolved ER-stress and the **unfolded protein response** (UPR) have been involved in IBD pathogenesis. In particular, genetic variants of *XBP1* gene, which is involved in UPR, have been associated with IBD [14]. Interestingly, alterations in *NOD2*, *ATG16L1* and *XBP1* activities have been all linked to Paneth cell dysfunction. This association may represent a convergent pathogenic pathway affecting antimicrobial responses [15].

Innate Immune Cells

Neutrophil-mediated clearance of mucosal microbes prevents activation and recruitment of macrophages. Due to their involvement in acute inflammation, **neutrophils** have been considered by some investigators as possibly central to the pathophysiology of Crohn's disease during the last years. Several neutrophil-associated defects have been described in patients with Crohn's disease, including impairment in migration to the site of inflammation and decreased phagocytic and bactericidal function [16].

Recent data has implicated **innate lymphoid cells** (ILCs) in the development of IBD. From a lymphocytic origin, ILCs belong to a diverse group of cells that comprise a new family of hematopoietic effector cells that include NK cells. Although a young field, Geremia *et al.* have demonstrated that ILCs are increased in the inflamed intestine of patients with Crohn's disease [17]. Further investigations are needed to elucidate the role of ILCs in intestinal inflammation.

Adaptive immunity

In order to mount an effective immune response against invading pathogens, a highly specific and long lasting immunity is also required. Adaptive immune cells, especially T-cells, can undergo a complex maturation process to finally adapt and respond to a specific type of antigen, generating different T-cell subsets. This plasticity must be highly regulated, thus an abnormal development of activated T-cell subsets may lead to an exacerbated immune response and the release of cytokines and chemokines that can subsequently affect the entire immune system. IBD pathogenesis has been clearly associated with an excessive pro-inflammatory immune response in the gut. The diagram in **Figure 2**, represents the different T-cell subsets and pro-inflammatory cytokines implicated in the development of intestinal lesions in IBD. In an inflammatory context, naïve T-cells differentiate into effector T helper cells (Th) namely Th1 and/or Th17. Subsequently, **Th1** cells produce large amounts of IFN-γ and TNF-α, both triggering apoptosis of epithelial cells. Intestinal activated macrophages, in turn, produce TNF-α that promotes differentiation of lamina propria stromal cells into activated myofibroblasts which produce high quantities of tissue-degrading matrix metalloproteinases (MMPs). On the other hand, **Th17** cells induce recruiting of neutrophils to the sites of active inflammation through the secretion of IL-17A. Besides, IL-21 is also released by Th17 cells and induces MMPs production by stromal cells which degrade the extracellular matrix and the basement membrane, thus inducing enterocyte apoptosis.

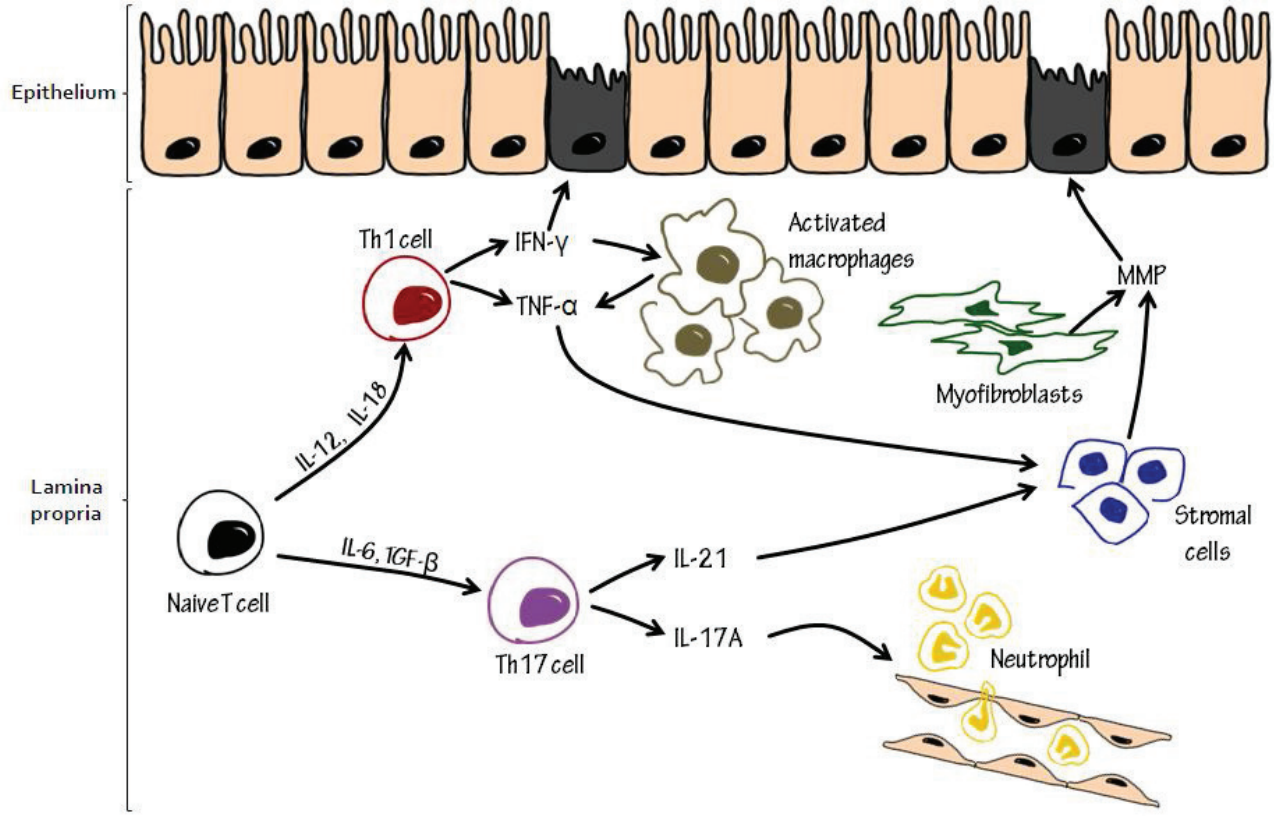


Figure 2. Adaptive response in the gut. *Adapted from [18], Autoimmunity Reviews 2014.*

Th1 cells

Th1 cells are induced by IL-12 and IL-18, and release high amounts of IFN-γ and TNF-α [19]. Crohn's disease is thought to be a Th1 mediated disease, causing an intestinal inflammation triggered by increased mucosal levels of IL-12 and IL-18 [20]. In fact, intestinal lamina propria activated-macrophages from Crohn's disease patients secrete abnormal levels of IL-12 [21]. Additionally, mucosal T-cells from Crohn's disease patients have been show to secrete higher amounts of IFN-γ and IL-2 than healthy individuals [22, 23].

However, mucosal T-cell immunoregulation changes with the course of the disease. Early Crohn's disease resembles an acute infectious process where mucosal T-cells appear to mount a typical Th1 response. As the disease

progresses, other cytokines such as IL-17 and IL-23 are thought to mediate late Crohn's disease [24].

Th17 cells

The induction of Th17 cells is driven by IL-6 and TGF-β. Th17 cells secrete copious amounts of IL-17A, IL-17F, IL-21, IL-22, and their expansion is promoted by IL-23 [25]. Interestingly, another subset of Th17 cells has been identified, Th1/Th17 cells are characteristic by the secretion of both IFN-γ and IL-17 [26]. Th17 and their signature cytokines have been widely studied in IBD. Indeed, numerous Crohn's disease susceptibility genes are involved in differentiation and expansion of Th17 cells, such as *IL-23R*, *IL12B*, *JAK2, STAT3* and *CCR6* [27]. Moreover, Crohn's disease patients have increased numbers of Th17 and Th1/Th17 cells in the lamina propria [28]. Higher IL-17A transcript levels are found in the gut mucosa of Crohn's disease patients compared to controls [29]. Although increased IL-17 mRNA is common to early and late Crohn's disease mucosa, exacerbated Th17 responses in the peripheral blood appear only in late disease. Thus, suggesting a role of Th17 in perpetuating the disease [30].

Regulatory T-cells

Regulatory T lymphocytes (T-regs) are generally defined by the expression of CD25 and Foxp3. To maintain the intestinal homeostasis, T-regs produce anti-inflammatory cytokines including IL-10 and TGF-β in order to suppress abnormal immune responses. The secretion of these cytokines in the gut mucosa, contributes to the generation of a suitable environment to induce tolerance by preventing activation of effector T-cells. In the context of IBD, a dysregulation of the anti-inflammatory activity may also be associated with the development of the disease. Actually, there is evidence demonstrating that T-regs are depleted in peripheral blood of patients with active IBD compared to controls [31]. So far, literature regarding T-regs and IBD, indicate that probably T-regs function itself is not impaired in IBD patients, but effector cells unresponsiveness to the action of T-regs may probably lead to the sustained inflammation [32].

Cytokines

Altogether, innate and adaptive immune cells can drive or shut down intestinal inflammation by producing different types of cytokines. The pleiotropic and pathogenic role of cytokines in IBD has been widely studied [6]. That being so, a relevant number of Crohn's disease treatments along the last years have taken advantage of cytokines as potential therapeutic targets. Without going any further, blockade of tumor necrosis factor (TNF) is currently being used as a standard therapy for Crohn's disease in the clinic [33].

As the inflammation progresses, there are different check points, where the presence or absence of a particular cytokine can change the course of the disease. For example, IL-1 family members, such **IL-1β** and **IL-18**, seem to be crucial during the initiation of colonic inflammation, where the recruitment of neutrophils and activation of antigen presenting cells (APCs) is essential. Activated-macrophages and $CD4^+$ T-cells from lamina propria produce **IL-6**; a pro-inflammatory cytokine that have been found to be increased in IBD patients [34]. IL-6 most relevant functions are prevention of T-cell apoptosis and stimulation of epithelial cells proliferation. Not only IL-6, but also **IL-12** and **IL-23** production by macrophages and DCs have been shown to be augmented in Crohn's disease patients. These cytokines orchestrate the crosstalk between innate and adaptive immunity and have a central role in driving early responses to microbes. While IL-12 drives Th1 cell differentiation and pro-inflammatory cytokine production, IL-23 promotes expansion of Th17 cells by binding IL-23R on the T-cell. Interestingly, *IL-23R* gene polymorphism was associated with Crohn's disease in 2006 by genome-wide association studies (GWAS) [35]. IL-23 has several functions, among others it increases **TNF-α** production by APCs and T-cells, one of the key cytokines for the pathogenesis and treatment of IBD. TNF-α may exert various pro-inflammatory functions that include induction of epithelial cell death, prevention of T-cell apoptosis, and production of pro-inflammatory cytokines and activation of fibroblasts. Lamina propria mononuclear cells from IBD patients produce large amounts of both membrane-bound and soluble TNF [36].

T-cell-derived cytokines are also implicated in the pathogenesis of IBD. Th1 effector T-cells produce high amounts of the pro-inflammatory cytokine **IFN-γ**, which activates macrophages and induces death of epithelial cells. Crohn's disease has been shown to be driven by an excessive Th1 response, based on studies that have found an increased production of IFN-γ and IL-2 by lamina propria T-cells in Crohn's disease patients compared to healthy individuals [37]. Although many cytokines have been clearly defined as pathogenic or non-pathogenic for IBD, the role of other cytokines remains still controversial. Indeed, this is the case of **IL-17A**, which is secreted by Th17 cells and induces neutrophil recruitment to the inflammatory site, and the up-regulation of a number of pro-inflammatory molecules and cytokines. Several studies regarding the role of IL-17A and **IL-17F** in IBD have been performed using different animal models. Although significant amount of data suggests the pathogenic role of IL-17A in IBD, there is also evidence showing a protective role of this cytokine in the development of the disease [38]. Furthermore clinical trials performed in order to neutralize the action of IL-17A in Crohn's disease patients were unsuccessful. Finally, **IL-21** is secreted by different type of T-cells and collaborates in the progression of intestinal inflammation, mainly by promoting Th17 expansion and inducing proliferation of epithelial cells and tissue destruction by MMPs.

In contrast, **IL-10** and **TGF-β** are the main anti-inflammatory cytokines involved in the regulation of inflammation in the gut by suppressing effector T-cells and pro-inflammatory cytokine production in APCs. Although a polymorphism in *IL-10* gene have been associated with IBD [39], no defects in TGF-β function have been described so far. However, effector T-cells from Crohn's disease patients have been shown to over-express *SMAD7*, which inhibits TGF-β signaling, therefore these cells become resistant to TGF-β-mediated suppression [32]. Th17 cells also produce **IL-22**, anti-inflammatory cytokine that controls epithelial proliferation, wound healing and the production of AMPs, although its function is still controversial.

Even though great efforts are being made to fully understand the role of cytokines in IBD, it is very difficult to unravel the complexity of mucosal cytokine

network. Factors including location and type of inflammation, immune cell plasticity, different pathogenetic mechanisms and shifting cytokine production patterns along the course of the disease, need to be considered as they may affect the cytokine function in patients with IBD.

Environmental factors

Several environmental factors have been associated with the pathogenesis of Crohn's disease namely diet, antibiotic use or microbial exposure in life; but smoking is the only factor that has been actually proved. Patients with Crohn's disease who smoke usually experience more severe symptoms and more frequent relapses compared with those who have the condition but do not smoke.

There is no evidence supporting the fact that diet may influence the course of the disease, but minor lifestyle changes can ameliorate the severity of the disease in some patients. Interestingly, changes in the gut microbiota have been associated with the pathogenesis of IBD. A comparison of bacterial composition of feces between Crohn's disease and healthy individuals showed less diversity and fewer numbers of non-redundant bacterial genes in Crohn's disease patients [40]. Whether this shift in the microbiome is cause or effect of the disease remains unclear. But apparently the gut bacterial composition and its interaction with the immune system play a role in disease pathogenesis, therefore it is currently an important scope of research regarding Crohn's disease.

Genetics

The inheritance pattern of Crohn's disease is unclear because many genetic and environmental factors are likely to be involved. However, this condition tends to cluster in families, and having an affected family member is a significant risk factor for the disease. As a multifactorial disease, Crohn's disease genetic variations are not enough to cause intestinal inflammation, but a combination with changes in the immune system and the presence of bacteria in the digestive tract may result in the development of the disease. Several studies have identified polymorphisms in specific genes involved in homeostatic

mechanisms. Meta-analyses of GWAS have now identified 163 SNPs conferring susceptibility to Crohn's disease, Ulcerative colitis, or both [39]. Additional research is needed to better understand how genetic variations in these chromosomal regions are related to the risk of developing Crohn's disease.

Treatment

The quality of life of a significant percentage of IBD patients is poor as a result of persistent disease activity, repeated surgery, adverse drug events and extra-intestinal complications. Therapeutic tools, including the most recent biological drugs, are not able to neither prevent this serious impact nor improve the long term prognosis of a significant proportion of IBD patients.

Current therapeutic approaches for treatment of Crohn's disease commonly use a step-up approach, using corticosteroids as first line, immunosuppressants such as azathioprine, mercaptopurine or methotrexate in patients with inadequate response to corticosteroids, and finally biologic drugs to treat patients failing immunosuppressants or in those with very severe disease. **TNF-α blockers** such as infliximab, certolizumab or adalimumab, reduce the amount of TNF-α able to bind to its receptor. *In vitro* studies suggest that TNF-α neutralization have several effects on the immune system that may contribute to the efficacy of these drugs observed in patients. In summary, some of the consequences of TNF-α blocking include an increase of TNFR2 release by monocytes, an increase of IL-10 synthesis and apoptosis of mononuclear cells, and inhibition of IL-6 production and leukocyte migration. A second class of biologic therapy, **α4-integrin blockers** such as natalizumab and vedolizumab, inhibit the ability of leukocytes to migrate to inflamed areas and reduce the frequency of relapses in Crohn's disease [41, 42]. Needless to say, all these treatments have usually several adverse events that may affect the quality of life of patients or even induce other immunological disorders due to the fact that these drugs target the immune system.

It is commonly observed that eventually a significant percentage of patients become refractory to all classes of available treatments, and their only option is

to undergo surgery. Hence new therapeutic targets and strategies are needed in order to better treat chronic inflammation, particularly Crohn's disease.

Cytokine or signaling molecule	Advantages as a target	Disadvantages as a target	Developmental stage	Therapeutic agent
IFN-γ	Plausible bioactivity seen in studies using mouse models and in ex vivo studies using cells from patients with IBD	Limited efficacy in clinical trials	Clinical trials (Phase II)	Fontolizumab
TNF	Central pro-inflammatory cytokine in IBD pathogenesis	Increased infection risk (lung infection and tuberculosis)	Approved for some indications in IBD and routinely used in the clinic	Infliximab, adalimumab, certolizumab and golimumab
TGF-β	Targeting SMAD7 restores the TGFβ-sensitivity of cells	Long-term toxicity and effects unclear	Clinical trials (Phase I)	*SMAD7* antisense oligonucleotides
IL-6R	Plausible bioactivity seen in studies using mouse models and in ex vivo studies using cells from patients with IBD	Response only seen in subgroups of patients; effectss on mucosal healing unclear	Clinical trials (Phase II)	Tocilizumab
IL-10	Plausible bioactivity seen in studies using mouse models	Limited or no efficacy in clinical trials	Clinical trials (Phase II)	Recombinant IL-10
IL-11	Immuostimulatory cytokine	Limited or no efficacy in clinical trials	Clinical trials (Phase II)	Recombinant IL-11
IL-12 and IL-23	Plausible bioactivity in mouse models and in ex vivo studies using cells from patients with IBD	Response of subgroups of patients only	Clinical trials (Phase II-II)	Ustekinumab and briakinumab (ABT874)
IL-17A	Plausible bioactivity in some models of inflammation	Aggravation of disease and effects on tissue homeostasis	Clinical trials (Phase II)	Secukinumab
JAK3	Targeting of several key cytokines simultaneously	Long-term toxicity unclear and not effective in a pilot study	Clinical trials (Phase II)	Tofacitinib

Table 1. Therapeutic targets in IBD, cytokines or cytokine signaling. *Adapted from [6], Nature Reviews Immunology 2014.*

Throughout the years, other promising therapies have emerged targeting T-cells (anti-CD3, CTLA-4 fusion protein), modulating regulatory T-cells (rhIL-10), targeting the pro-inflammatory cascade (anti-IL6, anti-IL-17, anti-IFNγ, anti-IL-12, anti-IL-23, anti-CD80/86), blocking cell recruitment (anti-ICAM1) or stimulating innate immunity (GM-CSF). Some of these therapies are already in clinical trials and some others have not been successful when translated from animal models to human trials (**Table 1**).

Besides all these treatments, clinical trials have also illustrated that autologous hematopoietic stem cells transplantation (HSCT) can be a reasonable option for patients with severe Crohn's disease who have failed standard treatments. The

aim of this therapy is to deplete auto-reactive T-cells and regenerate a non-conditioned immune system to luminal antigens. So far, encouraging results have been obtained and some patients are rapidly going into clinical remission [43]. Mesenchymal stem cells (MSCs) have also been used via the systemic route in IBD with promising results, taking advantage of their immune regulatory and regenerative properties. However, it is still early to draw firm conclusions; the next few years will be crucial for defining the role of stem cells therapy in the management of IBD [44].

Chapter II | Dendritic cells and Immune response

Human body is constantly exposed to a wide range of harmful pathogens such as bacteria, parasites and viruses. The immune system has evolved as protection against infectious agents, and it is specialized in distinguishing between invasive organisms and harmless antigens. The immune system is based on two distinct responses, the innate and the adaptive immune response. The **innate immune response** forms the first line of defense and includes anatomical barriers, secretory molecules and cellular components that initiate the non-specific immune response. It employs different cells such as macrophages, neutrophils, DCs and NK cells to rapidly detect and control infection. Activation of innate immune system is essential to initiate the immune response. The second line of defense against invading pathogens is known as **adaptive immune response**. In contrast to the innate immune response, adaptive immune system is highly specific and confers long lasting immunity. It involves pathogen-specific lymphocytes (B- and T-cells) that are directed against Ags and can lead to pathogen-specific memory. Adaptive immunity can be subdivided into cellular and humoral immune response. The activation of T and B-lymphocytes results in the production of antibodies, immunoglobulins with a high specificity for microbial motifs, the so-called humoral response.

Dendritic Cells

First identified by Ralph Steinman in the early 70's [45], DCs have long been recognized as highly potent APCs linking innate and adaptive immune responses. DCs are not only critical for the induction of primary immune responses, but may also be important for the induction of immunological tolerance, as well as for the regulation of the type of T-cell-mediated immune response. Human circulating DCs can be subdivided in two main subsets, namely **myeloid DCs** (myDCs) and **plasmacytoid DCs** (pDCs), which orchestrate different types of immune responses. On one hand, pDCs are thought to be of lymphoid origin and have a crucial role in antiviral immunity by producing high amounts of type I IFNs [46]. On the other hand, cells from

myeloid lineage give rise to myDCs which can be further subdivided on the basis of expression of distinct cell surface markers and functions (**Table 2**).

DC subset	Surface markers	Subtypes	Frequency	Toll-like receptors	C-type lectin receptors	Function
Plasmacytoid DCs (pDCs)	CD11c⁻ BDCA2⁺ BDCA4⁺ CD123⁺		0.3% of blood PBMCs	TLR1, TLR7, TLR9, TLR10	DEC-205 DCIR BDCA2	Antiviral immunity
Myeloid DCs (myDCs)	CD11c⁺ MHCII⁺ CD13⁺ CD33⁺ Lineage markers: (CD3,CD14, CD19, CD56)⁻	BDCA1⁺ (CD1c)	0.6% of blood PBMCs	TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR10	DEC-205 DCIR	Potent T-cell stimulators
		BDCA3⁺ (CD141)	0.04% of blood PBMCs	TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR10	DEC-205 CLEC9A	Cross-presentation

Table 2. Human DC subsets. Summarized from [47] and [48].

As discussed later, myDCs are specialized in microbial response and have the ability to orchestrate adaptive immunity by directing T-cell differentiation. Due to the extremely low frequency in which myDCs and pDCs are present in blood and tissues, research using primary blood DCs is complicated. Instead, many studies focusing on myDCs biology make use of monocyte-derived DCs, cultured from blood monocytes in the presence of IL-4 and GM-CSF, which share many of the phenotypic and functional features of naturally occurring myDCs [49].

Located in peripheral and lymphoid tissues, DCs are sentinels of the immune system and can respond to conserved structural motifs derived from microorganisms, so-called **PAMPs**, via specific receptors (**PRRs**). Thus, DCs are well-equipped to continuously monitor different tissues for the presence of microorganisms. For instance, in both the gut and lung, DCs send dendrites through the epithelial cell layer and directly sample the luminal surface while preserving epithelial barrier integrity [50, 51]. Indeed, they express a set of PRRs including Toll-like receptors (TLRs), C-type lectin receptors (CLRs) and the cytoplasmic NOD family, as well as RIG-like helicases [52]. However, not every TLR is expressed on each DC subset nor causes the same effects when activated. Thus, the differential expression pattern of TLRs between pDCs and

myDCs will define the specificity of the immune response against a certain pathogen (**Table 3**).

Pattern Recognition Receptor type	Location	Ligands	Effects
Toll-like receptors (TLRs)	Extracellular (TLR1, 2, 4, 5, 6 and 10)	LPS (TLR4) Flagellin (TLR5) Peptidoglycan (TLR2) Bacterial lipopeptides (TLR1/2 and 2/6)	Maturation Cytokine production Optimal Ag presentation Optimal CD4+ and CD8+ T cell activation Instruction of T cell differentiation
	Intracellular (TLR3, 7, 8 and 9)	RNA molecules (TLR3, 7 and 8) CpG DNA (TLR9)	
C-type Lectin receptors (CLRs)	Cell surface and endosomes (DC-SIGN, DEC-205, DCIR, Dectin-1, DNGR1, BDCA-2 and mannose receptor)	Carbohydrate structures	Ag internalization TLR signaling modulation Cytokine production Instruction of T cell differentiation DC trafficking, cellular interactions
NOD-like receptors (NLRs)	Cytoplasmic (NOD1, NOD2 and NALP)	Peptidoglycan moieties, PAMPs and host danger signals	Synergy with TLRs for cytokine release Instruction of T cell differentiation (pro) IL-1β and (pro) IL-18 processing
RIG-like helicases	Cytoplasmic (RIG-I and MDA-5)	Viral RNA	Type I interferon induction Antiviral immune response

Table 3. General features of the four main pattern recognition receptor families expressed by DCs. TLR1/2 indicates heterodimer of TLR1 and TLR2.

This broad variety of receptors enables DCs to recognize multiple “danger signals” and to discriminate between self and non-self antigens. As professional phagocytes, upon recognition of pathogens, DCs can engulf and process the antigenic peptides in order to present them through **major histocompatibility complex class** (MHC) molecules to T lymphocytes [53]. After PRR activation, the DCs undergo a process called **maturation** and migrate to secondary lymphoid organs where they can interact with T and B cells [54]. Mature DCs (mDCs) not only secrete cytokines but also express high levels of MHC and co-stimulatory molecules such as CD80, CD86 and CD40 which permits potent induction of effector lymphocyte responses. In the T-cell rich parafollicular areas of lymph nodes, several chemokines, such as CCL19 and CCL21, are produced. In order to respond to these chemokines and acquire the capacity to **migrate** toward lymphoid organs, mDCs up-regulate chemokine receptors such as CCR7 [55] (**Figure 3**).

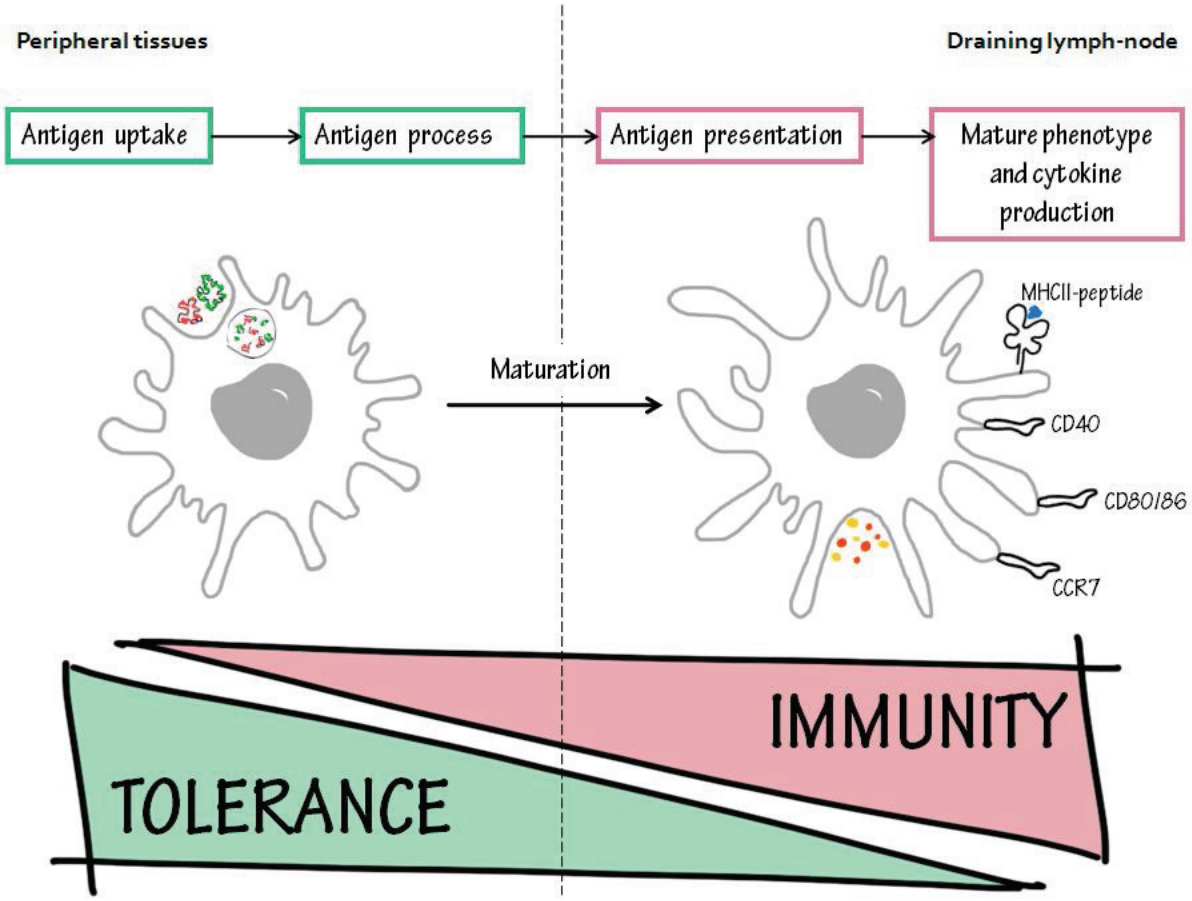


Figure 3. Maturation process of DCs. *Adapted from [56], Nature Reviews Immunology 2004.*

DCs and T-cell response

Upon infection or inflammation, mDCs instruct the T-cells in order to define the nature of the induced response. The encounter of a T-cell with its specific antigen is a process called **T-cell priming**. DCs have the ability to prime naïve $CD4^+$ and $CD8^+$ T-cells through the transduction of several signals. Therefore, the immunological outcome strongly depends on the activation state of DCs. Resting DCs or DCs receiving inhibitory signals, such as IL-10 or corticosteroids, induce immune tolerance via T-cell deletion or induction of regulatory T-cells, whereas mature DCs induce immunity. Furthermore the T-cell stimulatory potential of DCs also depends on the duration and combination of the stimulatory signals, being progressively acquired as the time of exposure increases [57].

Signal 1. Recognition of peptide-MHC complex.

As depicted in **Figure 4**, the major route for presentation of exogenous antigens that enter the DC via endocytosis or phagocytosis is via MHC class II. Following uptake, soluble and particulate antigens are directed to the MHCII compartments and presented to $CD4^+$ T-cells (**A**). Importantly, while MHCI-restricted Ag presentation is normally reserved for Ag derived from endogenous structures through proteasomal degradation (**B**), DCs have the capacity to present Ags derived from both the intra- and extracellular milieu in the context of MHCI. This process, called **cross-presentation**, enables efficient induction of $CD8^+$ T-cells and is essential for immunity against certain viruses and tumors [58] (**C**). DCs can activate both $CD4^+$ and $CD8^+$ T-cells and although many cell types have APC properties, DCs are superior in activating naïve T-cells. Hence, the antigen source and the method of antigen loading can direct an immune response towards a $CD8^+$, $CD4^+$ or combined immune response.

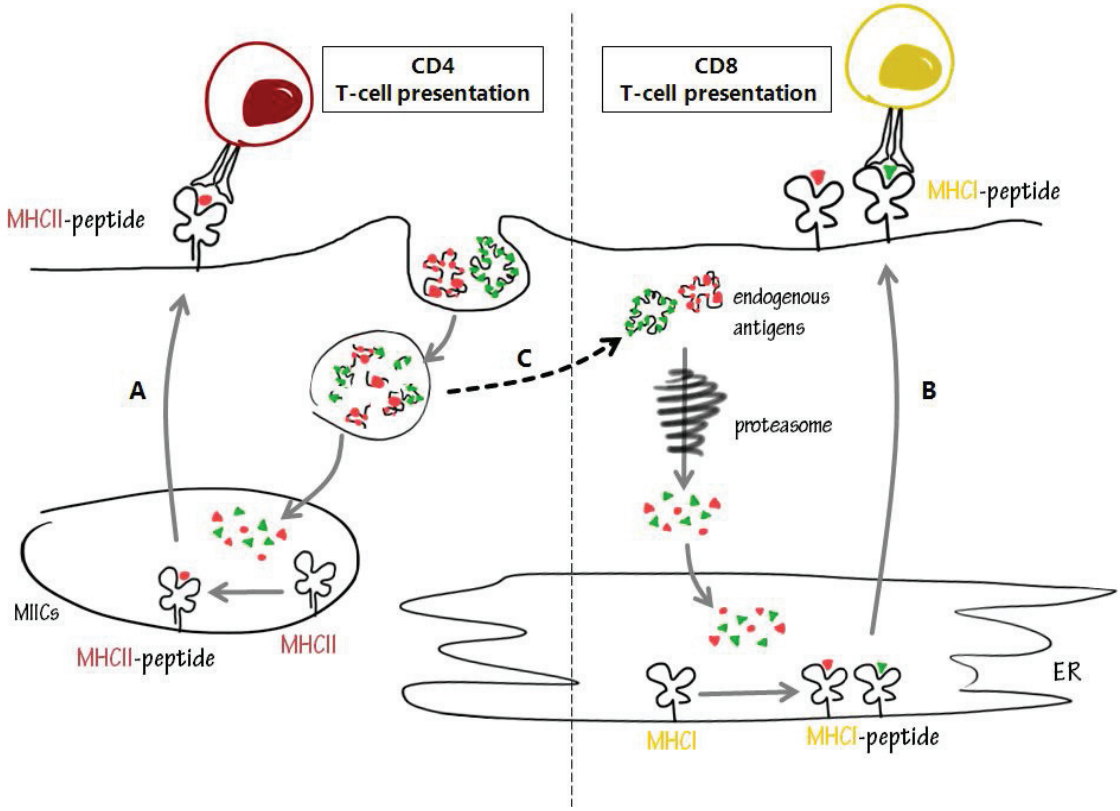


Figure 4. Different antigen-processing pathways for the MHC class I and class II molecules. ER: Endoplasmic Reticulum; MIICs: MHCII compartments. *Adapted from [59], Nature Reviews Immunology 2001.*

Signal 2. Stimulation via co-stimulatory molecules.

The interaction between **co-stimulatory molecules** expressed by DCs and their ligands expressed by T-cells is an essential event to sustain T-cell activation. The principal co-stimulatory molecules CD80 and/or CD86, intercellular adhesion molecule 1 (ICAM-1) and/or ICAM-2, and OX40-L interact with their respective T-cell counter-receptors, CD28, LFA-1 and OX40. These activation signals determine the stimulatory capacity of DCs and enable the amplification of T-cell response.

Signal 3. Cytokines

The third signal is determinant for the polarization of T-cells and the resulting class of immune response. DCs produce a broad variety of pleiotropic cytokines that can act on other cells (paracrine) or on the producing-cell itself (autocrine). These cytokines regulate **T-cell differentiation**, proliferation and survival at many stages of the immune response and are able to simultaneously control DC activation. The balance of these cytokines and the consequent immune response strongly depend on the conditions under which DCs are primed for the expression of the T-cell-polarizing signal 3. Moreover, DCs are also important for the regulation of tissue-selective lymphocyte trafficking [60]. In support of the notion that the migratory capacity of human T-cells can be affected by DC-related factors, such as vitamin metabolites and chemokines, some authors have considered the existence of a “signal 4” [61].

DCs and T-cell differentiation

After T-cell activation, DCs have also the ability to regulate the differentiation of **$CD4^+$ T helper cells** into different subsets that will become specialists dealing with a particular microorganism, the so-called effector T-cells (**Figure 5**). DCs receive signals from the environment, cytokines and PRRs that result in different “DC programs”. These differently programmed DCs express particular cytokine profiles and cell surface molecules that impact directly on naïve T-cells. The properties of distinct lineages are acquired via repression or induction

of transcriptional master regulators, namely T-bet [62] (Th1), GATA3 [63] (Th2) and RORγt [64] (Th17). For instance, in response to bacteria DCs produce IL-12p70 and thereby skew $CD4^+$ T-cells towards a pro-inflammatory **Th1** phenotype [65]. Polarization of naïve T-cells towards **Th2** is induced by extracellular parasites. Although the pathways leading to Th2 induction are still unclear, cytokines including IL-4 and IL-10 are thought to play a role as well as activation of Notch signaling pathways on the T-cell [66]. Finally, **Th17** subset can be induced upon IL-1β exposure of naïve T-cells, while IL-6 and IL-23 can potentially enhance their effector function [67].

Although the effector arm of the immune system is indispensable for the protection against microorganisms, it needs to be tightly regulated in order to prevent excessive inflammatory responses. **Regulatory T-cells** are crucial for prevention of these unwanted immune responses and can be subdivided into intrinsic and adaptive T-regs. What defines intrinsic or naturally occurring T-regs is their thymic origin and expression of Foxp3 [68]. After Ag-recognition, T-regs can inhibit proliferation and effector function of $CD4^+$ and $CD8^+$ effector T-cells in an Ag-independent, cell-contact dependent manner [69]. In contrast, adaptive or induced T-reg subsets, such as **Tr1** cells (type 1 regulatory T-cells), can be generated in the periphery via several mechanisms and exert their immunosuppressive function in a cell-contact dependent fashion or via secretion of anti-inflammatory cytokines like IL-10 and TGF-β [70].

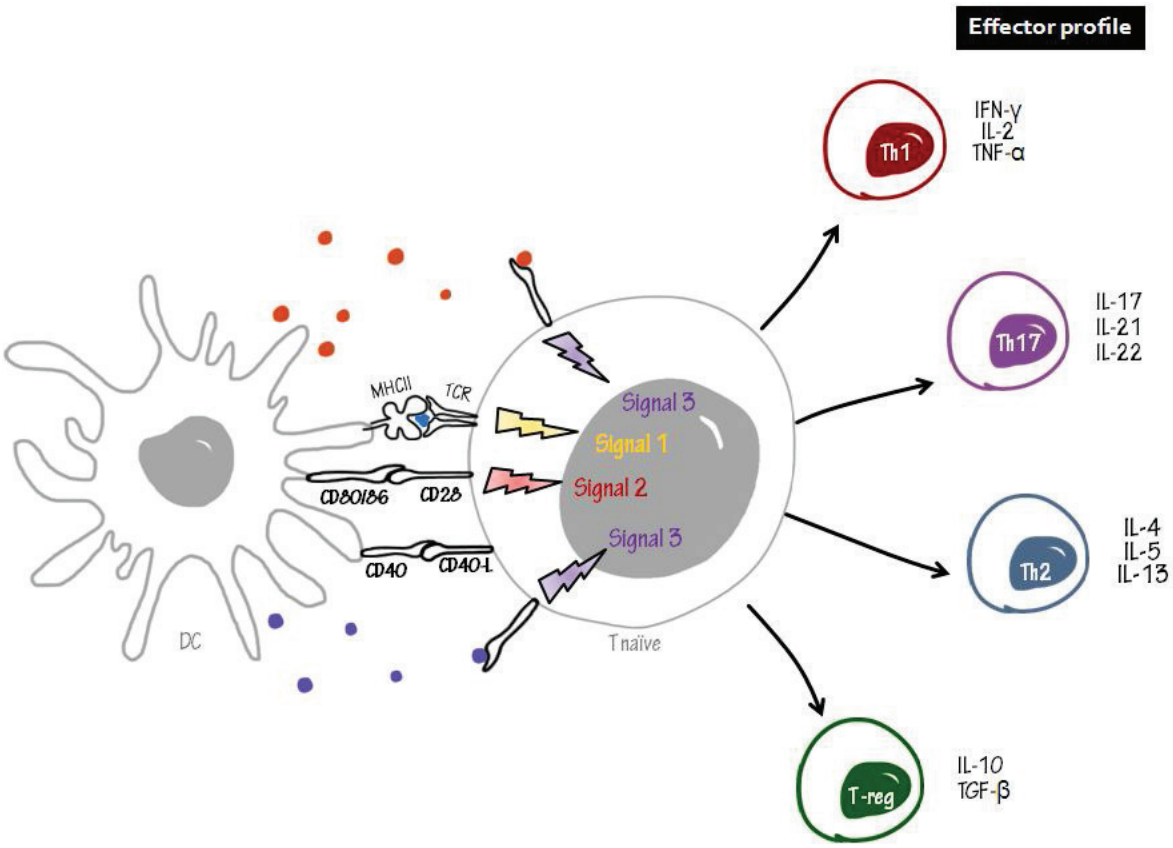


Figure 5. T-cell polarization requires 3 DC-derived signals. *Adapted from [71], Nature Reviews Immunology 2003.*

DCs and tolerance induction

Tolerance is a tightly regulated process that includes the elimination of auto-reactive T- and B-cells during development (central tolerance), as well as the down-regulation of their activation and induction of regulatory mechanisms in the periphery (peripheral tolerance) [72, 73]. A breakdown in self-tolerance mechanisms results in sustained adaptive immunity that responds to specific auto-antigens and may lead to the development of autoimmune diseases [74].

Besides their role as key inducers of protective immune responses, it is now recognized that DCs play an equally important role in induction and maintenance of tolerance to self-Ag [75]. Tolerance induction does not appear to be specific for a certain DC subset or restricted to “classical” immature DCs (iDCs) but reflects a phenotype skewed towards expression of inhibitory signals

(e.g. IDO, PDL-1, TRAIL, IL-10) rather than activation signals (e.g. CD80, CD86, OX40-L, IL-12). Due to the lack of expression of co-stimulatory molecules and MHCII, DCs are able to induce T-cell anergy, preventing T-cell activation. It has been described that DCs suboptimal antigen presentation, combined with the expression of IDO (indoleamine 2,3-dioxygenase) or FasL (CD95L) leads to inhibition of T-cell proliferation and T-cell deletion [76]. The induction of T-reg and Tr1 by DCs is another mechanism to induce peripheral tolerance (**Figure 6**) [77].

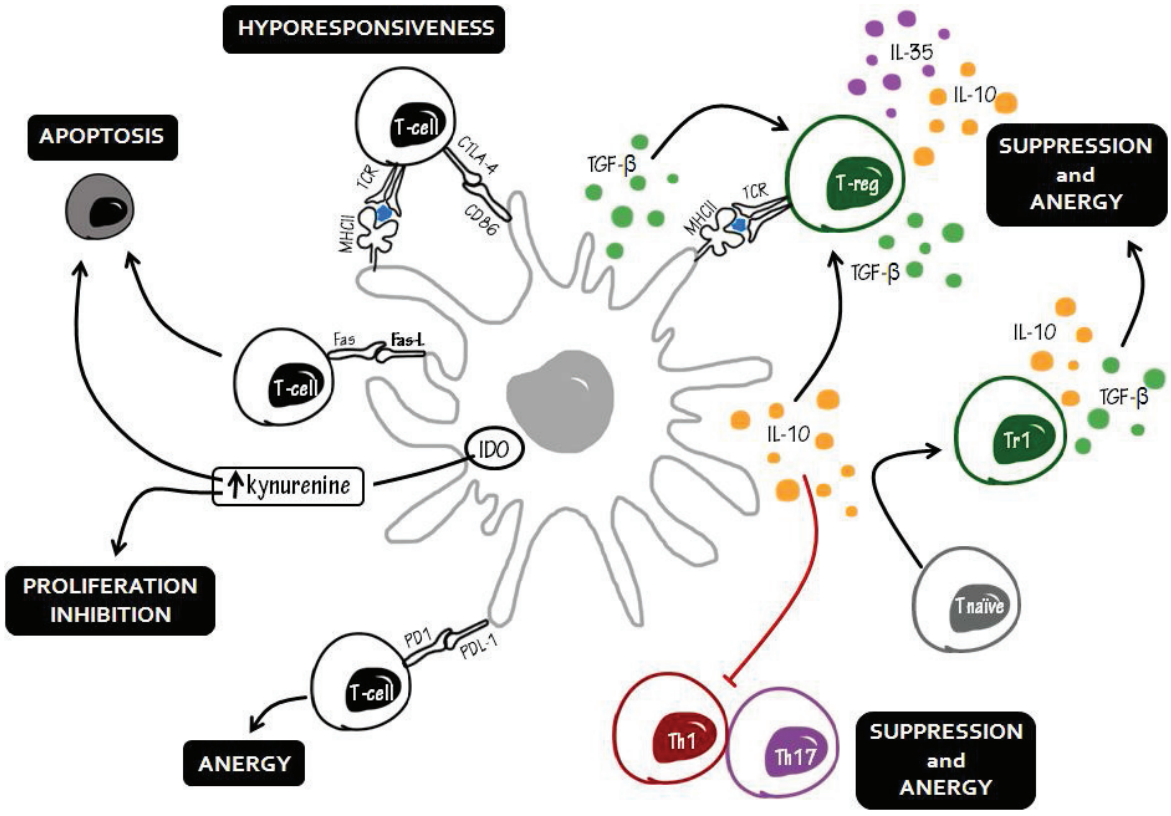


Figure 6. **Mechanisms to induce tolerance by DCs.** *Adapted from [78], Clinical and Developmental Immunology 2013.*

Mucosal DCs

How DCs induce immune responses to harmful stimuli, while preventing adverse reactions to harmless Ag (especially in tissues with high Ag load like the gut and lung) is still being explored. However, it has been shown that mucosal DCs, identified as $CD103^+$ DCs, have tolerogenic properties. Mucosal DCs promote T-reg differentiation [79], produce retinoic acid [80] and IDO [81], molecules that can drive T-regs and are known to be involved in the induction of tolerance in the gut. The ability of mucosal DCs to produce IL-10 and the lack of IL-12, together with the low expression of CD40, TLR2 and TLR4 make these cells suitable to be defined as the main regulators of immune tolerance in the intestinal tract. Indeed, the immunosuppressive cytokine IL-10 is a crucial mediator of tolerance in the gut and it has a non-redundant role in limiting inflammatory responses in the intestine. IL-10 can act on a variety of immune cells and its secretion is certainly involved in T-regs and Tr1 induction as well as regulating the local inflammatory immune response via antigen-presenting cells [82, 83]. Nevertheless, research in this area is complicated and a lot more needs to be elucidated regarding human DC subsets and tolerance in the gut.

Dendritic Cells: a potent tool for immunotherapy

The regulation of immunity and tolerance is not only determined by T-cell receptor specificity, but also by the context in which the antigens are presented to the immune system. DCs are professional APCs that play a crucial role in both initiation and modulation of the immune response. Depending on the activation state of DCs, they can either be immunostimulatory or tolerogenic. **Immunostimulatory DCs** stimulate immune responses by activating T- and B-cells. In contrast, **tolerogenic DCs** (tol-DCs) inhibit immune responses by the induction of anergic T-cells and activation of T-regs. In the settings of infection and cancer, microbes and tumors can exploit DCs to evade immunity, but DCs also can generate resistance, a capacity that is readily enhanced with DC-targeted vaccines. Due to their physiological properties and the availability of clinical grade reagents, immunostimulatory DCs have been safely and

successfully used in clinical trials designed to efficiently stimulate immune response against tumors or infectious diseases [84, 85]. DCs cultured directly from monocytes, $CD34^+$ progenitor cells, or directly isolated from blood can be loaded *ex vivo* with antigen, cultured in the presence of maturation stimuli and subsequently administered to patients. However, new insights have broaden the application of DCs for the treatment of autoimmune diseases, allergy and transplant rejection [86-88]. To date, only two clinical trials have taken advantage of tol-DCs properties to treat Type 1 Diabetes [89] and Rheumatoid Arthritis (RA) patients [90] respectively. Although it is still too early to draw any conclusion in relation to their clinical efficacy, these studies revealed that tol-DCs administration is safe and well tolerated, without no observable adverse events or toxicities.

After 20 years since the first human DC vaccination took place in 1996 [91], the field of DC based-immunotherapy have progressed slowly, specially in the field of cancer. The discovery of a method to differentiate DCs from monocytes *in vitro* in 1994 by Sallusto and colleagues was an important starting point to the development of DC-vaccination protocols [49]. Since then, many DC-based vaccination strategies have emerged to treat cancer in different clinical trials, providing enough information to improve efficacy along the years. As reviewed by Anguille *et al.*, although not all DC-vaccination studies were designed primarily to measure survival, an increasing number of trials indicate that DC-therapy could confer a survival benefit [92]. Altogether, these findings have contributed to accelerate the development of DC-based vaccines targeting autoimmune diseases in the past years (**Figure 7**).

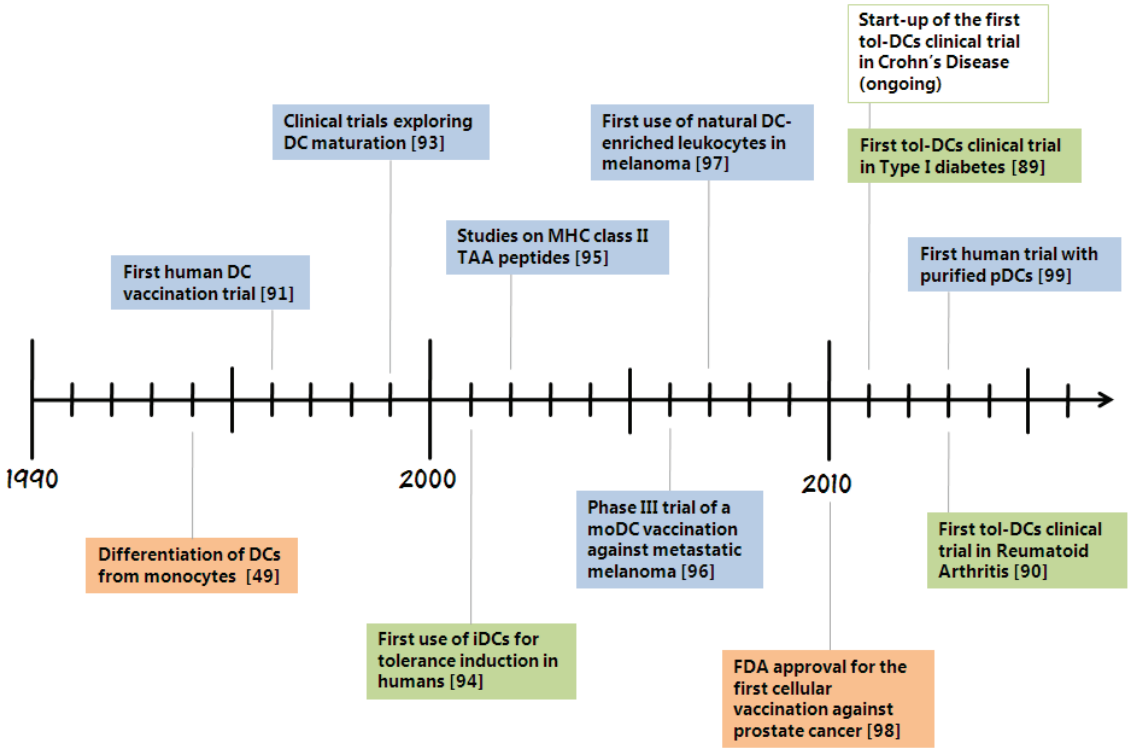


Figure 7. DC-vaccination chronogram. TAA: tumor associated antigens.

In order to fit its specific purpose, each DC vaccination study must be carefully designed and there are important issues to consider when designing effective DC vaccines:

Precursor isolation and differentiation into DC phenotype

Due to the low occurrence of naturally circulating DCs in blood, conclusive clinical evidence on their usability for immunotherapy is lacking. However, as reviewed by Wimmers *et al.*, DC vaccination with natural DCs (myDCs/pDCs) is currently performed in a limited number of medical centers, and even though some issues still need to be overcome, immunotherapy using circulating DCs have shown promising efficiency [99, 100].

Thus far, conventional cellular vaccination approaches have been carried out with *ex vivo* generated monocyte-derived DCs (moDCs). Monocytes can be easily isolated from peripheral blood by different strategies including adherence, immune-selection based on CD14 expression and counterflow elutriation. Culturing monocytes in the presence of GM-CSF and IL-4 promotes

differentiation into DCs. In general, sufficient DCs for a vaccination trial can be obtained from 500mL peripheral blood.

Maturation

It is now generally accepted that the function of DCs is determined by the signals that they receive during maturation. While *in vivo* maturation signals can be primarily derived either from the contact with invading pathogens or tissue injury, iDCs can be matured by incubation with PRRs agonists [101], **inflammatory cytokines** such as TNF-α or IL-1β, and prostaglandin E_2 (PGE_2) [102] as well as CD40L [103]. On the other hand, anti-inflammatory cytokines (IL-10, TGF-β), **immunosuppressive agents** (corticosteroids, vitamin D3, retinoic acid), mycophenolic acid (MPA) or certain PAMPs (Schistosomal lysophosphatidyl-serine), have all been shown to promote the tolerogenic function of DCs [104-107].

However, several studies have demonstrated that after induction of the tolerogenic phenotype, activation of tol-DCs might actually be a critical step in optimizing the re-stimulation and/or expansion of functional T-regs rather than in maintaining their immaturity [108, 109]. Therefore, maturation of DCs is of great importance for vaccination efficacy, as expression of CCR7 (induced during the maturation process) promotes the migration of injected DCs to the lymph nodes where the activation of T- and B-cells occurs [55].

Antigen loading

The choice of antigen source and loading method is a crucial step for the development of DC-based vaccines. To date, several approaches have been used to arm DC with target antigen for use in clinical trials. **Immunogenic peptides** derived from pathogens and tumors have been extensively used for loading of DCs to induce an immune response against those targets. The main advantage of using a defined peptide is to generate an immune response that is very specific for that epitope. In some cases the epitope is unknown, thus the DC loading with the **whole protein** is a possibility. In cancer, when immunogenic antigens have not yet been identified or a broad immune response against tumor cells is needed DCs can be loaded with **tumor lysates.**

This strategy may reduce the possibility of target escape by loss of epitope variants. Another approach is to use **RNA or DNA** isolated from tumors cells to deliver tumor antigens to DCs.

Throughout the years, several DC-vaccination studies have used different strategies of antigen-loading in order to induce immunity against melanoma, renal cell carcinoma or colon cancer among others [110].

Route of administration

In addition to questions regarding the source of DC, maturation stimuli or antigen loading, the choice of the appropriate route of administration is almost certainly going to have a profound influence on clinical outcome. Besides maturation-induced up-regulation of CCR7, the route of administration has a major impact on the migration of DCs to the T-cell rich zones in the lymph nodes. For instance, cells injected **intravenously** (i.v) collect in the lung and liver and appear to be less efficient in cancer patients. In contrast, other routes of injection such as **subcutaneous or intradermal** (i.d) have shown better DC migration to draining lymph nodes, despite a significant number of cells remain at the injection site. Lesterhuis *et al.* concluded that intradermal DC-vaccination results in superior antitumor T-cell induction when compared with intranodal vaccination in melanoma patients [111]. However, when prostate cancer patients were immunized with Ag-pulsed DCs by i.v, i.d or **intralymphatic** (i.l) injection; all patients developed Ag-specific T-cell immune responses regardless of the administration route [112]. Nevertheless, this study also showed differences in the quality of the response due to the route of administration. Interestingly, the possibility to inject DCs directly in the tumor or tissue injury allows cells to uptake a wide variety of tumor-associated antigens (TAAs) and therefore triggers a broad immune response against tumors. It has already been shown that **intratumoral** injection of DCs in cancer patients including breast cancer, prostatic cancer or gastrointestinal carcinoma patients is feasible and well tolerated [113-115].

It is worth noting that the number of injected cells as well as their maturation state may also influence the capacity of DCs to migrate to secondary lymphoid organs [116, 117].

Clinical-grade tol-DCs

Since maturation conditions determine the tolerogenicity of DCs, the development of tol-DC therapies for disorders that are characterized by a failure in immune tolerance have been promoted. The concept reinforcing tol-DCs therapy is that it specifically targets the pathogenic autoimmune response while leaving protective immunity intact. Clinical-grade tol-DCs can be defined by intermediate expression of co-stimulatory molecules and an anti-inflammatory cytokine profile. They induce T-cell hyporesponsiveness and have the ability to inhibit T-cell responses.

As clinical studies progress, the regulatory agencies require accomplishing the restrictive **good manufacturing practice** (GMP) guidelines when preparing DCs for immunotherapy [118]. When manufacturing clinical-grade tol-DCs, the final tol-DC product needs to conform to a list of predefined **quality control** (QC) criteria before its release to the clinical setting. To ensure consistency and quality of the cell product, aspects including sterility, viability, purity and functionality are taken into consideration [119]. Functional assays require at least 10 days to be completed; therefore they are unsuitable for establishing the latter QC. In the case of tol-DCs, a rapid read-out is needed, thus low expression of CD83, non-detectable production of IL-12 and high secretion levels of IL-10 were chosen as QC markers as they correlate with tol-DC function. Moreover, the stability of tol-DCs is an especially important consideration if they are going to be used for the treatment of autoimmune diseases that are characterized by chronic inflammation. Upon injection, tol-DCs should not differentiate into immunogenic DC in vivo when exposed to pro-inflammatory mediators.

The aim of tol-DCs therapy is to re-establish the balance of immunity and tolerance in autoimmune diseases. As depicted in **Figure 8**, autologous tol-DCs treatment consists in *ex vivo* generation of tol-DCs and re-infusion of tol-DCs to the patient (**Figure 8**).

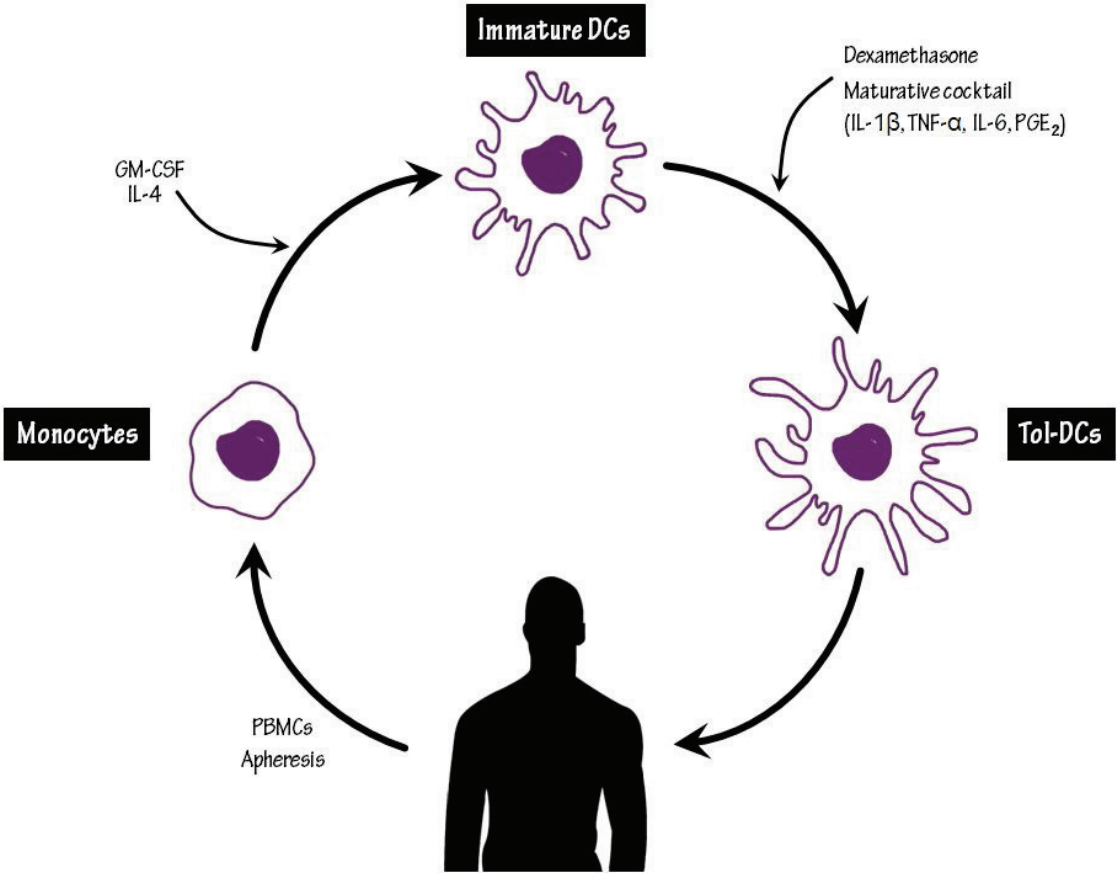


Figure 8. Schematic representation of tol-DCs vaccination protocol. Isolated monocytes from blood are cultured and differenciated into DCs by adding IL-4 and GM-CSF to the media. At day 3, addition of dexamethasone induces the tolerogenic profile, and at day 6 addition of the maturation cytokine cocktail potentiates the tolerogenic properties. *Adapted from [78], Clinical and Developmental Immunology 2013.*

The first clinical trial with tol-DCs was carried out by Giannoukakis *et al.* at the University of Pittsburgh, and the results were published in 2011 [89]. They conducted a Phase I study with tol-DCs in patients with type-1 diabetes. Autologous monocyte-derived DCs were treated *ex vivo* with anti-sense oligonucleotides targeting the CD40, CD80 and CD86 co-stimulatory molecules

and injected intradermally to the patients. DC treatment was well tolerated without any adverse events and it did not result in systemic immune suppression. To date, a few clinical trials are currently using tol-DCs therapy to treat autoimmune disorders such as RA [90], and the main conclusions that can be drawn are that intradermal injection of autologous tol-DCs is safe. Even though these trials were not designed to prove efficacy, they represent an important step forward in the field, and will pave the way for future tol-DCs trials.

Chapter III | TAMpering the immune response

Biology of the TAM receptors

TAM receptors and ligands

In the early 90s, the TAM group was among the last receptor tyrosine kinase (RTKs) subfamilies to be identified [120], and their biological role remained uncharacterized for several years. The name of the TAM family is derived from the first letter of its three members: **TYRO3, AXL, MERTK** [121]. Throughout the years, the analysis of engineered loss-of-function mutants in mice [122, 123], have helped elucidating the role of these receptors. Unlike many others RTKs, TAM receptors comprise a unique family that plays no essential role in embryonic development. Instead, they function as homeostatic regulators in adult tissues and organ systems that must be maintained in the face of continuous challenge, turnover, and renewal throughout life. In humans, constant homeostatic regulation must be carried out, frequently on a daily basis and for decades; otherwise the inability to maintain homeostasis may lead to death or disease, a condition known as homeostatic imbalance. A role for TAM regulation of tissue homeostasis is evident in the adult nervous, reproductive, and vascular systems; however it is in the regulation of the immune system that the TAMs play an especially prominent role [124].

In the immune system, TAM receptors are mainly expressed by professional phagocytes such as macrophages and DCs and also non-professional phagocytes including natural killer cells. However, TAM expression is not restricted to the immune cells; also sertoli cells, osteoclasts and retinal pigmental epithelium cells express TAMs. As depicted in **Figure 9**, the molecular structure of all three TAM receptors consists of a single-pass *trans*-membrane domain followed by a catalytically competent protein-tyrosine kinase in the C-terminal intracellular region. Ligand-binding regions of these receptors are located on the extracellular domain, each receptor have a defining arrangement of two tandem immunoglobulin-related domains and two fibronectin type III repeats in their N-terminus. TYRO3, AXL and MERTK share

more than 70% identity in their tyrosine kinase domain. TAMs signal as dimmers and are activated by the binding of two closely-related **ligands**, Growth Arrest Specific 6 (GAS6) and Protein S (PROS1). These two main ligands share Gla domains with several proteins of the blood coagulation cascade and were identified shortly after the identification of TAM receptors in 1995 [125, 126]. TAM ligands carry Gla domains in their N-terminus, followed by 4 EGF repeats and 2 laminin G domains in their C-terminal region. PROS1 also carries a thrombin sensitive region (TSR), and shares with GAS6 approximately 42% amino acid identity.

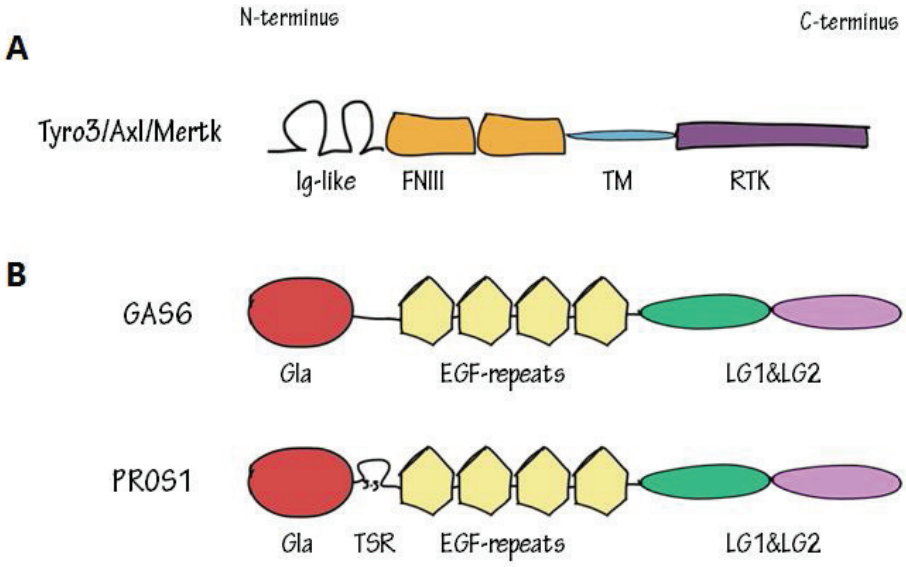


Figure 9. Schematic representation of TAM receptors and ligands protein structure. EGF: epidermal growth factor; FNIII: fibronectin III; LG: laminin G domain; RTK: receptor tyrosine kinase; TM: transmembrane; TSR: thrombin sensitive region. *Adapted from [127], Inflammatory Bowel Diseases 2014.*

Carboxilation of these Gla domains allows them to recognize and bind phosphatidylserine (PS), acting as bridge molecules to ensure the engulfment of ACs that expose PS on the surface of the cells (usually confined to the inner face of the plasma-membrane in living cells).

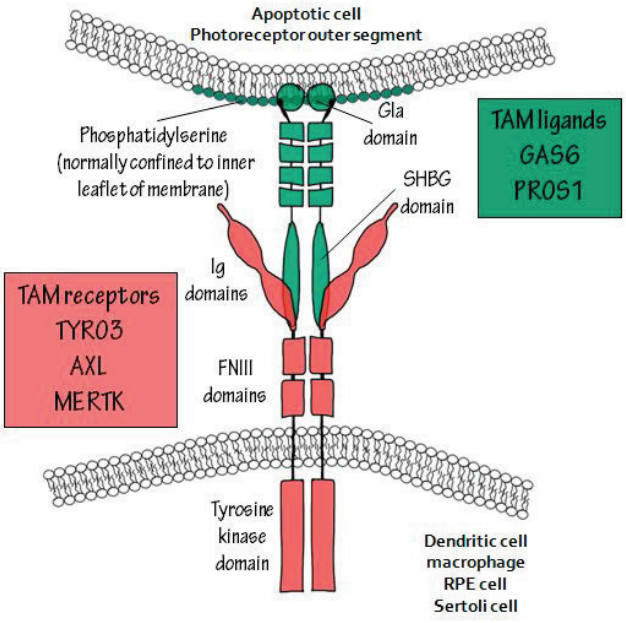


Figure 10. TAM receptors and ligands interaction.

Adapted [124], Nature Reviews Immunology 2008.

Both ligands carry at the C-terminus 2 laminin G domain that forms the sex hormone binding globulin-like domain (SHBG). This region binds the Ig domain of the TAM receptors, induce dimerization and triggers the activation of the receptors. Notwithstanding, three additional ligands for TAM receptors have been recently identified (tubby, tubby-like protein 1, and galectin 3), but their roles are still poorly understood [128, 129] (**Figure 10**).

MERTK

As mentioned above, the daily clearance of physiologically dying cells is essential for the regulation of homeostasis of the body, and TAM receptors function is strongly related to the **clearance of apoptotic cells** (ACs). Specifically, MERTK has been reported as a major macrophage AC receptor. Activation of MERTK by their soluble ligands -GAS6 or PROS1- bound to AC restricts the intensity of inflammatory cytokine production and immune responses mainly by inhibiting DC activation; thereby maintaining self-tolerance [130-132]. In humans, MERTK is expressed on DCs, NK cells, B-cells, M2c macrophages and platelets [133-135]. The biologically relevant cellular sources of GAS6 and PROS1 required for TAM activation remain to be determined. Although the role of MERTK and its ligands have become more apparent in the last years, most of the studies have been performed based on engineered loss-of-function mutants in mice [130, 131, 136], hence MERTK regulation in humans remains still unclear.

TAM inhibition of inflammation

Besides their role in the clearance of ACs, TAM receptors expressed in DCs and macrophages are fundamental for the regulation of the immune response [124]. Since 1999, the possibility of inactivating genes encoding the three TAMs in mice has helped understanding the function of these receptors. Observation of the immune system phenotypes of mice lacking all possible combinations of TAM receptors genes provided initial evidence for TAMs involvement in the regulation of the immune response [122, 123, 136]. Thus, the more number of inactivated TAM genes the more severe phenotype is observed in mice. Remarkably, **triple knock-out mice** (TKO), animals lacking the three TAM receptors, are fully viable and superficially normal at birth. However, TAM TKO mice eventually develop a broad-spectrum autoimmune disease, mainly characterized by lymphoadenophathy and splenomegaly [123] (**Figure 11**).

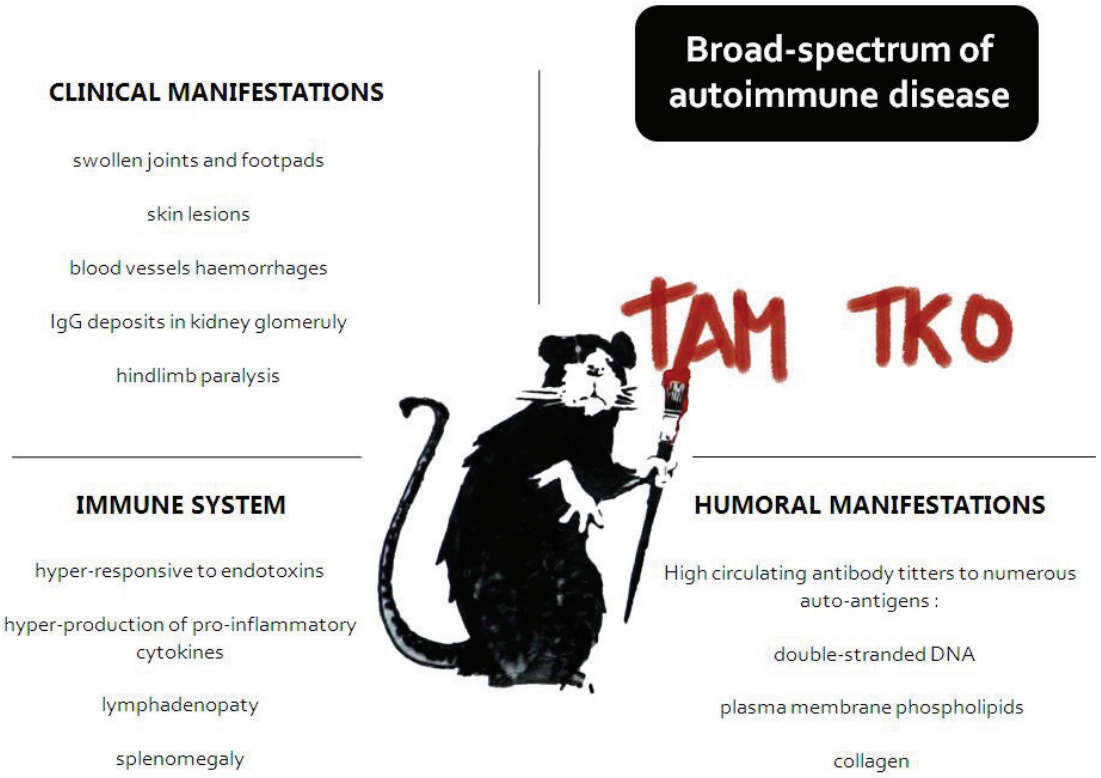


Figure 11. Broad-spectrum of autoimmune disease in TAM TKO mice. *Picture adapted from Banksy, Out of bed rat, Los Angeles.*

TAM mutant mice present a characteristic phenotype which probably results from the loss of TAM regulation of two related events: the phagocytosis of ACs by DCs and macrophages and the regulation of the innate inflammatory response of these APCs. As mentioned above, the importance of TAM receptors in the recognition and clearance of ACs has been largely investigated [131, 137, 138]. Recently, significant progress has been made in understanding the steps involved in prompt cell clearance *in vivo* [139]; particularly, the responses of the phagocytes that keep cell clearance events "immunologically silent" [140-142]. For instance, in the context of the intestinal immune system, mucosal APCs phagocyte apoptotic intestinal epithelial cells and are expert inducers of tolerance to self-antigens [143].

In 2007, Rothlin *et al*. further described that activation of the TAM receptors also **limits** the intensity and duration of **inflammatory responses**. The immune system must be tightly regulated in order to rapidly respond to bacteria, viruses and other pathogens to trigger an inflammatory response. Thus, it is conceivable that a mechanism that turns it off must be tied to a mechanism that turns it on. TAM receptors participate as pivotal inhibitors that prevent unrestrained inflammatory responses in APCs by inhibiting TLRs and cytokine receptors signalling [136]. These observations in TAM-deficient mice and analyses of TAM-mediated signal transduction have revealed multiple points in TLR signalling transduction cascade that are inhibited by TAMs (such as ERK1/2, NF-κB, and TRAF3/6). Moreover TAM receptors have also been found to inhibit TLR-induced production of inflammatory cytokines through the induction of SOCS proteins (suppressors of cytokine signaling). Several studies have shown that type I IFN receptors are the main inducers of SOCS proteins [144]. In this report, Rothlin *et al.* also demonstrated that the induction of SOCS proteins by IFNAR activation proceeds through and is dependent on TAM receptors. **Figure 12** summarizes these findings; TLR signalling induces cytokine receptors expression (**A**), in turn, TAM receptors are induced downstream of cytokine receptor signalling (IFNAR) in a STAT dependent manner (**B**). Subsequently, activation of TAM receptors in conjunction with cytokine receptors leads to the induction of SOCS genes and the suppression of both TLR and cytokine receptor signalling (**C**).

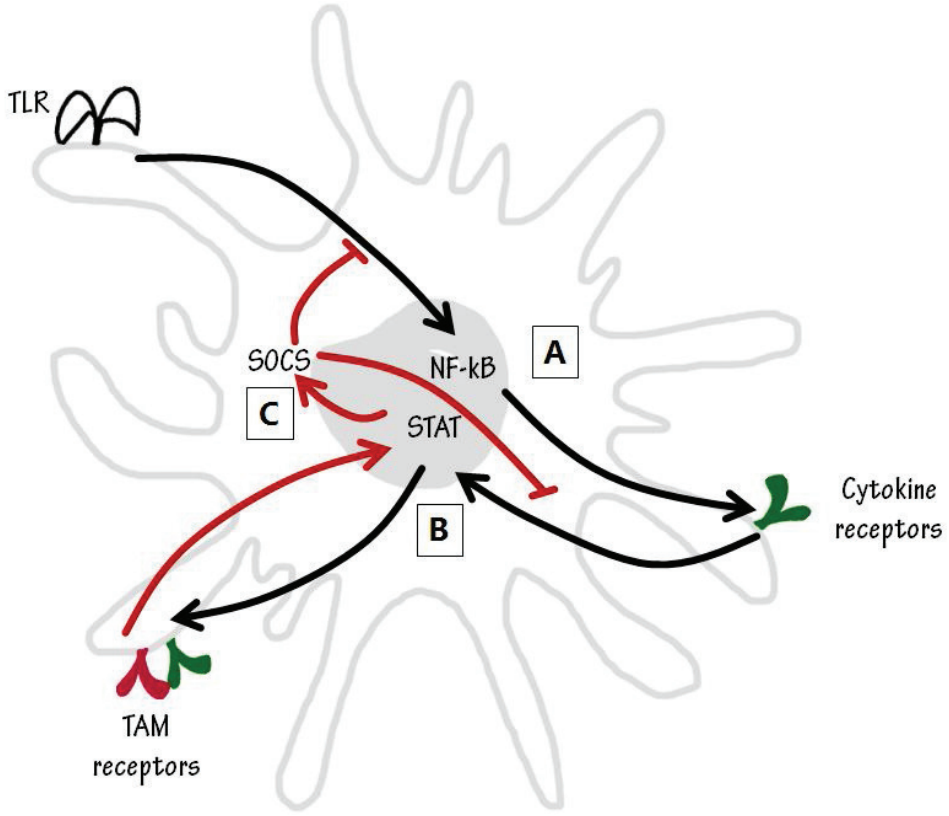


Figure 12. TAM receptors are potent inhibitors of the innate immune response.. *Adapted from [127], Inflammatory Bowel Diseases 2014.*

Given this unifying role of TAM signalling in the “silent” phagocytic removal of ACs, and in the inhibition of inflammatory responses, it would be interesting to further dissect TAM receptors dual function *in vivo* to better understand the regulation of these two tightly linked phenomena [130, 136].

TAM receptors and disease

TAM receptors and cancer

While TAM deficiency strongly contributes to autoimmune diseases, it is worth noting that TAM receptors are frequently over-expressed in human cancers, particularly AXL and MERTK. Actually, myeloid leukemia and lymphoblastoid lines were the source that allowed the cloning of the first cDNAs of these two receptors [133, 145]. The clinical association of their expression is often related with aggressive disease and poor survival outcome [146]. However, a definitive demonstration that **TAM over-expression** is fundamental for particular features of cancer development has not been made.

Over the last few decades, there has been a surge in papers that link TAM receptors and ligands to multiple aspects of cancer biology. Leukaemia, colorectal carcinoma or prostate cancer, among several others, have been reported to over-express TAM signalling components [147-149]. Although evidence indicates that TAMs can activate classic oncogenic pathways, such as PI3K and ERK, their role in tumor-tolerance induction might be also relevant due to their known function in regulating immune system and phagocytosis. Therefore, TAM receptors could contribute to tumor immune escape by counteracting signals for immunogenic cell death [150].

Interestingly, it has been reported that cancer progression in various model systems can be inhibited by interfering with TAM signalling through different molecular strategies [151-154]. However, the dissection of the pro-oncogenic and anti-oncogenic function of TAM receptors in cancer still needs to be addressed.

TAM receptors and autoimmunity

Autoimmune diseases are characterized by chronic inflammation and autoreactivity that arise when the immune system makes fundamental mistakes discriminating between self and non-self. As outlined above, TAM TKO mice display features of systemic autoimmunity. However, this impaired function is not restricted to mice mutant phenotypes. Several years investigating TAM

receptors in different autoimmune diseases, including Systemic lupus erythematosus (SLE), RA, Multiple sclerosis (MS) and IBD, suggests that **diminish TAM signaling** may contribute to human autoimmunity [155]. The pathogenesis of SLE has been associated with an impaired clearance of ACs in the germinal centers. Indeed, a recent analysis of a large cohort of SLE patients found low levels of PROS1, but not GAS6, in the circulation [156]. In addition, polymorphisms in the *MERTK* gene have been linked to SLE [157]. On the other hand, there is few literature relating low circulating levels of PROS1 to IBD, but still some studies have established this link [158-160]. Furthermore, a large genome-wide association study identified polymorphisms in the *MERTK* gene as risk factors for the development of MS [161, 162].

Besides TAM association with human autoimmune diseases, it is interesting to remark that the most commonly used drugs to treat the chronic inflammation, have recently shown to potentiate TAM signaling [163]. Therefore, drugs such as glucocorticoids (GC) or LXR agonists are able to up-regulate MERTK expression and consequently stimulate macrophage phagocytosis of ACs [164, 165]. Although these are promising findings regarding new potential targets to treat autoimmune diseases, more studies in humans are needed to fully understand the complexity of TAM system.

To conclude, TAM receptors have been strongly related to both autoimmunity (loss of function) and cancer (gain in function), suggesting that TAMs function on two sides of a common coin. These features make the TAM system a particularly favourable target for therapeutic intervention.

Hypothesis and objectives

Hypothesis and Objectives

The quality of life of a significant proportion of IBD patients is poor as a result of persistent disease activity and repeated surgery, among others. Current treatments for Crohn's disease, including the most recent biologicals, are not able to neither prevent this serious impact nor improve the long term prognosis of a significant proportion of IBD patients. Therefore, new therapeutic approaches are needed in order to modify or redirect the immune response of these patients.

We **hypothesize** that administration of *ex-vivo* generated autologous tol-DCs to Crohn's disease patients may arrest Th1 lymphocyte proliferation and therefore may restore specific tolerance against non-pathogenic antigens in the gut. The **overall objective** of this thesis was to generate and characterize tol-DCs for the purpose of implementing an autologous immunotherapy treatment for Crohn's disease patients. To achieve these objectives, the following **specific aims** were defined:

1. Generation and characterization of tol-DCs in GMP conditions.

    a) Standardization of a protocol in order to induce a tolerogenic phenotype to monocyte-derived DCs from Crohn's disease patients.

    b) Characterization of the tolerogenic properties of tol-DCs.

    c) Evaluation of the stability of the tolerogenic properties.

    d) Evaluation of the activation profile of tol-DCs when exposed to Gram-negative enterobacteria.

2. Understanding the mechanisms underlying the tolerogenic function of DCs.

    a) Identification of a biomarker for tol-DCs.

    b) Perform functional studies to understand the potential effect of this biomarker in the induction of tolerance.

Results

Results I

Gram-negative Enterobacteria Induce Tolerogenic Maturation In Dexamethasone-conditioned Dendritic Cells

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***PLoS One* (2012);7(12)**

OPEN ACCESS Freely available online

PLOS ONE

Gram-Negative Enterobacteria Induce Tolerogenic Maturation in Dexamethasone Conditioned Dendritic Cells

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Abstract

Dendritic cells have been investigated in clinical trials, predominantly with the aim of stimulating immune responses against tumours or infectious diseases. Thus far, however, no clinical studies have taken advantage of their specific immunosuppressive potential. Tolerogenic DCs may represent a new therapeutic strategy for human immune-based diseases, such as Crohn's disease, where the perturbations of the finely tuned balance between the immune system and the microflora result in disease. In the present report, we describe the generation of tolerogenic DCs from healthy donors and Crohn's disease patients using clinical-grade reagents in combination with dexamethasone as immunosuppressive agent and characterize their response to maturation stimuli. Interestingly, we found out that dexamethasone-conditioned DCs keep their tolerogenic properties to Gram-negative bacteria. Other findings included in this study demonstrate that the combination of dexamethasone with a specific cytokine cocktail yielded clinical-grade DCs with the following characteristics: a semi-mature phenotype, a pronounced shift towards anti-inflammatory versus inflammatory cytokine production and low T-cell stimulatory properties. Importantly, in regard to their clinical application, the tolerogenic phenotype of DCs remained stable after the elimination of dexamethasone and after a second stimulation with LPS or bacteria. All these properties make this cell product suitable to be tested in clinical trials of inflammatory conditions including Crohn's disease.

Citation: Cabezón R, Ricart E, España C, Panés J, Benitez-Ribas D (2012) Gram-Negative Enterobacteria Induce Tolerogenic Maturation in Dexamethasone Conditioned Dendritic Cells. PLoS ONE 7(12): e52456. doi:10.1371/journal.pone.0052456

Editor: Phillip A. Stumbles, Murdoch University, Australia

Received September 6, 2012; **Accepted** November 19, 2012; **Published** December 27, 2012

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Funding: This work was supported by grant SAF 2009-07272 from the Ministerio de Ciencia e Innovacion, grant TRA-097 from the Ministerio de Sanidad y Politica Social, and by Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBERehd). DB-R is supported by CIBERehd and by the Instituto de Salud Carlos III, RC is funded by a FI fellowship from the Generalitat de Catalunya. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Dendritic cells (DCs) represent the most potent antigen-presenting cells linking innate and adaptive immune responses. DCs express a set of receptors involved in pathogen recognition. Known as pattern-recognition receptors (PRR), they include Toll-like receptors (TLR), C-type lectins and the cytoplasmic NOD family, as well as RIG-I and MDA-5 molecules [1]. Interaction of these receptors with their specific ligands leads to DC differentiation to an activated state. Their role in the immune system is crucial, either by initiating effective immune responses or by inducing tolerance, depending on the presence or absence of danger associated molecular patterns within endocytosed particles [2].

Due to their physiological properties [3] DCs have been safely and successfully used in clinical trials aimed at stimulating an efficient immune response against tumors in humans [4,5]. However, only one recent study has taken advantage of their specific tolerogenic properties by utilizing CD40, CD80 and CD86 antisense transfected DCs to treat diabetic patients [6]. The tolerogenic properties of immature autologous DCs have already been documented in healthy human volunteers, providing proof of principle that systemic antigen-specific T-cell tolerance can be achieved using this approach in humans [7]. However, an important concern when designing DC-based immunotherapy protocols is whether immature DCs might inadvertently receive *in vivo* maturation signals in an inflammatory microenvironment, either from pro-inflammatory cytokines and/or pathogen-derived molecules or whole microorganisms [8]. An alternative to the use of immature DCs is to generate tolerogenic DCs (tol-DCs). The addition of immunosuppressive agents, pharmacological modulation, or inhibitory cytokines during the process of DC differentiation from monocytes influences the functional properties of the resulting cells [9,10]. Recently, a study between clinical-grade DCs compared the phenotypic characterization of human DCs using different tolerogenic agents [11]. These studies demonstrate that activation of tol-DCs might actually be a critical step in optimizing the re-stimulation and/or expansion of functional Tregs rather than in maintaining their immaturity [12,13]. Alternative activated DCs differentially regulated naïve and memory T cells; specifically, naïve T cells were sensitized and polarized towards a low IFN-γ/high IL-10 cytokine profile, whereas memory T cells were anergized in terms of proliferation and cytokine production [14]. The studies described above were carried out using animal

models or DC lines [15,16]. However, the use of reagents that fail to fulfil GMP requirements, such as LPS, cytokines or fetal calf/bovine serum [17], makes this approach unfeasible for human trials [18]. An important obstacle to overcome in translating this method to a human setting is the need for reproducible, high-quality stable tol-DCs [19]. Furthermore, given the importance of genetic predisposition in the majority of immune mediated inflammatory disorders, it needs to be proven that tol-DCs produced from patients' monocytes have the same tolerogenic functions as those of healthy controls.

In this study, we characterized the tolerogenic properties of monocyte-derived DCs from healthy donors and Crohn's disease patients generated under clinical-grade conditions. In addition, we evaluated not only the stability of the tolerogenic phenotype after washing out all of the factors, but also the activation profile of those cells when exposed to different Gram-negative enterobacteria a physiologic stimuli that tol-DCs will likely encounter after administration to patients. This approach takes advantage of the complexity of the microbes that provide, at the same time, a variety of stimuli for innate receptors to elicit polarizing cytokines.

Materials and Methods

Generation of Human DCs and Cell Cultures

The present study was approved by the Ethics Committee at the Hospital Clinic of Barcelona. Buffy coats were obtained from Banc de Sang i Teixits and written informed consent was obtained from all blood donors. PBMC from Crohn's disease patients were obtained with written informed consent to participate in the study. DCs were generated from the peripheral blood samples as previously reported [4]. In summary, PBMCs were allowed to adhere for 2 h at 37°C. Non-adherent cells peripheral blood lymphocytes (PBLs) were gently removed, washed, and cryopreserved. The adherent monocytes were cultured in X-VIVO 15 medium (BioWhittaker, Lonza, Belgium) supplemented with 2% AB human serum (Sigma-Aldrich, Spain), IL-4 (300 U/ml), and GM-CSF (450 U/ml) (Both from Miltenyi Biotec, Madrid, Spain) for 6 days in order to obtain immature DCs (iDCs). The maturation cocktail consisted of IL-1β, IL-6 (both at 1000 IU/ml), TNF-α (500 IU/ml) (CellGenix, Freiburg, Germany) and Prostaglandin E2 (PGE2, 10 μg/ml; Dinoprostona, Pfizer) and was added on day 6 for 24 h. Mature DCs (mDCs) were harvested and analyzed on day 7. Dexamethasone (10^{-6} M; Fortecortin, MERCK, Spain) was added on day 3. For cell stability, DCs were washed and further stimulated for 24 h with 100 ng/ml LPS (Sigma Aldrich) or 1 μg/ml of recombinant soluble CD40 ligand (Bender Medsystems, Vienna, Austria). We did not observe differences in viability and yield between iDCs, mDCs and tol-DCs generation. The protocol and reagents for tol-DC generation are fully compatible with cGMP regulations and it has been approved by Agencia Española del Medicamento y Productos Sanitarios.

Heat-killed *Escherichia coli*, *Protheus mirabillis*, *Klebsiella pneumoniae and Salmonella thyphimurium* were incubated at 1:10 (DC:bacteria) ratio with DCs for 24 h. After co-incubation, supernatant was collected for cytokines determination and DCs phenotype was then analyzed.

Flow Cytometry

To characterize and compare the phenotype of the DC populations, flow cytometry was performed. The following mAbs or appropriate isotype controls were used: anti- CD14 (eBioscience, San Diego, CA), CD80, CD83, CD86 (BD-Pharmingen), CCR7, MHC class I (W6/32 a generous gift from Dr. Ramon Vilella, Dept of Immunology Hospital Clinic de Barcelona) and FITC-labeled MHC class II (BD-Pharmingen). Primary antibodies were followed by staining with PE-labelled goat-anti-mouse (from BD Pharmingen™). Flow cytometry was performed using a FACSCalibur™ with CellQuest software (BD Biosciences) and data were analyzed using WinMDI software (version 2.9; http://facs.scripps.edu/software.html), FACSCanto II, and analyzed with BD FACSDiva 6.1™ software.

T-cell Stimulation

For co-culture experiments, PBLs and naïve $CD4^+$ T cells were isolated from healthy individuals using the $CD4^+$ and naïve $CD4^+$ T isolation kit (Miltenyi Biotec, Spain), according to the manufacturer's instructions. The allo-response was tested in a mixed lymphocyte reaction; allogeneic T cells were co-cultured with DCs differently generated in a 96-well microplate. For Ag-specific T-cell responses, 1 μg/ml of tetanus toxoid (TT) (Sigma-Aldrich, Spain) or 10 ng/ml of superantigen toxic shock syndrome toxin-1 (TSST-1) (Sigma-Aldrich, Spain) loaded DCs were co-cultured with autologous T lymphocytes in a 96-round well microplate. For the proliferation assay, a tritiated thymidine (1 μCi/well, Amersham, UK) was added to the cell cultures on day six and an incorporation assay was measured after 16 h. For some experiments T cells were labelled with CFSE and plated in fixed amounts of 10^5 cells/well. T-cell proliferation was determined by the sequential dilution of CFSE fluorescence in positive cells, as detected by flow cytometry. TT-specific cell lines were generated by adding 1 μg/ml of TT to PBMCs for one week and further cell expansion with 50 IU/ml of IL-2 for an extra week.

Anergy Induction

For anergy induction, $1*10^6$ of highly (>98%) purified naïve $CD4^+$ $CD45RA^+$ T cells were co-cultured with DCs (iDCs, mDCs and tol-DCs) in a 6-well plate for 1 week (ratio 1:10; DC:T). After extensive washing, T cells were expanded and rested in the presence of IL-2 and IL-7 for an additional week. T lymphocytes were washed and re-stimulated by co-culturing $1*10^5$ T cells with matured DCs from the original donor at 1:20 ratio in 96-well plates. After 6 days, plates were pulsed with ^{3}H-thymidine and measured as described above.

Cytokine Production

DC supernatants were collected and frozen after 24 h of activation. IL-10, IL-12p70, IL-23 and TNF-α from the DCs supernatants and IFN-γ and IL-10 from the T-cell cultures were analyzed by ELISA according to the manufacturer's guidelines.

mRNA Isolation, cDNA Synthesis, and Real-time PCR

Total RNA was isolated from DCs using an RNeasy Mini Kit (Qiagen, Germany). RNA was transcribed to cDNA using a High-Capacity cDNA Archive RT kit (Applied Biosystems, USA), and was then used to perform quantitative real-time PCR in triplicate wells with a TaqMan Universal PCR Master Mix (Applied Biosystems) containing IL-10 and IL-12p35 and ß-actin (TaqMan primers and probes; Applied Biosystems). PCRs were performed using an Applied Biosystems 7500 Fast Real-Time PCR System sequence detection system. mRNA content (x) was calculated using the formula $x = 2^{-\Delta Ct}$ (where ΔCt = Ct target gene-Ct housekeeping gene) were calculated for each gene and setting using ß-actin as a housekeeping gene. Fold-increase expression of target genes in mDCs or in tol-DCs was determined relative to iDCs.

Statistical Analysis

Results are shown as the mean ± SD. To determine statistical differences between the means of two data sets, the paired or independent sample two-tailed Student *t*-tests were used. Statistically significant difference was set at $p<0.05$.

Results

Tolerogenic DCs Display a Semi-mature Phenotype

The presence of dexamethasone during DC diferentiation partially impaired the upregulation of co-stimulatory molecules such as CD80 (38% reduction, $p<0.001$), the maturation marker CD83 (40% reduction, $p<0.001$), and the HLA-DR (39% reduction, $p<0.05$) compared with fully mDCs (**Figure 1A**). CD86 was highly expressed on iDCs and we did not observe any significant changes in the expression of CD86 upon activation in tol-DCs compared to mDCs. Consistently, similar phenotypic results were obtained by stimulation of dexamethasone-treated DCs with TLR ligands, such as LPS (data not shown), as elsewhere described [20,21,11]. The maturation of DCs resulted in a tightly regulated production of pro- and anti-inflammatory cytokines, depending on the type of stimuli. In accordance with the tolerogenic phenotype shown in **Figure 1A**, tol-DC cytokine secretion resulted in significantly higher production of the anti-inflammatory cytokine IL-10 (mean = 510±453 pg/ml) compared with either iDCs (68±69 pg/ml, $p<0.001$) or mDCs (51±59 pg/ml, $p<0.001$) (**Figure 1B**). The inflammatory cytokines IL-12p70 and IL-23 remained undetectable in the supernatants of either tol-DCs or mDCs, which is coherent with the absence to TLR-L on the maturation cocktail [22,23]. In order to confirm these results, we analyzed the transcripts of these cytokines by real-time PCR. mRNA levels for the pro-inflammatory cytokine IL-12p35 were significantly reduced in tol-DCs compared to mDCs (**Figure 1C**), whereas the RNA levels of IL-10 exhibited a significant six-fold increase in tol-DCs compared with mDCs, thus corroborating our results at the protein level.

Tolerogenic DCs Show Reduced T-cell Stimulatory Capacity

To determine the functional properties of clinical-grade tol-DCs, we analyzed their T-cell stimulatory capacity. Tol-DCs induced a lower proliferative allo-response (mean cpm = 40.879, $p<0.05$) compared to mDCs (cpm = 74.651), whereas the response to iDCs was also low (mean cpm = 23.634, $p<0.001$ vs mDCs) as expected, **Figure 2A**. We also investigated the capacity of tol-DCs to present exogenous antigen to autologous T cells. As depicted in **Figure 2B**, tol-DCs exhibited a reduced antigen-presenting capacity to autologous T cells compared with control DCs, when the latter were loaded with either the superantigen toxic shock syndrome toxin-1 (TSST-1) or tetanus toxoid (TT). Thus, tol-DCs were poorer stimulators of allo- or antigen-specific T-lymphocyte responses (in allogeneic and autologous settings) than mDCs.

Tolerogenic DCs Generate Antigen-specific Anergic T cells

To evaluate the ability of tol-DCs to induce $CD4^+$ T-cell hyporesponsiveness, allogeneic highly purified $CD4^+$ naïve T cells (purity 98% $CD4^+CD45RA^+$) were initially primed for 14 days during the first round with iDCs, mDCs or tol-DCs (initial challenge) and then were re-stimulated (re-challenged) with iDCs or fully competent mDCs from the original donor. T cells exposed to tol-DCs exhibited a reduced capacity to proliferate as well as reduced IFN-ÿ secretion when re-challenged with fully competent mDCs. In contrast, T cells exposed to control DCs proliferated and secreted IFN-γ to a high degree (**Figure 3A**). To confirm the capacity of tol-DCs to mitigate effector T cells, tetanus toxoid (TT)-specific T cell lines were re-stimulated with TT loaded or control (non-loaded) mDCs. Whereas T cells primarily exposed to mDCs vigorously responded to TT, as measured by T-cell proliferation and IFN-γ production (**Figure 3B**), those exposed to tol-DCs showed a significantly reduced proliferation and an absolute inability to induce IFN-γ during a secondary response to TT-loaded DCs.

Tolerogenic DCs are Stable and Resistant to Further Stimulation

To address the stability of tol-DCs, dexamethasone and cytokines were carefully washed away and the DCs were re-stimulated with secondary maturation stimulus. Tol-DCs were refractory to further stimulation with LPS (**Figure 4A**, data from **n = 6** independent experiments) and CD40L (**n = 4**), maintaining a stable semi-mature phenotype. Interestingly, tol-DCs retained their ability to further produce high levels of IL-10, but failed to generate IL-12 or IL-23 following stimulation with LPS (**Figure 4B**) data not included for negative IL-12 and IL-23), we did not detect any cytokine after CD40L stimulation. Furthermore, tol-DCs re-challenged with LPS or CD40L were unable to induce a proliferative T-cell response (**Figure 4C**). In addition, the lower levels of IFN-γ cytokine secretion by T cells stimulated with LPS-treated tol-DCs compared with mDCs (mean 6332±1514 vs 1700±700 pg/ml $p = 0.07$) suggest inhibition of the Th1-type response (**Figure 4C**).

Tolerogenic Response of Dexamethasone-conditioned DCs to Gram-negative Bacteria

Whole microorganisms contain multiple PAMPs capable of stimulating DCs by different pathways. This capacity exemplifies a more physiological setting, versus the use of restricted TLR agonists or exogenous recombinant cytokines. DCs were incubated with Gram-negative heat-inactivated *Escherichia coli (E. coli)*. Interestingly, the presence of dexamethasone during DCs differentiation profoundly influenced cell maturation, exhibiting strong inhibitory effect on their phenotype (**Figure 5A**) with significant reduction in CD83, CD86 and MHC class I and II expression, when compared with DCs without *E. coli*. Importantly, it caused a robust inhibition of pro-inflammatory cytokines (IL-12p70, IL-23 and TNF-α), increased IL-10 secretion (**Figure 5B**), and modified the immune response of T lymphocytes (**Figure 5C**) inhibiting T cell proliferation and Th1 induction. The production of IFN-γ by T cells was inhibited (mean 21550±11782 pg/ml vs 7869±6198 pg/ml; $p = 0.07$) when DCs were conditioned with dexamethasone previously to *E. coli* stimulation. We did not detect any IL-10 in the supernatant of activated T cells.

Tolerogenic DCs are Stable and Resistant to Further Gram-negative Bacteria

To address the stability of tol-DCs, dexamethasone and maturation cytokine cocktail were carefully washed away as described above and DCs were incubated with *E. coli* for further 24 h without dexamethasone or other factors present in the culture. Tol-DCs were refractory to further stimulation with Gram-negative bacteria. Interestingly, tol-DCs produced significantly higher levels of IL-10 in response to *E. coli* than mDCs (mean 1252±694 vs 249±306 pg/ml; $p = 0.01$) even after DC maturation with a cytokine cocktail, whereas the levels of pro-inflammatory cytokines were hardly detected (**Figure 6A**). Fur-

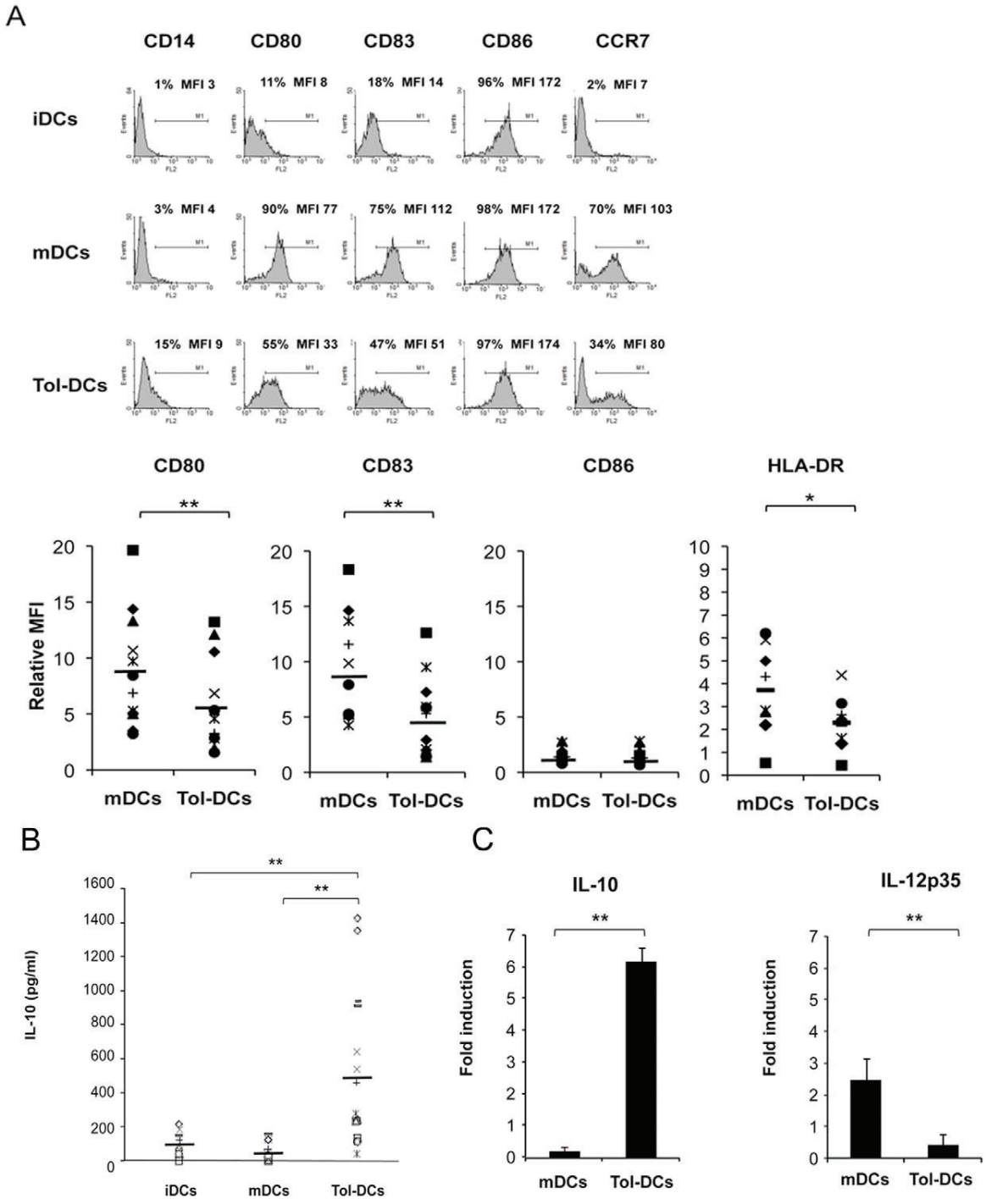

Figure 1. Dexamethasone modulates cytokine cocktail-induced DC maturation. (**A**) Phenotypic analysis of untreated (iDCs), cytokine-activated (mDCs) and 10^{-6} M dexamethasone cytokine-activated dendritic cells (Tol-DCs) was performed by flow cytometry. Representative histogram data set from 12 independent experiments is shown. Maturation associated molecules are depicted in the lower graph as mean fluorescent intensity of expression (MFI) of mDCs and Tol-DCs relative (fold-change expression) to iDCs. (**B**) IL-10 and IL-12p70 were measured in supernatants harvested from DCs. Concentration of IL-10 (in pg/ml) is shown (n = 15). In none of the conditions analyzed were IL-12p70 or IL-23 produced (lowest detection limit 7.6 pg/ml). (**C**) Transcripts levels of IL-10 and IL-12p35 were determined by real-time PCR using β-actin as the endogenous reference gene. Data represent fold-change induction relative to iDCs (n = 3). Student's *t*-test: $*p<0.05$, $**p<0.001$.
doi:10.1371/journal.pone.0052456.g001

thermore, when we evaluated the capacity of DCs to generate Th1 response we observed that tol-DCs induced significant lower IFN-γ levels compared to mDCs (**Figure 6B**). The results obtained with *E. coli* were further confirmed and strengthened when

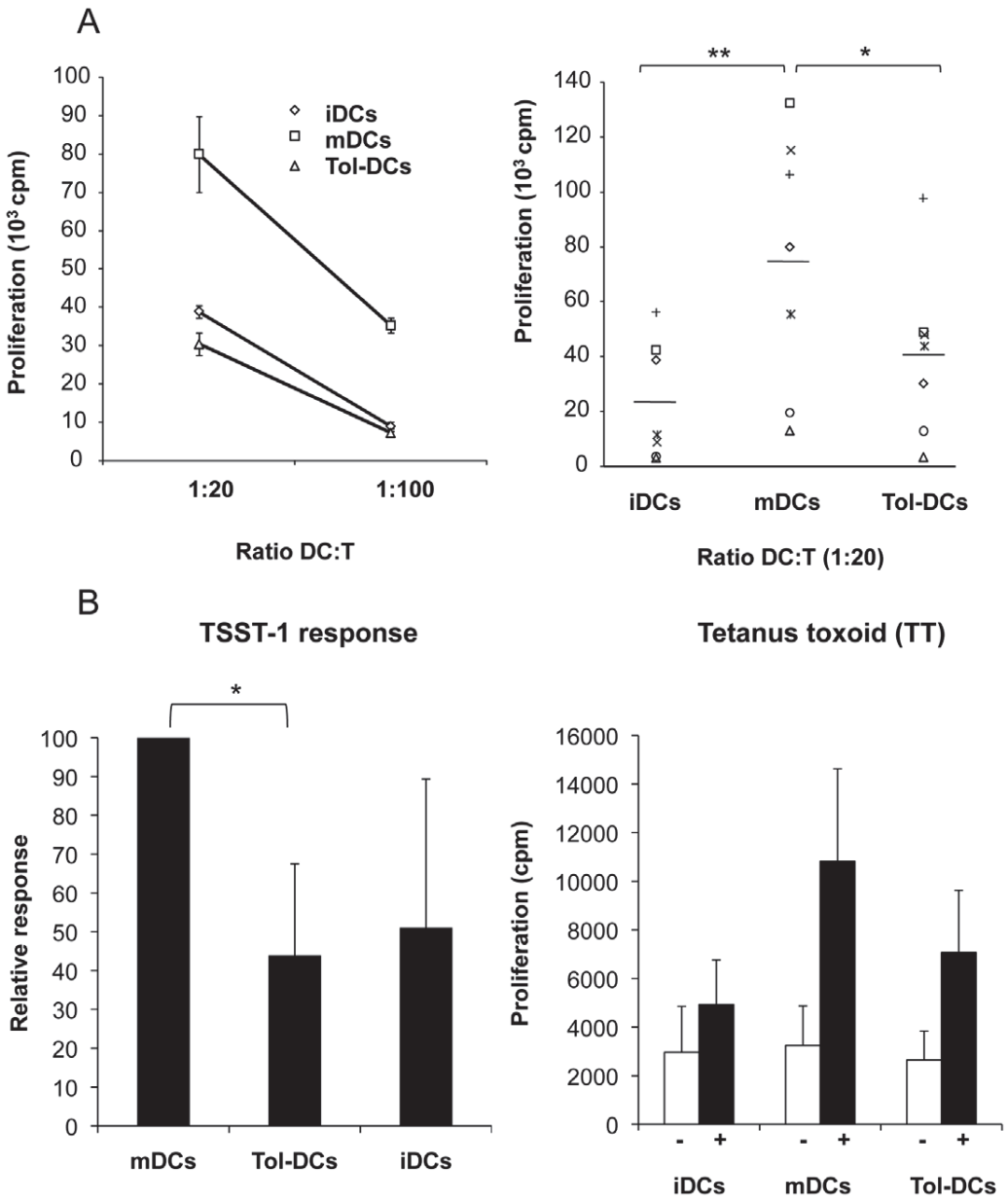

Figure 2. Tol-DCs have a reduced capacity to stimulate T lymphocytes. (**A**) DCs were cultured with allogeneic PBL at different ratio (1:20 or 1:100) for seven days. Upper-left panel data represent the mean ± SD of a representative experiment carried out in triplicate of the seven (upper-right graph) that were independently performed. (**B**) Antigen-specific T-cell responses. $CD4^+$ T cells we cultured with autologous DCs pre-loaded with the superantigen TSST-1 (left graph) or with tetanus toxoid (+ presence and – absence of TT) at a 1:20 ratio for seven days. T-cell proliferation was determined in triplicate by ^3H thymidine incorporation. Data represent the mean ± SD of n = 3 independently performed experiments. Student's *t*-test: *$p<0.05$, **$p<0.001$.
doi:10.1371/journal.pone.0052456.g002

different Gram-negative enterobacteria. *P. mirabillis*, *K. pneumoniae and S. thyphimurium* were incubated with dexamethasone-conditioned DCs (**Figure 7A**) or with tol-DCs (dex-DCs plus maturation cocktail) (**Figure 7B**) after washing out the immunosuppressive agent and cytokines. Although, mDCs and tol-DCs stimulated with bacteria provoked a comparable T cell proliferative response, the IFN-γ secretion was significantly reduced in both culture conditions (no IL-10 was detected in any condition) (**Figure 7**). These results show the incapacity of dex-DCs or tol-DCs to generate Th1 response measured by IFN-γ production revealing the stability of the tolerogenic properties, even after strong and activation induced by Gram-negative bacteria.

DCs from Crohn's Disease Patients can be also Educated towards a Tolerogenic Phenotype

In order to validate the tol-DCs generation in the context of an inflammatory disease, DCs from Crohn's disease patients were generated and analysed. As depicted in **figure 8A**, tol-DCs generated from Crohn's disease patients showed a statistically significant impairment in the upregulation of CD80, CD83 and

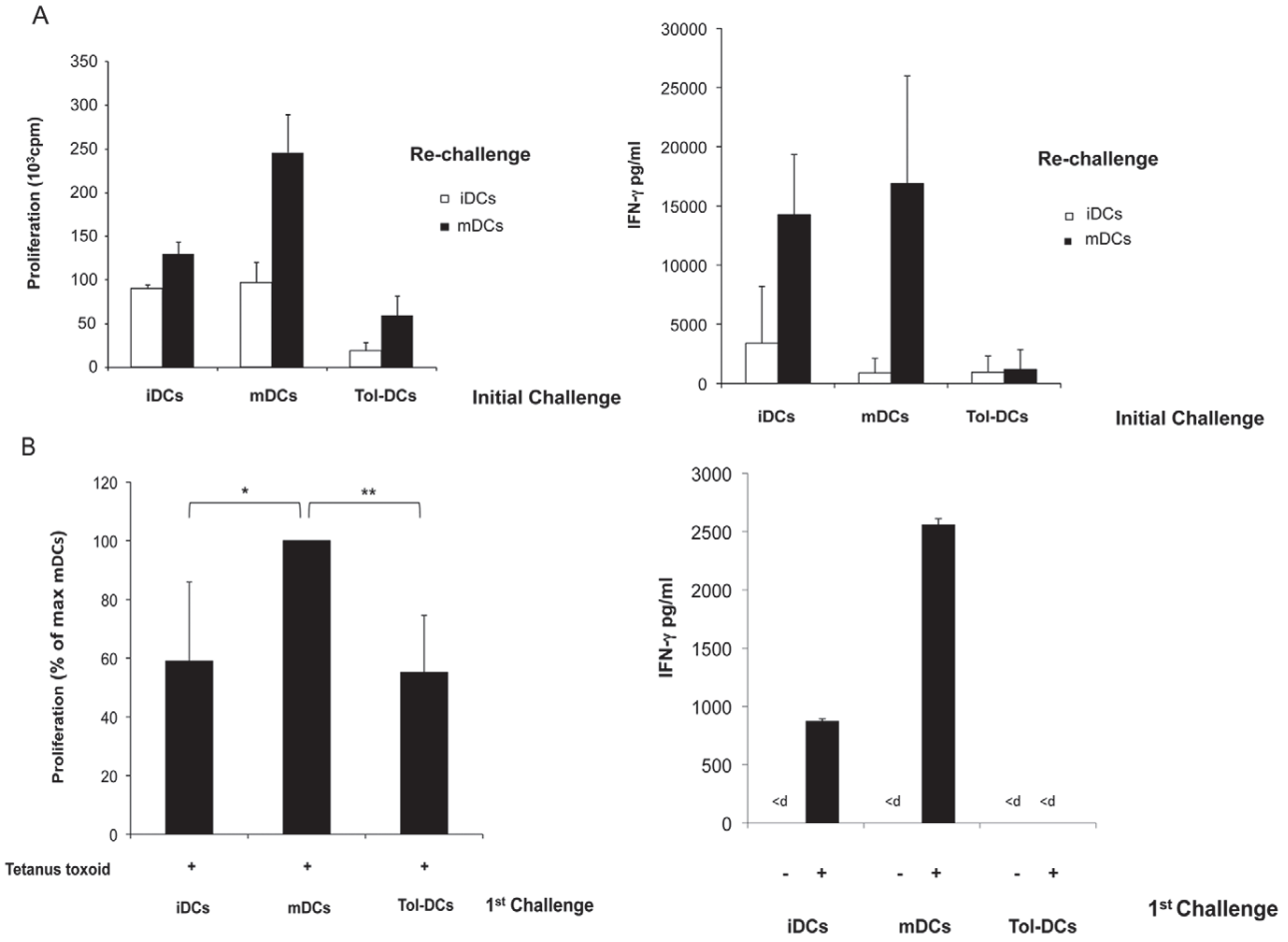

Figure 3. Tol-DCs induce anergic T cells. (**A**) Naïve $CD4^+$ $CD45RA^{++}$ T cells were primarily primed with allogeneic iDCs, mDCs or tol-DCs for 7 days. After 5 days, anergy induction was examined by re-stimulation of primed $CD4^+$ T cells with iDCs or mDCs from the original donor. (**B**) TT-specific $CD4^+$ T cells were primed with TT-loaded autologous iDCs, mDCs or tol-DCs for 6 days (initial challenge). After *in vitro* expansion with TT loaded-DCs anergy induction was examined by re-stimulation of TT-specific $CD4^+$ T cells with mDCs loaded (+) with TT at a 1:20 ratio. Data represent the mean ± SD of n = 5 experiments that were independently performed. Proliferation was normalized relative to mDCs loaded with TT (100%) for each independent experiment. Cytokines were determined in the supernatant of cell cultures by ELISA (<d; below detection limit; IFN-γ data represent mean ± SD of n = 3).
doi:10.1371/journal.pone.0052456.g003

HLA-DR compared to iDCs, with no CD86 modification. Interestingly, the levels of IL-10 were significantly increased in the supernatants of tol-DCs of Crohn's disease patients compared to mDCs and iDCs (**figure 8B**) and did not produce pro-inflammatory cytokines like IL-12 or IL-23 (data not included). Furthermore, T cells exposed to tol-DCs from Crohn's disease patients exhibited a significantly reduced capacity to proliferate (mean cpm = 20561±13058 vs 38181±18177; p = 0.037) compared to mDCs, as well as reduced IFN-γ secretion when co-cultured with fully competent mDCs (**figure 8C**). These results show the ability to generate tol-DCs in patients with Crohn's disease.

Discussion

The generation of reproducible and stable clinical-grade tolerogenic DCs is a critical step towards developing therapeutic trials for the treatment of human disorders such as allergies, autoimmune diseases, chronic inflammation, and transplant rejection [19] [24]. The addition of immunosuppressive agents, pharmacological modulation, or inhibitory cytokines when DCs are being generated from monocytes influences the functional properties of the resulting DCs [9,10]. Several agents, including glucocorticoids [25] such as dexamethasone [26,27], mycophenolic acid [28], vitamin D3 (1α,25-dyhydroxyvitamin D_3) [29], retinoic acid [30], the combination of dexamethasone and vitamin D3 [31], or IL-10 [32] have been used to render DCs resistant to maturation [33].

Tolerogenic DCs have been shown to induce T-cell anergy [34], suppress effector T cells, and promote the generation of regulatory T cells (Tregs) [14,35]. Interestingly, some studies [14] have reported that the maturation of dex-conditioned DCs with LPS potentiates the tolerogenic phenotype of DCs.

We performed a detailed phenotype analysis in order to compare iDCs and fully mature DCs with tol-DCs from healthy donors and patients with Crohn's disease and address the stability of tol-DCs. DCs conditioned with dexamethasone displayed a semi-mature phenotype, which is consistent with the tolerogenic DC phenotypes described elsewhere [36]. We also observed an alteration in the DC maturation process; characterized by low-

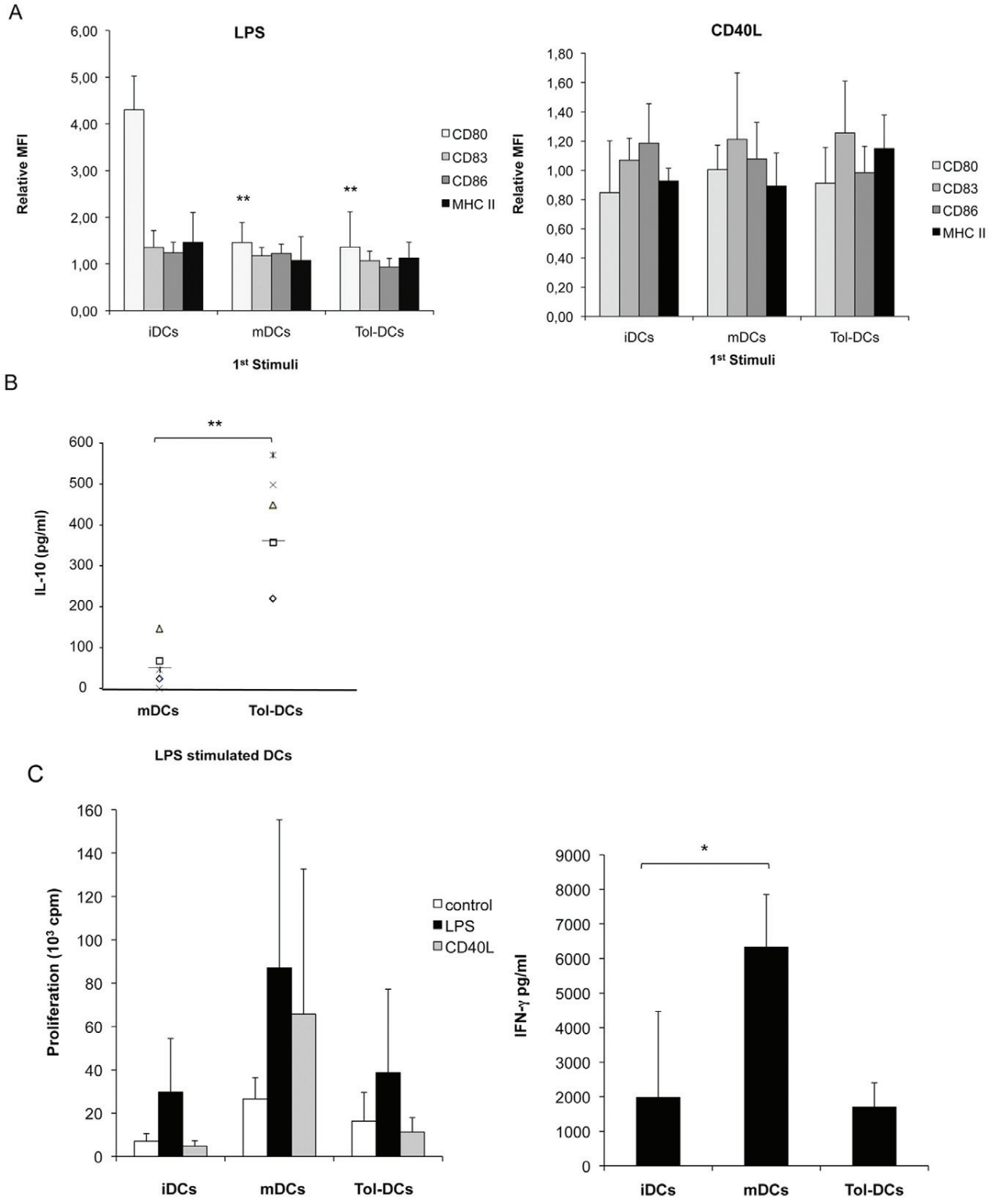

Figure 4. Tol-DCs possess a stable phenotype. DCs were carefully washed to eliminate cytokines and dexamethasone, and viable DCs were further re-challenged with 100 ng/ml of LPS or 1 μg/ml of soluble CD40L as second stimuli. After 24 h, the phenotype (**A**) was analyzed by flow cytometry. Data represent relative MFI increase induced by LPS (n = 6) or CD40L (n = 4) compared to unstimulated iDCs, mDCs or tol-DCs as control. (**B**) IL-10 concentration is shown in pg/ml. IL-12p70 and IL-23 were not detected (detection limit = 7.8 pg/ml). Student's *t*-test: *$p<0.05$, **$p<0.001$. (**C**) Tol-DCs do not recover the ability to stimulate T cells after re-challenge. T-cell proliferation was determined in triplicate by ^{3}H-thymidine incorporation. IFN-γ and IL-10 production in the supernatant was analyzed.
doi:10.1371/journal.pone.0052456.g004

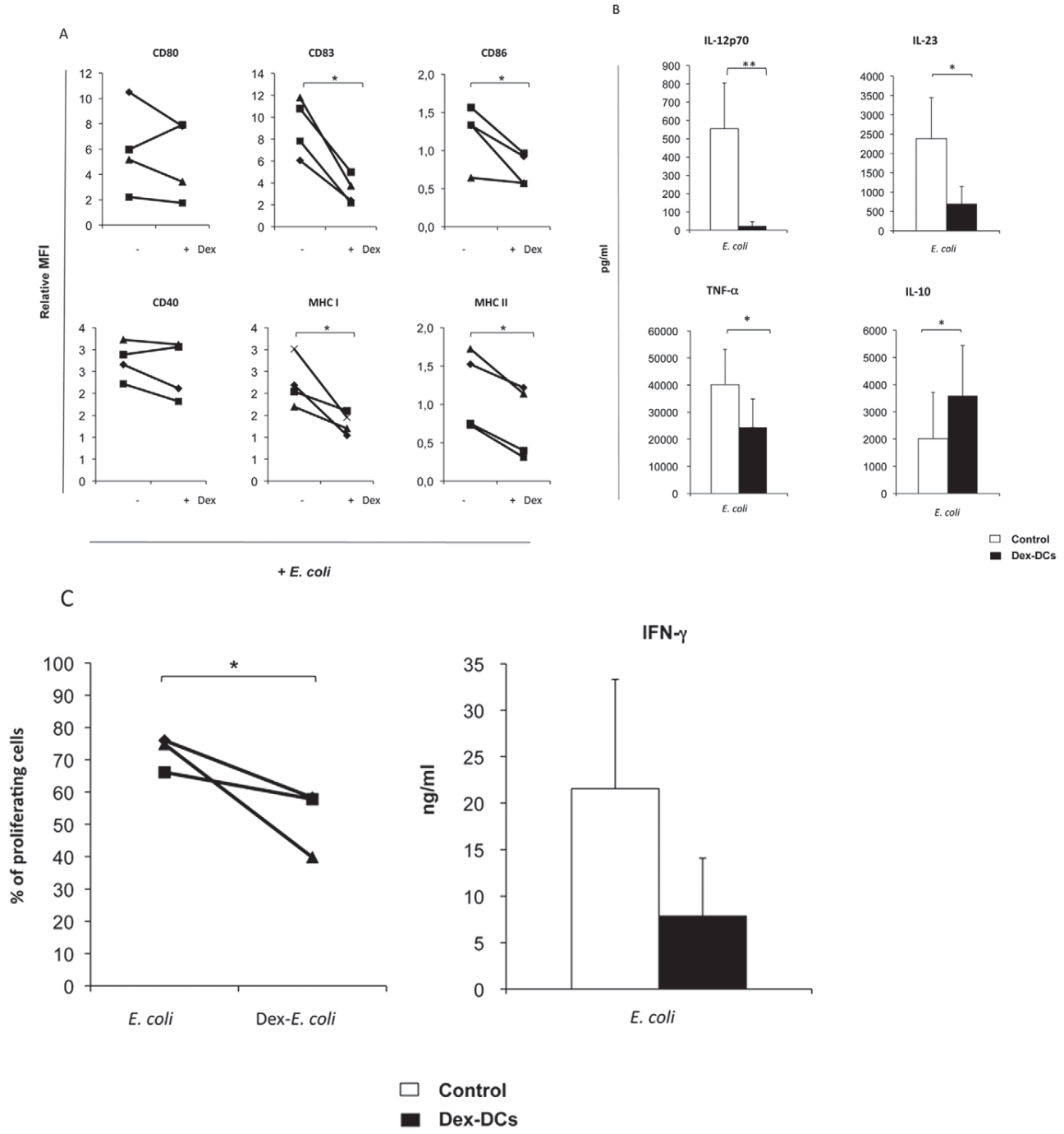

Figure 5. Gram-negative bacteria do not break the tolerogenic properties of dexamethasone-DCs. Heat-killed bacteria were added at ratio 1:10 for 48 h to mo-DCs treated with dexamethasone or untreated as a positive control. **A**. Phenotypic analysis revealed statistically significant reduction of CD83, CD86, and MHC I and class II expression. Maturation associated molecules are depicted as mean fluorescent intensity of expression (MFI) of *E. coli* stimulated-DCs relative (fold-change expression) to control DCs without *E. coli*. (**B**) Cytokines produced by *E. coli*-stimulated DCs. Reduction of IL-12p70 (95.9%; $p<0.05$), IL-23 (70.5%; $p<0.05$) and TNF-α (40%; $p<0.05$) and elevation of IL-10 (78% increase; $p<0.05$) in Gram-negative treated DCs. (**C**) Gram-negative stimulated DCs were cultured after being carefully washed with allogenic PBLs (ratio 1:20) for 7 days. The % of proliferating cells was measured by CFSE dilution using flow cytometry. Significant allo-response inhibition of *E. coli* dex-DC (inhibition 28%; $p<0.05$) compared to control DCs. IFN-γ secretion was analyzed in the supernatant by standard ELISA. Results represent the mean and standard deviation of three independent donors. Student's *t*-test: $*p<0.05$, $**p<0.001$.
doi:10.1371/journal.pone.0052456.g005

intermediate CD80, CD83, CCR7, MHC class I and MHC class II expression. The high levels of CD86 on DCs can be explained by the presence either of human serum or steroids in the culture [37]. Indeed, dexamethasone has been shown to increase CD86

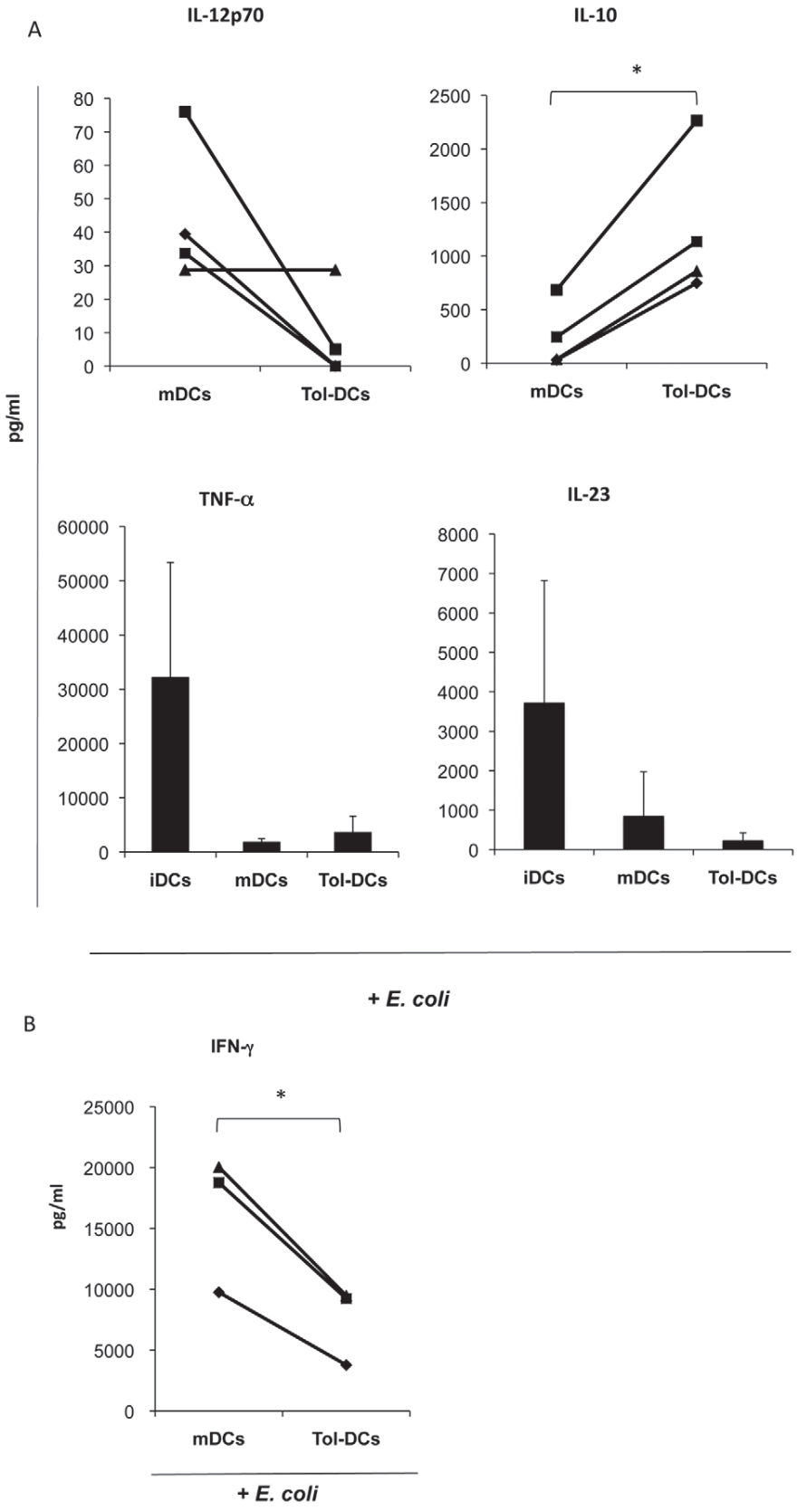

Figure 6. Gram negative *E. coli* induces tolerogenic activation on Tol-DCs. DCs were carefully washed to eliminate cytokines and dexamethasone at day 7, and viable DCs were further re-challenged with *E. coli* (ratio 1:10) without cytokines or dexamethasone. (**A**) Tol-DCs (dex

matured-DCs) produced significant higher levels of IL-10 whereas levels of pro-inflammatory cytokines were very low compared with mDCs or iDCs in response to *E. coli* (n = 4, from each donor, iDCs, mDCs and tol-DCs were generated in parallel). (**B**) The production of IFN-γ was evaluated in the supernatant of allogenic T cells cultured for 7 days with *E. coli* stimulated mDCs or tol-DCs. IFN-γ production was significantly (p = 0.024) reduced in T cells stimulated with tol-DCs plus *E. coli*. IL-10 was not detected in any condition (data not included). Student's *t*-test: *$p<0.05$, **$p<0.001$.
doi:10.1371/journal.pone.0052456.g006

expression through GILZ (glucocorticoid-induced leucine zipper) induction [38]. Furthermore, interactions involving CD80/86 are needed in order to expand Tregs, as was revealed when Treg expansion was inhibited via the use of CD86-blocking antibodies [39]. CCR7 mediates the migration of peripheral DCs to lymph nodes [40]. Although CCR7 expression is induced on DCs by PGE2 [41], we were unable to detect CCR7 expression in tol-DCs by increasing PGE2 concentration (unpublished results). Our data clearly demonstrate that a phenotypic description alone without functional studies appears insufficient for ascertaining the nature of tol-DCs. Comparisons between different tolerogenic agents have revealed the differences among these so-called tol-DCs [11,33]. The cytokine balance determines the type of T-cell effector response when DC-T cell interaction occurs. Pro-inflammatory cytokines like IL-12p70 and IL-23 were absent in tol-DCs at both the protein and mRNA transcripts levels. Interestingly, levels of IL-10 in response to maturation stimuli, which is one of the most important anti-inflammatory cytokines having powerful tolerogenic properties, were significantly higher in tol-DCs compared with mDCs. The balance between IL-12/IL-10 might be crucial both for the induction of tolerance and for Th1 inhibition.

Tol-DCs exhibited a low stimulatory capacity in an allogeneic-mixed leucocyte reaction, as well as skewed T-cell polarization toward an anti-inflammatory phenotype. Importantly, this immunosuppressive function was also observed in autologous settings when superantigen TSST-1 or TT antigens were used as recall antigens. DCs can be manipulated to induce T-cell anergy and regulatory T-cell activity depending on the maturation level and the interaction with naïve $CD4^+CD45RA^+$ or memory T cells. The induction of anergy on naïve T cells could represent another mechanism of tolerance induction. In our study, we demonstrate that naïve T cells expanded with tol-DCs were unable to

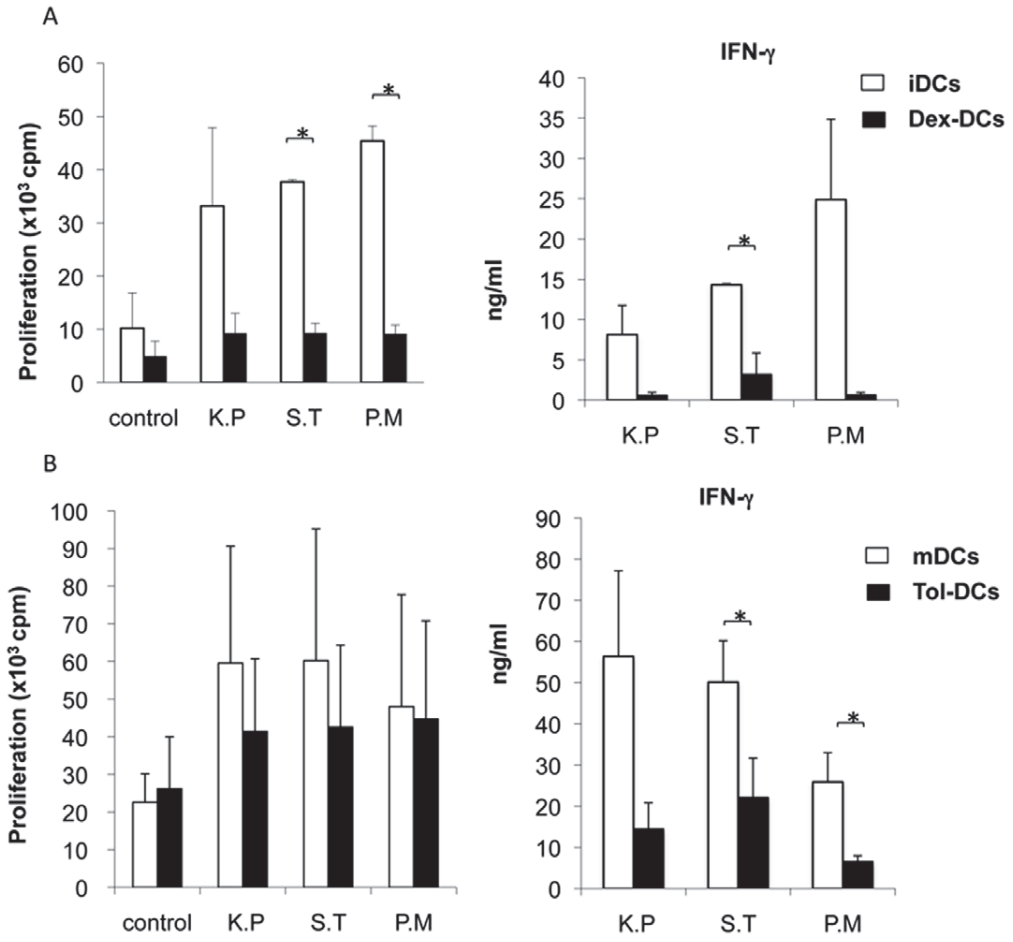

Figure 7. Tol-DCs interaction with Gram-negative enterobacteria inhibits Th1 response. Tol-DCs were treated as described in figure 5 and 6. Proliferative response and IFN-γ production induced by Gram-negative enterobacteria (*P. mirabillis, K. pneumoniae and S. thyphimurium)* stimulation of dex-DCs (**A**) and tol-DCs (dex matured-DCs) (**B**) were evaluated in allogeneic T cell culture. IFN-γ production was reduced in T cells stimulated with tol-DCs plus Gram-negative enterobacteria. IL-10 was not detected. Data represent mean ± SD of four independent experiments. Student's *t*-test: *$p<0.05$.
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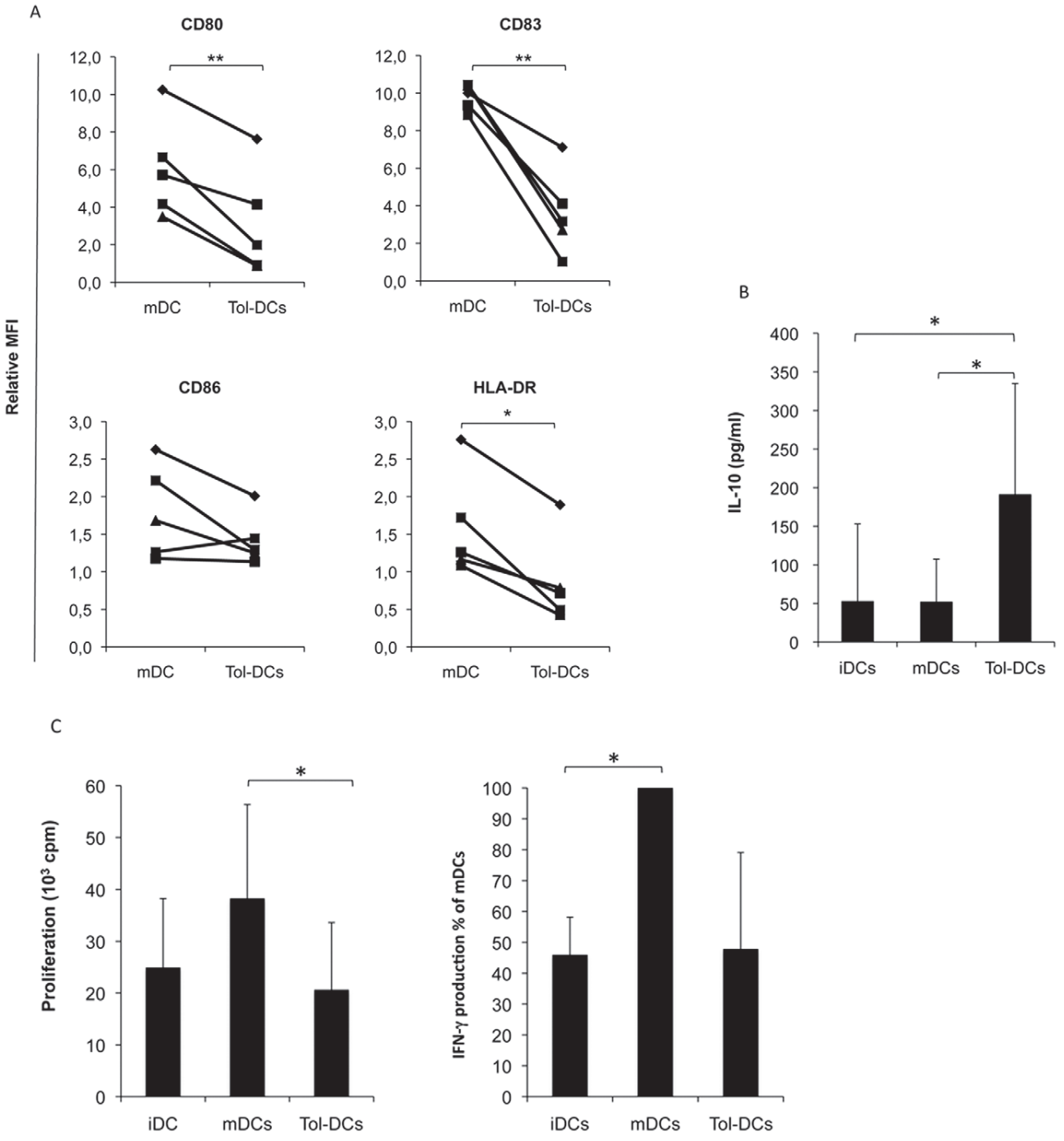

Figure 8. Crohn's disease patients' DCs are educated towards tolerogenic phenotype. (**A**) Maturation associated molecules upregulation in DCs from Crohn's disease patients are depicted as mean fluorescent intensity of expression (MFI) in mDCs and tol-DCs relative to iDCs (fold-change expression). (**B**) IL-10 was measured in supernatants harvested from DCs. Concentration of IL-10 (in pg/ml) is shown as mean ± SD (n = 6). (**C**) Proliferative response and IFN-γ production induced by tol-DCs from patients were evaluated in allogeneic T cell culture. Both, proliferation and IFN-γ production were reduced in T cells stimulated with tol-DCs compared to mDCs (data represent mean± SD (n = 4)). IFN-γ production was normalized relative to mDCs (100%) for each independent experiment (n = 3). Student's *t*-test: $*p<0.05$.
doi:10.1371/journal.pone.0052456.g008

proliferate, even after further stimulation with fully mature DCs from the same donor. Interestingly, we observed the same pattern of inhibition when TT was used as specific antigen. While TT induces strong IFN-γ secretion following interaction with mDCs [42], in our study tol-DCs completely inhibited such Th1 polarization. Increasing evidence suggests that mature DCs that lack the ability to deliver signal 3 preferentially promote the differentiation of $CD4^+$ T cells into IL-10 producing T cells (reviewed by Joffre O et al. [22]). Interestingly, our results reveal that tol-DCs have the capacity to tolerize memory T cells, which

are generally viewed as very difficult cell type to tolerize. However, we failed to generate *de novo* Treg (Foxp3 positive) from purified naïve $CD4^+$ T lymphocyte when cultured with tol-DCs.

An important concern to be considered when designing DC-based immunotherapy protocols is their stability. In this regard, it is important to point out that tol-DCs maintained their tolerogenic properties (particularly relevant for IL-10 production) once the immunosuppressive agent was removed from the culture and the DCs were further stimulated with LPS or CD40L.

It is important to stress that the tolerogenic effects of dexamethasone were evident after adding whole microorganisms (Gram-negative enterobacteria), taking into account the presence of multiple PAMPs capable of stimulating DCs by various pathways [43,44]. Interestingly, it has been recently described how glucocorticoids alter DC maturation in response to TLR7 or TLR8 through a mechanism involving GR transcriptional activity [45]. These results indicate that the response to commensal bacteria is directly related to any pre-conditioning DCs receive, underscoring the importance of the interaction between DCs and their surrounding environment [46]. Although pre-conditioning might entail some risk of infection in treated patients, it may also constitute a critical component in the treatment of immune-mediated inflammatory disorders, particularly of those in which an inappropriate response to commensal bacteria is believed to play a role, such as inflammatory bowel diseases. The clinical relevance of such interaction between enterobacteria with clinical-grade tol-DCs would take place in the inflamed lamina propria of IBD patients in the context of a cellular-based therapy. Importantly, we confirm for the first time that this protocol could be used for the production of tol-DCs from Crohn's disease patients, in line with studies in other immune-based diseases like rheumatoid arthritis [47] or multiple sclerosis [48]. This is a key aspect for considering this form of cell therapy in Crohn's disease, because it might have occurred that genetic variants conferring susceptibility for Crohn's disease might alter the biology of DCs.

In conclusion, we herein report that DCs generated by the addition of dexamethasone in combination with a cocktail of pro-inflammatory cytokines yield clinical-grade DCs with tolerogenic properties. Tol-DCs remain stable after Gram-negative bacteria interaction. These properties may serve as the basis for modulating abnormal immune responses and for developing effective strategies for the treatment of immune-mediated diseases.

Acknowledgments

We would like to thank Dr. Xavier Romero Ros and Dr. Elisabeth Calderón-Gómez for discussion and critical reading of the manuscript and the DC.CAT group (the Catalan group for DCs studies) for suggestions.

We would like to thank Dr. Jordi Vila and Elisabet Guiral for providing the microorganisms included in this study.

Author Contributions

Conceived and designed the experiments: RC JP DB-R. Performed the experiments: RC CE DB-R. Analyzed the data: RC ER JP DB-R. Wrote the paper: RC JP DB-R.

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Results II

MERTK as Negative Regulator of Human T-cell Activation

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Journal of Leukocyte Biology (2015) Jan 26. pii: jlb.3A0714-334R. [Epub ahead of print]

Epub ahead of print February 5, 2015 - doi:10.1189/jlb.3A0714-334R

JLB

Article

MERTK as negative regulator of human T cell activation

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RECEIVED JULY 14, 2014; REVISED DECEMBER 9, 2014; ACCEPTED DECEMBER 22, 2014. DOI: 10.1189/jlb.3A0714-334R

ABSTRACT

The aim of this study was to test the hypothesis whether MERTK, which is up-regulated in human DCs treated with immunosuppressive agents, is directly involved in modulating T cell activation. MERTK is a member of the TAM family and contributes to regulating innate immune response to ACs by inhibiting DC activation in animal models. However, whether MERTK interacts directly with T cells has not been addressed. Here, we show that MERTK is highly expressed on dex-induced human tol-DCs and participates in their tolerogenic effect. Neutralization of MERTK in allogenic MLR, as well as autologous DC–T cell cultures, leads to increased T cell proliferation and IFN-γ production. Additionally, we identify a previously unrecognized noncell-autonomous regulatory function of MERTK expressed on DCs. Mer-Fc protein, used to mimic MERTK on DCs, suppresses naïve and antigen-specific memory T cell activation. This mechanism is mediated by the neutralization of the MERTK ligand PROS1. We find that MERTK and PROS1 are expressed in human T cells upon TCR activation and drive an autocrine proproliferative mechanism. Collectively, these results suggest that MERTK on DCs controls T cell activation and expansion through the competition for PROS1 interaction with MERTK in the T cells. In conclusion, this report identified MERTK as a potent suppressor of T cell response. *J. Leukoc. Biol.* 97: 000–000; 2015.

Introduction

DCs are the most potent APCs connecting innate to adaptive immune responses. DCs are crucial in promoting proinflammatory responses against pathogenic microbes and tumors, in addition to establishing and maintaining tolerance to self- or harmless antigens [1]. Cellular therapies based on DCs have been used to treat different pathologic conditions with the aim of inducing a specific immune response in cancer patients or infectious diseases [2]. Immunogenic DCs are phenotypically well characterized, and their cytokine secretion profile and functional responses are firmly described and established. Up-regulated, costimulatory molecules or maturation-associated receptors are currently used as standard biomarkers to determine DC activation status. Recently, the interest in developing tol-DCs and their potential role in ameliorating autoimmune or immune-based diseases and transplantation have paved the way to apply these cells in clinical protocols [3, 4]. Although great efforts are being made to understand fully tol-DC physiology, as well as to identify specific molecules that mediate their tolerogenic function, no appropriate biomarker for these cells has been identified so far. Furthermore, the signaling pathways that program human DCs into a tolerogenic state are poorly understood. As a result of their attractive and potential role to be applied in clinical trials in human diseases [5], identification of tol-DC markers and characterization of the mechanisms involved in mediating tolerance are of utmost importance.

DNA microarray technology has been used to study the maturation, as well as the effect, of immunosuppressive agents on mouse and human DCs [6, 7]. Here, after gene-expression profile characterization of human tol-DCs cultured with dex and a MC of cytokines, as described previously [8], we identified MERTK as one of the most up-regulated molecules in tol-DCs.

Receptor tyrosine kinases of the TAM family [9, 10] are molecules involved in tempering the immune response in murine macrophages and DCs [11]. Specifically, MERTK have

Abbreviations: AC = apoptotic cell, DC = dendritic cell, dex = dexamethasone, Fla2 = flagellin 2, GAS6 = growth arrest-specific 6, GR = glucocorticoid receptor, iDC = immature dendritic cell, MC = maturation cocktail, mDC = mature dendritic cell, Mer-Fc = rMer tyrosine kinase Fc, MERTK = Mer tyrosine kinase, MFI = mean fluorescence intensity, PROS1 = protein S, qPCR = quantitative PCR, TAM = Tyro-3, Axl, and Mer, tol-DC = tolerogenic dendritic cell

The online version of this paper, found at www.jleukbio.org, includes supplemental information.

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been reported as a major macrophage AC receptor. Activation of MERTK by their soluble ligands—GAS6 or PROS1—bound to AC restricts the intensity of inflammatory cytokine production and immune responses mainly by inhibiting DC activation, thereby maintaining self-tolerance [12–14]. It has been described that MERTK regulates murine DC production of BAFF [15]. In humans, MERTK is expressed on DCs, NK cells, B cells, M2c macrophages, and platelets [16–18]. Although the role of MERTK and its ligands has become more apparent in the last few years, most of the studies have been performed based on engineered loss-of-function mutants in mice [11, 13]; hence, MERTK regulation in humans still remains unclear. Recently, it has been described that activated human T lymphocytes are able to produce PROS1, which in turn, regulates DC activation and the subsequent immune response [19]. However, whether MERTK regulates T cell activation directly has not been investigated.

In this study, we sought to identify the mechanisms underlying the tolerogenic properties of human tol-DCs and identified MERTK to be highly up-regulated in these cells, contributing to their immunosuppressive function. In addition, although MERTK is up-regulated under tolerogenic conditions, non-tol-DCs also expressed MERTK. Our results revealed that MERTK represents a novel immune-regulatory receptor within the Ig superfamily by directly inhibiting T lymphocyte activation in humans. Thus, MERTK regulates human T cell activation and expansion by sequestering the TAM ligand PROS1 and limiting its proproliferative effect on T cells. Furthermore, the modulation of MERTK activity could be a promising therapeutic approach when control of the immune response is required.

MATERIALS AND METHODS

Generation of human DCs

The present study was approved by the Ethics Committee at the Hospital Clinic of Barcelona, and the authors declare no violation of the Helsinki Doctrine on human experimentation. Buffy coats were obtained from Banc de Sang i Teixits (Barcelona, Spain), and written informed consent was obtained from all blood donors. Monocyte-derived DCs were generated from the peripheral blood samples of healthy volunteers, as reported previously [8]. In summary, PBMCs were allowed to adhere for 2 h at 37°C. Nonadherent cells—PBLs—were gently removed, washed, and used for $CD4^+$ naïve T cell isolation. The adherent monocytes were cultured in X-VIVO 15 medium (BioWhittaker, Lonza, Belgium), supplemented with 2% AB human serum (Sigma-Aldrich, Madrid, Spain) and IL-4 (300 U/ml) and GM-CSF (450 U/ml; Miltenyi Biotec, Madrid, Spain) for 6 days to obtain iDCs. The MC consisted of IL-1β and IL-6 (both at 1000 IU/ml) and TNF-α (500 IU/ml; CellGenix, Freiburg, Germany) and PGE_2 (10 μg/ml; Dinoprostona; Pfizer, New York, NY, USA) and was added on day 6 for 24 h. mDCs were harvested and analyzed on day 7. dex (10^{-6} M; Fortecortin; Merck, Madrid, Spain) was added on day 3. We did not observe differences in viability and yield among iDC, dex-iDC, mDC, and tol-DC generation. RU-486 was used in some experiments and added 2 h before dex addition at different concentrations (10, 50, 150, 300 ng/ml; RU-486; Mifepristone). Ethanol was used as a vehicle control, and it did not affect viability or DC phenotype.

RNA isolation

Total RNA was isolated by use of RNeasy Mini Kit columns with on-column DNase I treatment (Qiagen, Hilden, Germany). RNA yield and purity were measured by use of the NanoDrop ND-1000 spectrophotometer and the Agilent 2100 bioanalyzer.

Microarray analysis

Microarray experiments were conducted on baseline 6 iDC- and 6 mDC- and 3 dex-iDC- and 6 tol-DC-treated samples by use of Affymetrix Human Genome U133 Plus 2.0 arrays, containing 54,675 probes for 47,000 transcripts (Affymetrix, Maumee, OH, USA). Raw data were normalized by use of the robust multiarray algorithm [20]. Thereupon, we select 31,436 probes after a filtering step, excluding probes not reaching an average log2 signal intensity of 5. For the detection of differentially expressed genes, a linear model was fitted to the data, and empirical Bayes-moderated statistics were calculated with use of the limma package from Bioconductor (Seattle, WA, USA). Adjustment of *P* values was done by the determination of false discovery rates by use of the Benjami-Hochberg procedure [21]. Microarray raw data (.cel files) and processed data have been deposited in the Gene Expression Omnibus of the National Center for Biotechnology Information and are accessible through GEO Series accession number GSE56017.

Real-time qPCR

Microarray expression of selected DC genes was confirmed in aliquots of the same RNA samples by use of qPCR. RNA was reverse transcribed to cDNA by use of the High-Capacity cDNA RT Kit (Applied Biosystems, Carlsbad, CA, USA). Reverse transcription was carried out in a 96-well thermocycler (Veriti 96W, Applied Biosystems) in the following conditions: 25°C, 10 min; 37°C, 120 min. TaqMan real-time PCR was used to detect transcripts of *MERTK* and *IL-2*. Primers and probes for each sequence were obtained as inventoried TaqMan gene-expression assays (Applied Biosystems). B-ACTIN was used as a reference gene.

Purified RNA from a naïve $CD4^+$ T cell was reverse transcribed to cDNA by use of the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA). *MERTK* mRNA expression was evaluated by qPCR by use of the KAPA SYBR Fast qPCR kit (KapaBiosystems, Wilmington, MA, USA), and reactions were performed on a Stratagene Mx3000 system. Eukaryotic translation elongation factor 1 α1 was used as a housekeeping gene. Amplified products were checked by dissociation curves.

Flow cytometry

MERTK expression, by flow cytometry, was performed with the use of purified or allophycocyanin directly conjugated α-MERTK mAb (2 μg/ml; R&D Systems, Minneapolis, MN, USA) and the appropriate isotype control (Santa Cruz Biotechnology, Heidelberg, Germany; and R&D Systems). Primary antibodies were followed by staining with PE-labeled goat anti-mouse (BD Biosciences, Franklin Lakes, NJ, USA). For intracellular detection of MERTK, cells were fixed with 2% paraformaldehyde, permeabilized with saponin-based permeabilization buffer, and stained with allophycocyanin, directly conjugated α-MERTK mAb (2 μg/ml; R&D Systems).

Activation of $CD4^+$ T cells was analyzed by use of CD4, CD69, CD25, and CD44 antibodies (BD Biosciences). Viability of cells was checked by use of LIVE/DEAD kit (Life Technologies, Carlsbad, CA, USA).

Flow cytometry was performed with the use of BD FACSCanto II and LSR-II (BD Biosciences) or Stratedigm S1000EX (Stratedigm, San Jose, CA, USA) and analyzed with BD FACSDiva 6.1 or FlowJo 7.6.1.

T cell cultures

MLR. Naïve $CD4^+$ T cells were isolated from human PBLs by use of naïve $CD4^+$ T cell Isolation Kit II (Miltenyi Biotec), following the manufacturer's instructions. Allogeneic, naïve $CD4^+$ T cells were cocultured with DCs differently generated in a 96-well microplate at a 20:1 ratio. Purified α-MERTK mAb (R&D Systems) and the appropriate isotype control were added to the culture at 5 μg/ml. In some experiments, polymyxin B (Sigma-Aldrich) was added to the culture at 10 μg/ml.

Naïve $CD4^+$ T cell cultures. Naïve $CD4^+$ T cells were stimulated with α-CD3 antibody (BD Biosciences) and human Mer-Fc (R&D Systems; both prebound to the microplate for 1 h at 37°C, 1 μg/ml). Human rCD36-Fc was used as a negative control (1 μg/ml; R&D Systems). In some experiments, other stimuli were added to the culture, such as α-CD28 antibody (1 μg/ml; BD

Biosciences), human rIL-2 (50 IU/ml; eBioscience, San Diego, CA, USA), α-CD3/CD28 beads at a 1:1 ratio (Life Technologies), or human PROS1 (50 nM; Haematologic Technologies, Essex Junction, VT, USA). For blocking experiments, purified α-MERTK mAb (R&D Systems) or purified α-PROS1 (PS7) mAb (Santa Cruz Biotechnology) was used. For rechallenge experiments, naïve $CD4^+$ T cells were stimulated for 7 days with α-CD3 antibody (1 μg /ml), harvested and washed with PBS, and rechallenged with α-CD3 antibody (0.5 μg/ml) and Mer-Fc (1 μg/ml). T cell activation was analyzed by flow cytometry after 4 days stimulation with α-CD3 antibody and Mer-Fc (1 μg/ml; see Flow cytometry above).

Antigen-specific T cell cultures. For antigen-specific T cell responses, 2 μg/ml bacterial antigen (Fla2; kindly provided by Prometheus Laboratories) was added to PBMC of a CFSE (Invitrogen, Carlsbad, CA, USA)-labeled Crohn's disease patient, cultured, and expanded for 2 weeks in the presence of IL-2 (20 UI/ml). $CFSE^-$ $CD4^+$T cells were sorted by use of BD FACSAria II and restimulated in the presence of Fla2 antigen and autologous, irradiated PBMCs for antigen-specific expansion. After 12 days, Fla2-specific T cells were harvested and cultured with α-CD3 antibody (0.5 μg/ml) and Mer-Fc (1 μg/ml) for 3 days.

T cells were cultured in X-VIVO 15 medium (BioWhittaker), supplemented with 2% AB human serum (Sigma-Aldrich) unless specified otherwise.

Proliferation assay

For all of the experiments, proliferation assay was performed by use of tritiated thymidine (1 μCi/well; Amersham, Cambridge, United Kingdom), and supernatants were obtained and frozen properly. The [^{3}H]thymidine incorporation took place during the last 16 h of culture. Proliferation was also tested by intracellular Ki-67 staining (BD Biosciences) by use of commercial permeabilization and fixation buffers (Invitrogen) and quantified by flow cytometry.

Cytokines

Culture supernatants were collected and frozen at −20°C. IFN-γ and IL-2 were analyzed by ELISA, according to the manufacturer's guidelines.

Western blotting

Cell lysates and Western blot studies were performed by use of standard procedures. Polyvinylidene difluoride membranes were incubated with α-MERTK polyclonal antibody (R&D Systems) and reprobed with actin (Sigma-Aldrich). After washes, membranes were incubated with HRP-conjugated secondary antibody. Proteins were detected by ECL (ImageQuant LAS 4000; GE Healthcare Life Sciences, Barcelona, Spain) by use of ECL Western blotting detection reagent (GE Healthcare Life Sciences).

Statistical analysis

Data are plotted as mean ± SEM. Statistical analysis was performed by use of 2-tailed Student's *t*-test: $*P < 0.05$; $**P < 0.001$; and $***P < 0.0001$.

RESULTS

MERTK up-regulation in human DCs is controlled by dex

We analyzed microarray gene expression data on in vitro dex-induced human tol-DCs [8] and identified differentially expressed genes in tol-DCs compared with control DCs that could potentially be involved in tolerance induction. Based on the heat map included in **Fig. 1A**, we identified *MERTK*, a member of the tyrosine kinase family known as TAM, as one of the most 50 up-regulated genes expressed in monocyte-derived tol-DCs. In vitro dex treatment increased *MERTK* mRNA expression in iDCs and mDCs by 5.1- and 20.2-fold, respectively, validating the microarray data by qPCR (Fig. 1B). mRNA results were confirmed at the protein level, and MERTK was found to be expressed in in vitro-generated DCs (iDCs, 17.1 ± 3.3%; mDCs, 15.4 ± 3.8%), and the addition of dex resulted in its significant up-regulation (dex-iDCs, 74.4 ± 5.2%; tol-DCs, 59.6 ± 6.9%), as detected by flow cytometry and Western blot (**Fig. 2A** and **B**). Expression kinetics showed >50% of $MERTK^+$ DCs at day 3 upon dex treatment (Supplemental Fig. 1A). It is important to highlight that the majority of MERTK protein was intracellularly detected in the absence of dex (Supplemental Fig. 1B). Moreover, dex-induced MERTK up-regulation was dose

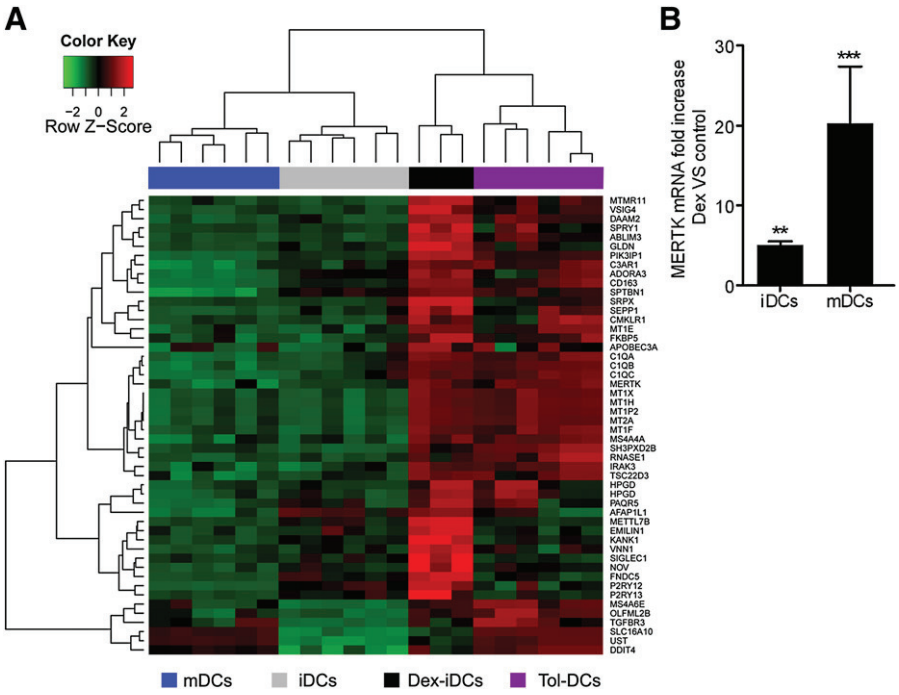

Figure 1. *MERTK* is expressed in human DCs and up-regulated upon dex treatment. (A) Heat map showing clustering (by use of correlation distance and complete method) of the most significant genes among comparisons between untreated human DCs (iDCs), MC-treated DCs (mDCs), dex-treated DCs (dex-iDCs), and dex plus MC-treated DCs (tol-DCs). Results are expressed as a matrix view of gene expression data (heat map), where rows represent genes, and columns represent hybridized samples. The intensity of each color denotes the standardized ratio between each value and the average expression of each gene across all samples. Red pixels correspond to an increased abundance of mRNA in the indicated blood sample, whereas green pixels indicate decreased mRNA levels. (B) Transcripts levels of *MERTK* were determined by real-time PCR by use of *β-ACTIN* as the endogenous reference gene. Data represent dex-treated fold-change induction means ± SEM relative to control DCs [iDCs vs. (VS) dex-iDCs, $n = 3$; mDCs vs. tol-DCs, $n = 8$]. Statistical analysis of *MERTK* expression data of iDCs versus dex-iDCs and mDCs versus tol-DCs was performed with 2-tailed Student's *t*-test: $**P < 0.001$; $***P < 0.0001$.

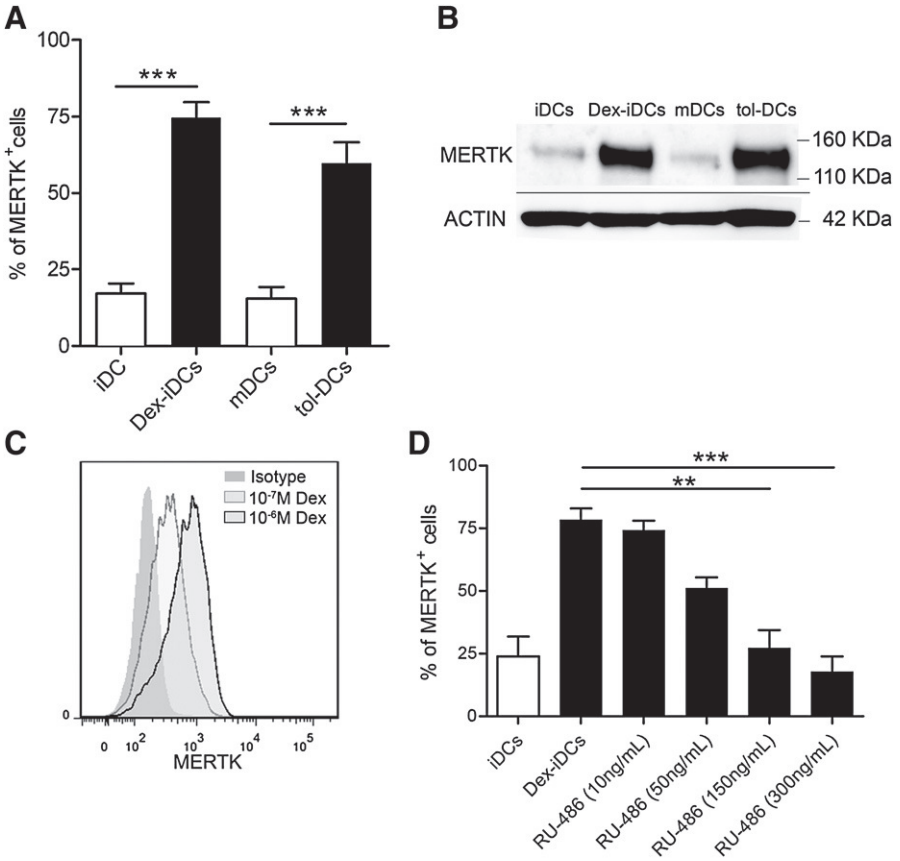

Figure 2. MERTK expression in human DCs is controlled by dex. (A) Flow cytometric analysis of surface MERTK expression on human monocyte-derived DCs ($n \geq 8$). (B) Representative Western blot of MERTK expression in human DCs ($n = 3$). (C) Representative flow cytometry histogram of dex dose-dependent up-regulation of MERTK on DCs: 10^{-7} M (gray line; 56%), 10^{-6} M (black line; 85%), and isotype control (gray, filled histogram). (D) Inhibition of MERTK up-regulation in dex-treated DCs by use of different doses of RU-486 (GR inhibitor). Data are plotted as means ± SEM, and statistical analysis was performed with 2-tailed Student's *t*-test: $^{**}P < 0.001$; $^{***}P < 0.0001$.

dependent (Fig. 2C), and it was inhibited by RU-486, a specific GR inhibitor (Fig. 2D). We confirmed the involvement of GR in MERTK regulation by use of other glucocorticoids (Supplemental Fig. 1C). When other immunosuppressive agents were tested (vitamin D3, IL-10, and retinoic acid), none of them induced up-regulation of MERTK expression in DC (data not shown).

The blockage of MERTK in DC–T cell interaction increases naïve CD4$^+$ T cell response

To evaluate the function of MERTK, purified naïve CD4$^+$ T cells were cocultured with DCs in the presence of blocking α-MERTK mAb. Interestingly, T cell proliferation was enhanced significantly in the presence of α-MERTK mAb compared with the isotype control (**Fig. 3A**), revealing a role of MERTK in controlling the immune response. Moreover, when blocking MERTK, IFN-γ production was increased significantly, in concordance with proliferation data (Fig. 3B). Similar results were obtained when whole PBLs were used (Supplemental Fig. 2A and B). The maturation status of DCs did not modify the results observed. When mDCs were incubated with naïve CD4$^+$ T cells in the presence of blocking α-MERTK mAb, proliferation and cytokine secretion were also increased (data not shown). Nonspecific T cell activation, as a result of endotoxin contamination of MERTK antibody, was ruled out by adding polymyxin B to the culture (Supplemental Fig. 2C). To confirm that blocking antibodies were not interfering with T cells, DCs were preincubated with α-MERTK, and unbound antibodies were washed out before culturing MLR experiments. Preincubation of DCs with MERTK-blocking antibodies, followed by wash, also induced increased T cell proliferation and cytokine secretion (data not shown). Furthermore, MERTK not only regulated the magnitude of the alloresponse but also controlled autologous immune response. When *Escherichia coli*-activated DCs were incubated with autologous naïve CD4$^+$ T cells in the presence of α-MERTK mAb, T cell proliferation was also increased (Supplemental Fig. 2D), suggesting that MERTK expression in human DCs plays an important role in regulating naïve T cell activation.

MERTK inhibits naïve CD4$^+$ T cell activation

To investigate the direct function of MERTK in T cell activation, we used a Mer-Fc to mimic the effect of MERTK expressed on human DCs. Stimulated, naïve CD4$^+$ T cells with α-CD3 mAb were cultured in the presence of Mer-Fc for 7 days. Surprisingly, Mer-Fc significantly suppressed naïve CD4$^+$ T cell proliferation (67%) and IFN-γ production (from 963 ± 363 to 204 ± 116 pg/ml; **Fig. 4A** and **B**). An irrelevant rFc protein (CD36-Fc) did not alter T cell proliferation or cytokine production (Supplemental Fig. 3A). When Mer-Fc was blocked by use of α-MERTK mAb, the proliferative response to α-CD3 was restored (Supplemental Fig. 3B), confirming the direct suppressive effect of MERTK on T cell activation. To explore the mechanism by which Mer-Fc inhibited T cell activation, we analyzed IL-2 secretion in response to α-CD3. Consistent with our previous data, IL-2 production was reduced significantly when T cells were incubated with Mer-Fc

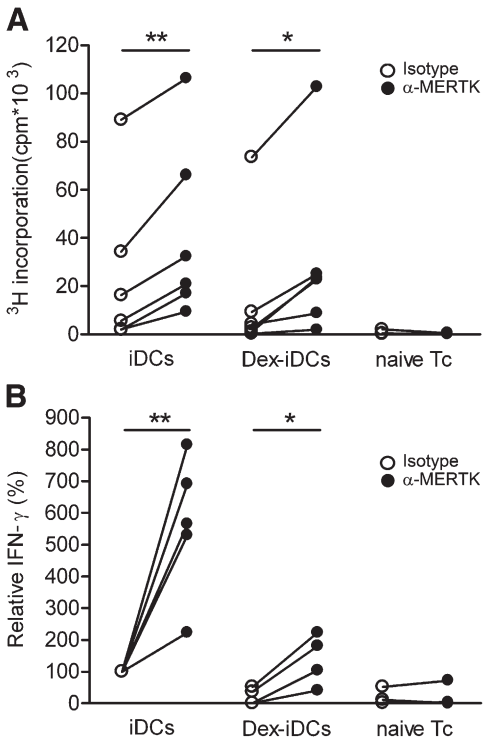

Figure 3. The blockage of MERTK increases allogenic T cell response. DCs were cocultured with naïve CD4$^+$ T cells (ratio 1:20), and T cell activation was measured after 7 days. (A) Proliferation of T cells cultured with iDCs, dex-iDCs, or alone (naïve Tc) in the presence of α-MERTK mAb (black-filled dots) or with an isotype control (empty dots); $n \geq 5$. (B) Relative IFN-γ production in MLR supernatant in the presence of α-MERTK mAb (black-filled dots) compared with isotype control (empty dots); $n \geq 4$. For normalization, T cell IFN-γ production, induced by iDCs in the presence of isotype control antibody of each experiment, was set at 100, and relative IFN-γ production in the presence of α-MERTK mAb was calculated. Data are plotted as means ± SEM, and statistical analysis was performed with 2-tailed Student's *t*-test: $^*P < 0.05$; $^{**}P < 0.001$.

(from 266 ± 65 to 100 ± 30 pg/ml; Fig. 4C). *IL-2* mRNA was down-regulated rapidly after overnight stimulation with α-CD3 and Mer-Fc (Supplemental Fig. 3C), which correlated with the low levels of IL-2 cytokine production in the supernatants. To investigate further the mechanism of MERTK-mediated suppression, the expression of early TCR activation markers (CD69, CD44, and CD25) was measured after T cell activation. In the presence of Mer-Fc, percentage of positive cells and MFI of all activation markers were diminished (Fig. 4D) without compromising cell viability (Supplemental Fig. 3D). To test further the inhibitory potential of this receptor, we added soluble α-CD28 mAb or rIL-2 to naïve CD4$^+$ T cell culture, described previously in Fig. 4A. Remarkably, in the presence of stronger costimulatory signals, such as α-CD3α-CD28 stimulation or exogenous IL-2, T cell inhibition by Mer-Fc was still significant (Fig. 4E and Supplemental Fig. 3E and F).

MERTK suppresses antigen-specific memory T cell activation

To evaluate the ability of MERTK to suppress previously activated CD4$^+$ T cells, we stimulated naïve CD4$^+$ T cells with α-CD3 mAb in vitro for 1 wk, washed cells, and rechallenged with α-CD3 mAb plus rMer-Fc. In line with our previous results, Mer-Fc significantly suppressed 77% of T cell proliferation (**Fig. 5A**) and reduced IFN-γ (86%) and IL-2 (83%) production (Fig. 5B and C) of activated T cells. To test the immunosuppressive capacity of Mer-Fc in memory T cells, sorted flagellin-specific CD4$^+$ T cells from Crohn's disease patients (unpublished results) were expanded and rechallenged with α-CD3 mAb plus Mer-Fc. Interestingly, a proliferation assay revealed again a potent suppressive effect of MERTK (38%) in this model of memory T cell response, shown in Fig. 5D. Not only proliferation but also their ability to produce IFN-γ (58%) was strongly impaired by Mer-Fc (Fig. 5E). The same results were observed with tetanus toxoid-specific T cells isolated from healthy donors (data not shown).

PROS1 regulates T cell proliferation through MERTK

To investigate whether the expression of MERTK is regulated during T cell stimulation, naïve CD4$^+$ human T cells were activated by use of α-CD3/CD28 beads, and MERTK expression was evaluated. As shown in **Fig. 6**, *MERTK* mRNA levels were increased after 72 h of stimulation (Fig. 6A). We confirmed the expression of MERTK at protein level by flow cytometry (Fig. 6B).

Based on the fact that MERTK and PROS1 are expressed upon T cell activation, we hypothesized that PROS1 could favor T cell proliferation. To assess whether PROS1 was involved in CD4$^+$ T cell suppression mediated by MERTK on DCs, we neutralized soluble PROS1 from the culture media by use of mAb. Interestingly, our results revealed that the blockage of PROS1 abrogated T cell proliferation (82%) and IFN-γ production (from 375 ± 67 to 99 ± 58 pg/ml) induced by α-CD3 stimulation (**Fig. 7A** and **B**). As PROS1 is present in human serum, we performed experiments in serum-free media to confirm the proproliferative effect of PROS1 on T cells. In these settings, the addition of human PROS1 to activated naive CD4$^+$ T cells for 5 days showed a significant increase of proliferation by use of [^{3}H]thymidine incorporation and Ki-67 intracellular staining (Fig. 7C and D). We did not observe any changes in T cell proliferation when PROS1 was added to unstimulated T cells (data not shown). Moreover, PROS1 was able to rescue the proliferation of activated T cells in the presence of Mer-Fc (Fig. 7E). To understand better the mechanism by which PROS1 was regulating T cell proliferation, we neutralized MERTK on activated T cells and added PROS1 to the culture. As shown in Fig. 7F, we remarkably found that the blocking of MERTK on T cells significantly reduced the ability of PROS1 to induce proliferation of activated T cells.

DISCUSSION

Arising from the importance of tol-DCs being currently used in clinical trials to treat human immune-based diseases [22], it would be of great interest to define better the molecules and mechanisms that mediate their tolerogenic function. In taking advantage of in vitro generation of dex-induced tol-DCs [8], we identified MERTK as a highly up-regulated receptor expressed in

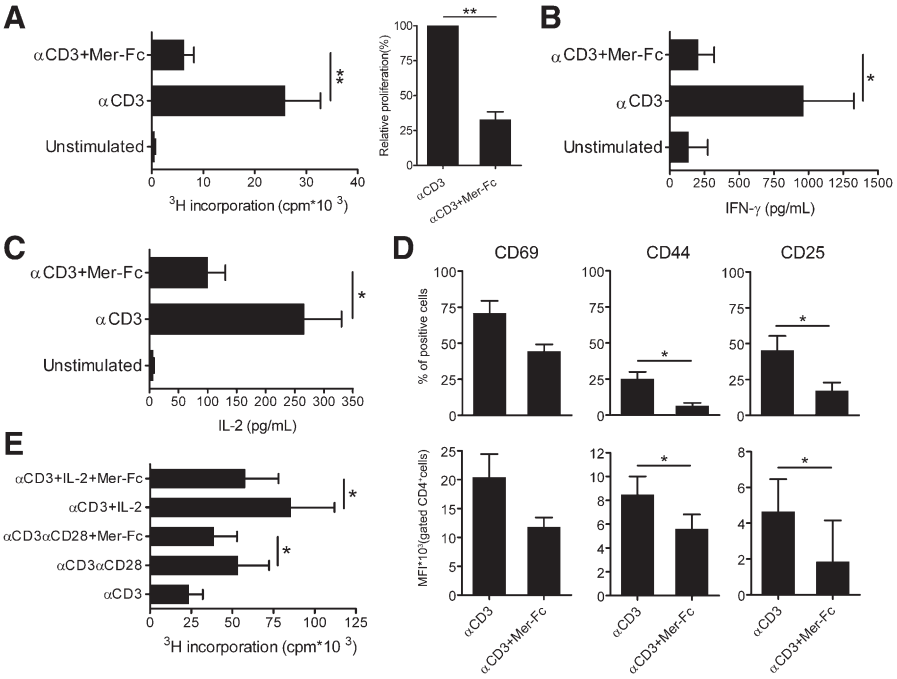

Figure 4. Mer-Fc inhibits naïve CD4$^+$ T cell activation and proliferation. (A, left) Proliferation of naïve CD4$^+$ T cell measured by [^{3}H]thymidine incorporation ($n = 11$). (Right) Relative proliferation; maximum proliferation (T cell proliferation induced by α-CD3 activation) was set at 100, and relative proliferation (T cell proliferation induced by α-CD3 plus Mer-Fc) was calculated per experiment. (B) IFN-γ and (C) IL-2 production in culture supernatants ($n \geq 9$). (D) Expression of T cell activation markers (CD69, CD44, and CD25) analyzed by flow cytometry after stimulation of naïve CD4$^+$ T cells with α-CD3 or α-CD3 plus Mer-Fc for 4 days ($n = 4$). (Upper) Percentage of positive cells compared with isotype control. (Lower) MFI of 4 independent experiments. (E) Proliferation of naïve CD4$^+$ T cell upon α-CD3α-CD28 stimulation or exogenously added rIL-2 plus Mer-Fc; $n \geq 4$. Data are plotted as means ± SEM, and statistical analysis was performed with 2-tailed Student's *t*-test: $^*P < 0.05$; $^{**}P < 0.001$.

monocyte-derived tol-DCs. Our results show that MERTK is expressed in human DCs, and it is up-regulated specifically at mRNA and protein level by dex, therefore, establishing MERTK as a characteristic feature to identify tol-DCs generated with glucocorticoids, even though its expression is not restricted to tol-DCs, as revealed with the MERTK presence in iDC and mDCs. These results are in agreement with the presence of GR binding sites in the *MERTK* gene locus [23]. Interestingly, its expression was induced by a broad variety of corticosteroids but not by other immunosuppressive agents, such as IL-10, vitamin D3, or retinoic acid. The fact that other immunosuppressant drugs did not induce MERTK up-regulation revealed a specific expression pattern of molecules, according to each tolerogenic agent used in the generation of tol-DCs. Besides, it also explains the diversity of functional consequences that may lead to tolerance induction [24].

MERTK belongs to the tyrosine kinase family known as TAM (including 3 members: TYRO-3, AXL, and MERTK). It has been shown previously that TAM receptors are notably expressed by monocytes and their derivatives, emphasizing the involvement of MERTK on the clearance of apoptotic bodies [13, 14]. TAM receptors have also been described as pleiotropic-negative regulators of TLRs and cytokine receptor signaling in murine DCs [11]. It has been described how steroids regulate expression of MERTK and PROS1 and enhance their activity in AC clearance in human macrophages [25], as well as in DCs [26]. Although the importance of MERTK in the engulfment and efficient clearance of AC in humans has been investigated, little is known about the immunomodulatory role of MERTK in humans, as it may differ from animal models. In this report, we reveal an unknown function of MERTK in regulating T cell response. Interestingly, by adding α-MERTK blocking mAb to MLR or an autologous response, CD4$^+$ T cell proliferation and IFN-γ production were enhanced significantly, revealing a role of MERTK in controlling a naïve T cell response. Although MERTK is highly expressed in dex-iDCs, we observed a more pronounced effect of blocking the

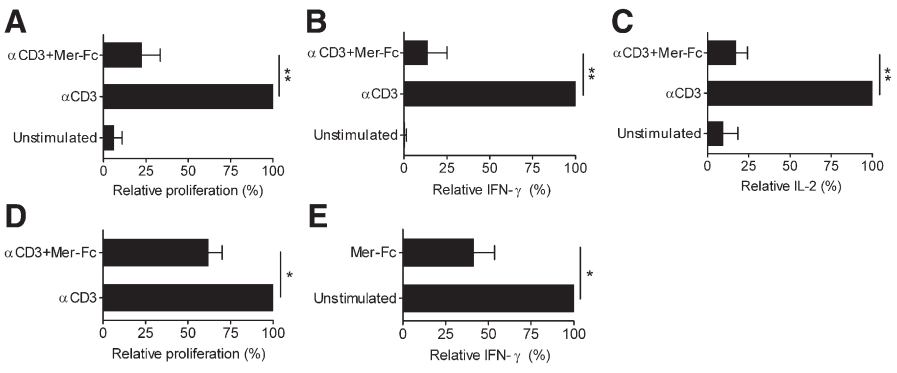

Figure 5. Mer-Fc inhibits antigen-specific memory T cell response. (A) Stimulated CD4$^+$ T cells were harvested, washed, and rechallenged further with α-CD3 and Mer-Fc for 72 h; $n = 4$. Relative proliferation was measured by [^{3}H]thymidine incorporation. (B) Relative IFN-γ and (C) relative IL-2 production in culture supernatants ($n \geq 3$). (D) Sorted Fla2-specific CD4$^+$ T cells were stimulated with α-CD3 and Mer-Fc for 72 h; $n = 3$. Relative proliferation was measured. (E) Relative IFN-γ production in culture supernatants; $n = 3$. All graphs show relative values normalized as described previously in Fig. 4. Data are plotted as means ± SEM, and statistical analysis was performed with 2-tailed Student's *t*-test: $^*P < 0.05$; $^{**}P < 0.001$.

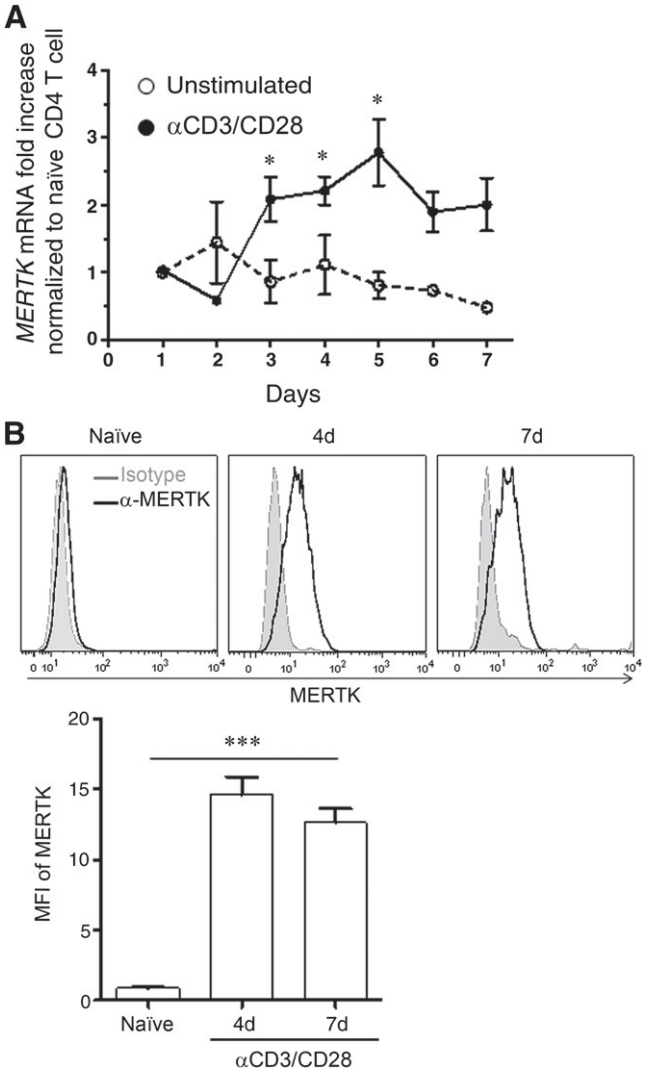

Figure 6. MERTK is expressed in activated human T cells. (A) Naïve $CD4^+$ T cells were isolated and in vitro stimulated with α-CD3/CD28 beads in a 1:1 ratio. Cells were harvested at different time-points, and *MERTK* mRNA was evaluated by qPCR. Fold induction was normalized to acutely isolated, naïve $CD4^+$ T cells ($n = 5$). (B) Surface MERTK expression on acutely isolated, 4 days (4d) and 7 days (7d) postactivated, naïve $CD4^+$ T cells was evaluated by flow cytometry. Representative histogram and MFI analysis of 4–6 independent samples are shown. Data are plotted as means ± SEM, and statistical analysis was performed with 2-tailed Student's *t*-test: $*P < 0.05$; $***P < 0.0001$.

receptor in iDCs. This could be explained by the lower immunogenic capacity of dex-iDCs as a result of the glucocorticoid-induction of other inhibitory mechanisms, such as IL-10 [8]. Although high MERTK expression is observed in dex-iDCs, the tolerogenic properties of these cells may impede the evaluation of the inhibitory effect of MERTK itself. Nevertheless, the fact that the blocking of MERTK in dex-iDCs alloresponse results in an enhanced proliferation suggests that this receptor contributes to their tolerogenic properties.

A pronounced suppressive effect of GAS6 [27] and PROS1 [19] (natural ligands) on DCs via MERTK has already been shown. However, the role of MERTK in regulating T cell activation has not yet been explored. Our results demonstrate the importance of MERTK in controlling T cell proliferation and cytokine production; henceforth, we wondered whether MERTK is involved in T cell-priming regulation.

MERTK induction on DCs constitutes a self-regulatory mechanism to restrain an ongoing immune response [11]. Notwithstanding, a neutralizing effect of MERTK on T cell proliferation would highlight the importance of this receptor in regulating the adaptive immune response. To test the direct function of MERTK in T cell activation, we used Mer-Fc to mimic the effect of MERTK expressed on human DCs. Remarkably, we demonstrate that MERTK suppresses memory and naïve $CD4^+$ T cell activation, proliferation, IFN-γ, and IL-2 secretion by use of human rMer-Fc. Naïve T cell IL-2 production is essential for $CD4^+$ and $CD8^+$ T cell growth, proliferation, and differentiation. Indeed, one of the most rapid consequences of T cell activation is the de novo synthesis of IL-2; followed by expression of a high-affinity IL-2R, it permits the expansion of effector T cell populations activated by antigen [28]. We hypothesize that MERTK on DCs might be inhibiting T cell expansion, impairing IL-2 production to negatively regulate T cell proliferation, thus leading to an intrinsic negative feedback to down-regulate CD25 (α subunit of IL-2R) and IFN-γ production on T cells. This may contribute to maintain the physiologic balance of immune activation against pathogens yet evading exacerbated inflammation. It is important to highlight that MERTK capacity to inhibit $CD4^+$ T cells (in a cell-nonautonomous manner) has not been described previously. Wallet et al. [29] investigated the effect of NOD mice DCs lacking MERTK in mediating AC-induced inhibition of DC activation and therefore, T cell proliferation. However, these authors did not observe a direct effect of MERTK in T cell activation, independently of AC engulfment by DCs, revealing significant differences of MERTK function between human and mice [29].

Interestingly, we provide further evidence that MERTK is able to regulate negatively the immune response, even though T cells had been activated previously by α-CD3 or in antigen-specific memory T cells from Crohn's disease patients recently characterized by our group (unpublished data). The fact that MERTK suppresses the proliferation of these specific populations contributes to speculate in novel therapies to treat autoimmune or immune-based diseases considering TAM receptors, in particular, MERTK, as potential candidates. Indeed, a recent publication showed the therapeutic efficacy of TAM tyrosine kinase agonists in collagen-induced arthritis by the administration of plasmids coding for GAS6 and PROS1 [30].

MERTK overexpression has been reported in a variety of human cancers, including B- and T-acute lymphocytic leukemia, indicating the involvement of MERTK in intrinsic cell proliferation [31]. Surprisingly, in contrast to what has been reported in mice, the expression of MERTK by human T cells may represent an autonomous mechanism of regulation that remains to be understood.

Although 2 main ligands have been described for MERTK—GAS6 and PROS1 [32]—the ubiquitous location of these pleiotropic proteins in vivo makes it difficult to study their role in TAM receptors-mediated-immune regulation, particularly

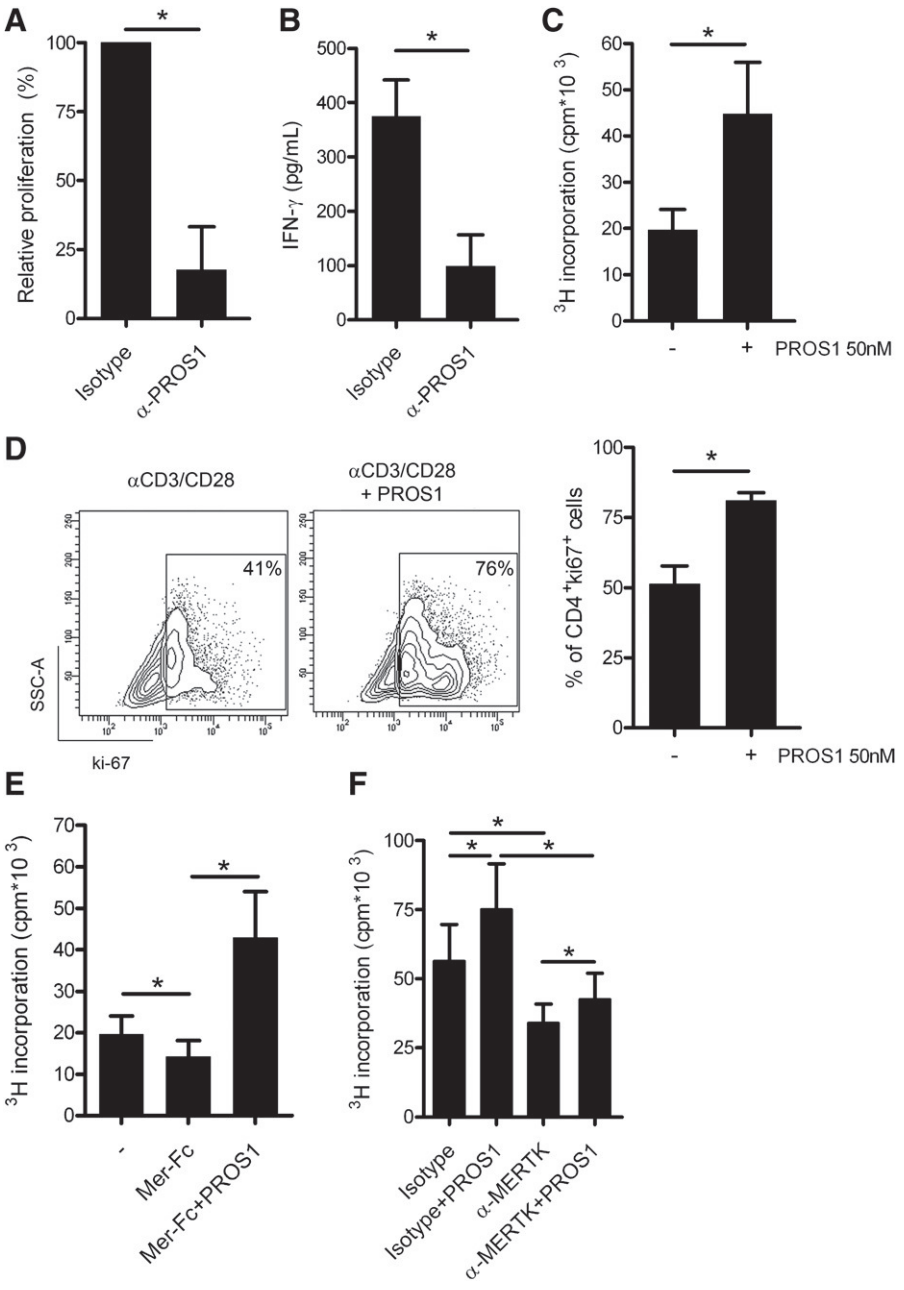

Figure 7. PROS1 regulates T cell proliferation through MERTKR. (A) Relative proliferation of α-CD3-stimulated, naïve $CD4^+$ T cells treated with isotype control or α-PROS1 mAb. Maximum proliferation (T cell proliferation induced by α-CD3 activation, treated with isotype control) was set at 100, and relative proliferation (T cell proliferation induced by α-CD3 plus α-PROS1) was calculated per experiment; $n = 4$. (B) IFN-γ production in culture supernatants ($n = 5$). (C) Naïve $CD4^+$ T cells were cultured in serum-free media and activated with α-CD3/CD28 beads. Human PROS1 (50 nM) was added to the culture for 5 days, and T cell proliferation was measured by [^{3}H]thymidine incorporation; $n = 5$. (D) Naïve $CD4^+$ T cells were cultured in serum-free media and activated with α-CD3/CD28 beads. Human PROS1 (50 nM) was added to the culture for 5 days, and T cell proliferation was evaluated by Ki-67 intracellular staining. Flow cytometry plots show representative Ki-67 staining, gated on live $CD4^+$ cells. (Right) Percentage of $CD4^+Ki\text{-}67^+$ cells; $n = 3$. SSC-A, Side-scatter-area. (E) Naïve $CD4^+$ T cells were cultured in serum-free media and activated with α-CD3/CD28 beads. Human PROS1 (50 nM) and Mer-Fc were added to the culture for 5 days, and T cell proliferation was measured by [^{3}H]thymidine incorporation; $n = 5$. (F) Naïve $CD4^+$ T cells were cultured in serum-free media and activated with α-CD3/CD28 beads. α-MERTK mAb or isotype control was added to the culture for 30 min before addition of human PROS1 (50 nM). After 5 days, T cell proliferation was measured by [^{3}H]thymidine incorporation; $n = 6$. Data are plotted as means ± SEM, and statistical analysis was performed with 2-tailed Student's t-test: $^*P < 0.05$.

in humans. Recently, Carrera Silva et al. [19] reported that activated human $CD4^+$ T cells produce PROS1 that acts locally at the DC–T cell interface limiting DC activation. It has been shown that overexpression of GAS6 and PROS1 is correlated with poor prognosis in a variety of cancers [33, 34]. We wondered whether PROS1 was involved in $CD4^+$ T cell suppression mediated by MERTK on DCs to asses that we neutralized soluble PROS1 from the culture media by use of mAb. Proliferation was abrogated by α-PROS1 mAb, suggesting that PROS1 has a proproliferative effect in activated T cells. It is tempting to speculate that MERTK expressed by DCs might be neutralizing PROS1 and therefore, avoiding the autocrine effect on T cells mediated by MERTK. PROS1, produced by hepatocytes and endothelial cells, is found in large amounts in the blood [35]. We demonstrate further that the addition of human PROS1 to serum-free media-cultured T cells enhanced T cell proliferation significantly through MERTK expressed on T cells. These findings confirmed the direct proproliferative effect of PROS1 on T cells.

We hypothesize that the availability of soluble PROS1 during DC–T cell interactions will define the result of the immune response. Although this concept seems counterintuitive, our results reveal that MERTK function varies depending on the expressing cell type. Thereby, this receptor restrains activation on DCs, and by contrast, it has a proproliferative function on T cells. Carrera-Silva et al. [19] showed that T cell-derived PROS1 functions locally at the DC–T cell interface and engages TAM

signaling within DCs to limit their activation. It is well known that MERTK is up-regulated in human APCs upon tolerogenic treatment, and several studies showed its anti-inflammatory role, especially in AC clearance [36]. On the other hand, MERTK is ectopically expressed or overexpressed in hematologic and epithelial malignant cells acting as a tumor oncogene [37]. Recently, Knubel et al. [38] described that specific MERTK inhibition profoundly limits human glioma growth, and several other studies are considering the inhibition of MERTK as a therapeutic approach to treat cancer. However, the mechanisms by which increased MERTK signaling contributes to tumor malignancy remain unknown. Interestingly, our results reveal a hitherto-unknown dual function for MERTK, depending on whether it is expressed in human DCs or T cells.

Collectively, our study gives novel insights into the molecular basis for regulating T cell activation, considering MERTK as a key player for the suppression of a T cell response. We demonstrate that MERTK expression on tol-DCs contributes to their tolerogenic function by directly regulating the adaptive immune response. MERTK is identified as a novel immune-suppressive receptor, which is expressed in human DCs and up-regulated by glucocorticoids. Therefore, it is conceivable that MERTK on the DC membrane suppresses T cell responses by neutralizing PROS1 produced by human T cells and inhibiting an autocrine and proproliferative effect of PROS1 in MERTK-expressing T cells. Interestingly, experiments by use of Mer-Fc demonstrated that the new inhibitory function of MERTK is not dependent on the activation state of DCs.

Additionally, MERTK expression in cancer cell lines, beside its role as a tumor oncogene [37, 39], could be involved in silencing T cell responses as a mechanism for tumor-immune escape. The proposed immune-evading effect of MERTK in tumors is in agreement with the immunosuppressive effect demonstrated in the current study. We provide evidence that this receptor not only acts regulating DC activation [19], but it also regulates naïve and memory T cell responses. Targeting this molecule may provide an interesting approach to induce or inhibit tolerance effectively for the purpose of immunotherapy.

AUTHORSHIP

R.C. designed and performed research, analyzed and interpreted data, and wrote the manuscript. E.A.C.S. performed research and analyzed and interpreted data. G.F.-G., A.E.E., E.C-G., and C.E. performed research. J.J.L. contributed vital analytical tools. E.R. and J.P. interpreted data. C.R. designed research and interpreted data. D.B.-R. designed research, analyzed and interpreted data, and wrote the manuscript.

ACKNOWLEDGMENTS

This research was supported by Grant SAF 2009-07272 from the Ministerio de Ciencia e Innovación, Grant TRA-097 from the Ministerio de Sanidad y Politica Social, Grant FIS PI13/01585 from Instituto Carlos III (to D.B.-R.), and a grant from the U.S. National Institutes of Health National Institute of Allergy and Infectious Diseases (R01 AI089824; to C.R.). D.B-R. is supported by Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBERehd) and by the Instituto de Salud Carlos III. R.C. is funded by a FI fellowship (Ayuda de personal investigador novel) from the Generalitat de Catalunya. Bacterial antigen (Fla2) was kindly provided by Prometheus Laboratories.

DISCLOSURES

The authors have no conflicting financial interests.

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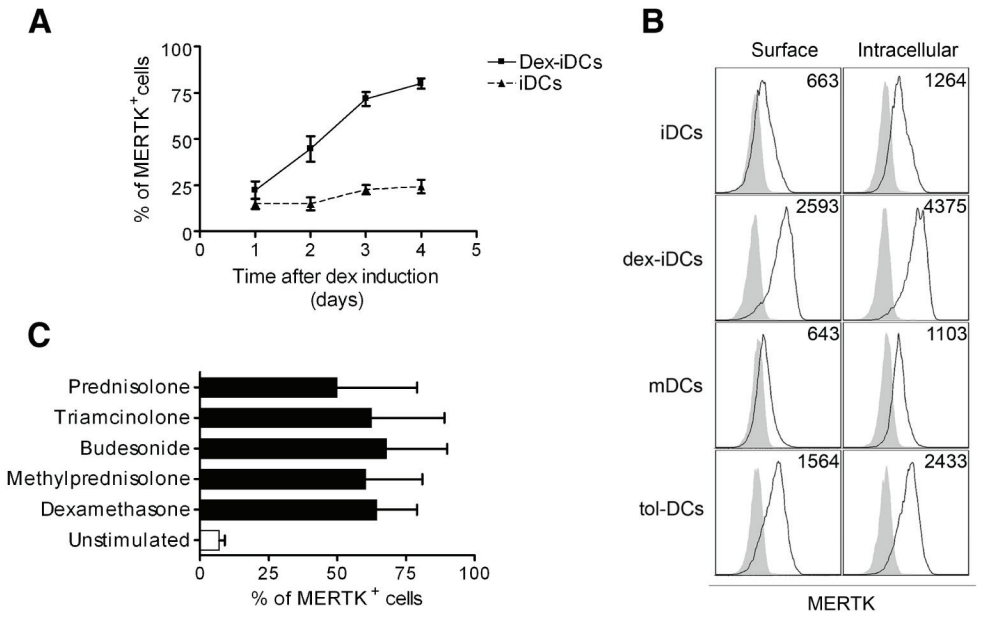
KEY WORDS:
tolerogenic dendritic cells · suppression · TAM receptors

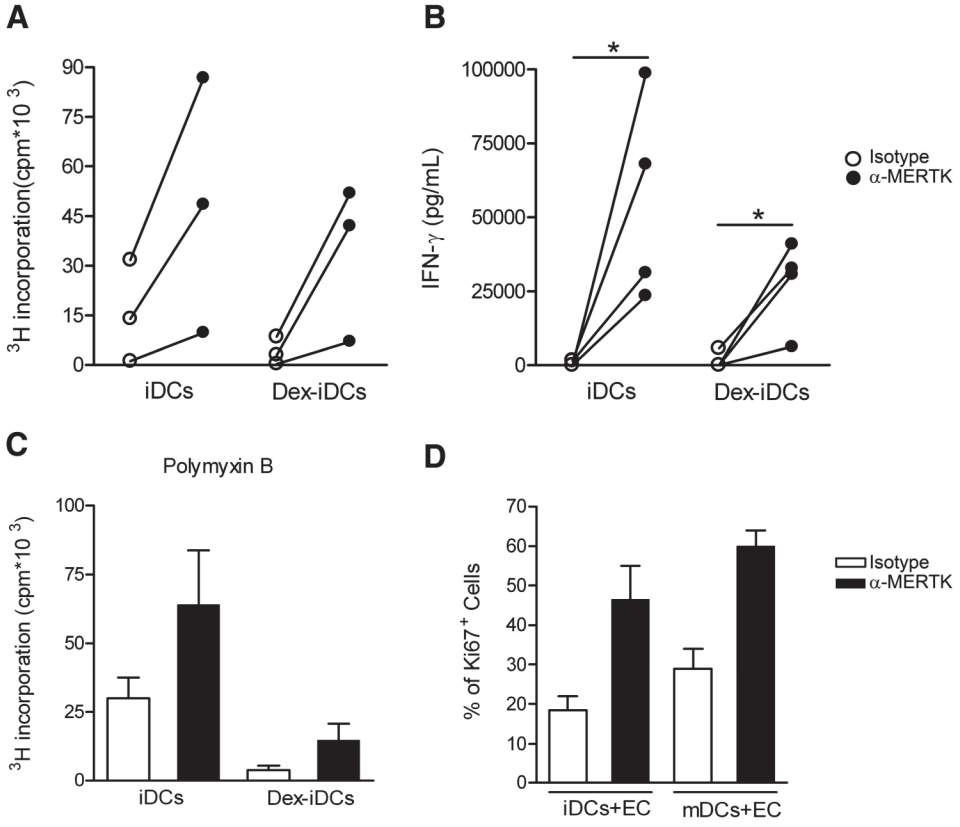
Results II

MERTK as Negative Regulator of Human T-cell Activation

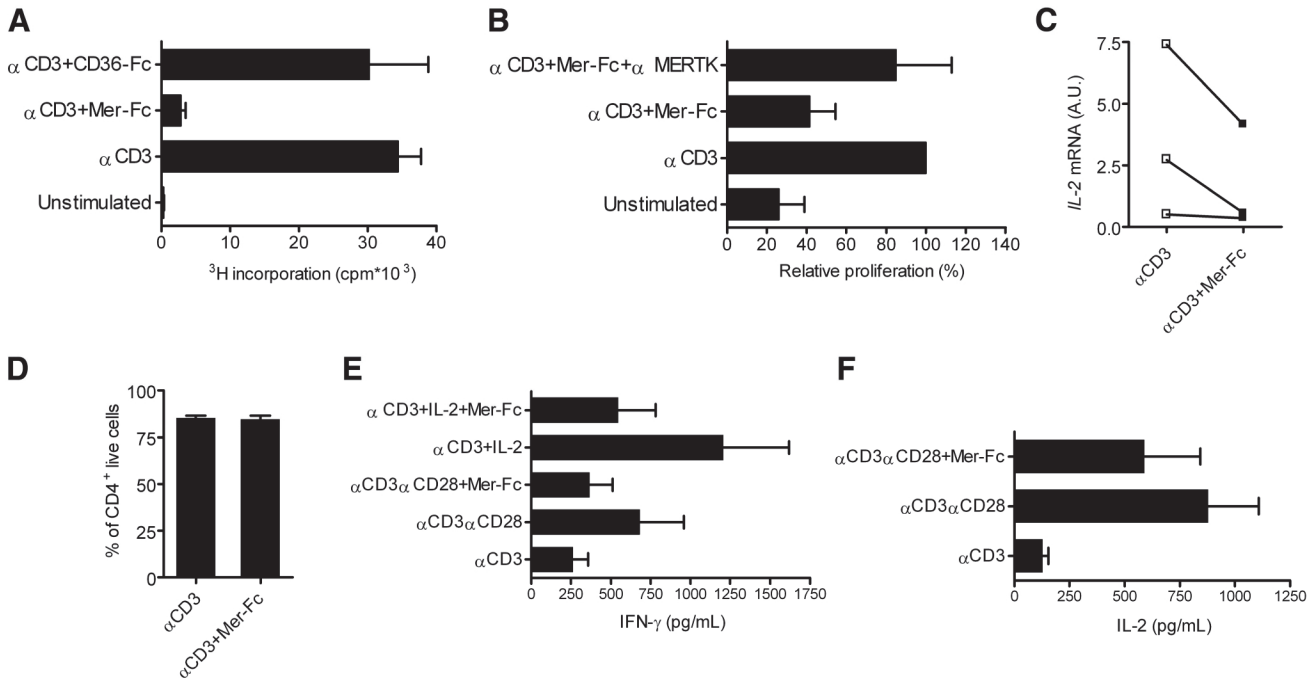
Supplementary data



Supplementary Figure 1. (A) MERTK expression kinetics. Dex up-regulation of MERTK in human DCs during 4 days after dex treatment (black line) compared to untreated iDCs (dotted line). Expression was measured by flow cytometry in each time point for every independent experiment (n=4). (B) Representative histograms of surface (left panel) and intracellular (right panel) MERTK staining in human DCs. Each histogram shows MERTK expression (black histogram) and its isotype control (grey filled histogram). Numbers indicate MERTK MFI. Isotype controls showed MFI < 250. (C) Different glucocorticoids up-regulate MERTK expression measured by flow cytometry (n=2). Data are plotted as means ± SEM, and statistical analysis was performed with two-tailed Student's t-test: *$p<0.05$, **$p<0.001$, ***$p<0.0001$.



Supplementary Figure 2. DCs were co-cultured with PBLs (ratio 1:20), and T cell activation was measured after 7 days. (A) Proliferation was increased when α-MERTK mAb was added (black filled dots) in comparison to isotype control (empty dots), n=3. (B) Increased IFN-γ production in MLR supernatant after 7 days by adding α-MERTK mAb (black filled dots) in comparison to isotype control (empty dots), n=4. (C) Polymyxin B was added to the MLR culture. Proliferation was increased when α-MERTK mAb was added (black bars) in comparison to isotype control (white bars). (D) Autologous naïve $CD4^+$ T cell proliferative response was measured by intracellular Ki67 staining. DCs were previously activated with heat killed *E.Coli* for 24h (ratio 1:10) and incubated with autologous naïve $CD4^+$ T cells for 12 days. Graph shows increment of the percentage of proliferating Ki-67^+ cells after incubation with α-MERTK mAb (black bars) compared to isotype control (white bars), n=2. Data are plotted as means ± SEM, and statistical analysis was performed with two-tailed Student's t-test: *$p<0.05$, **$p<0.001$, ***$p<0.0001$.



Supplementary Figure 3. Naïve $CD4^+$ T cell were stimulated with α-CD3 plus Mer-Fc, proliferation and production of cytokines were measured. (A) An irrelevant Fc (CD36-Fc) was added to the culture and proliferation was measured in order to discard unspecific effect on T cells due to the presence of the Fc chimera. (B) Mer-Fc effect was blocked using α-MERTK mAb (n=5). (C) *IL-2* mRNA levels after ON stimulation of naïve $CD4^+$ T cell (n=3). A.U. (Arbitrary Units) relative to *β-ACTIN* gene. (D) Naïve $CD4^+$ T cell viability after 4 days of stimulation (n=4). (E) IFN-γ and (F) IL-2 production upon stimulation of naïve $CD4^+$ T cell (n≥3). Data are plotted as means ± SEM, and statistical analysis was performed with two-tailed Student's t-test: *$p<0.05$, **$p<0.001$, ***$p<0.0001$.

Results summary

Results summary

Results I | Gram-negative Enterobacteria Induce Tolerogenic Maturation In Dexamethasone-conditioned Dendritic Cells

In this study, we described the generation of tol-DCs from healthy donors and Crohn's disease patients by use of clinical-grade reagents in combination with dexamethasone as immunosuppressive agent and characterized their functional properties. Our main findings demonstrated that the combination of dexamethasone with a specific cytokine cocktail yields clinical-grade DCs with the following characteristics: a semi-mature phenotype, a pronounced shift towards anti-inflammatory versus inflammatory cytokine production and low T-cell stimulatory properties. This characteristic tolerogenic profile is maintained when tol-DCs are activated using heat-inactivated Gram-negative bacteria (*E.Coli*) as maturative stimulus. Whole microorganisms contain multiple PAMPs capable of stimulating DCs by different pathways. Our results clearly showed a strong inhibitory effect on DC phenotype, a robust inhibition of pro-inflammatory cytokines, increased IL-10 secretion, and inhibition of T-cell proliferation and Th1 induction.

Interestingly, we showed that tol-DCs have reduced immunogenic capacity in autologous, allogeneic and antigen-specific T-cell responses. We further evaluated the ability of tol-DCs to induce $CD4^+$ T-cell hypo-responsiveness. Our results demonstrated that T-cells or antigen-specific T-cells previously cultured with tol-DCs are anergic exhibiting a reduced capacity to proliferate as well as reduced IFN-γ secretion when re-challenged with fully competent mDCs.

With regard to tol-DCs clinical application, we importantly found that their tolerogenic properties remain stable after washing out dexamethasone and subsequent re-stimulation with LPS, CD40L or different Gram-negative enterobacteria strains. All these properties led us to conclude that this cell product is suitable to be tested in clinical trials of immune-based diseases such as Crohn's disease.

Results II | MERTK as Negative Regulator of Human T-cell Activation

The purpose of this work was to identify a biomarker for tol-DCs that could potentially be involved in their tolerogenic properties. In this study, we showed that MERTK receptor is highly expressed on clinical grade dexamethasone-induced human tol-DCs and contributes in their tolerogenic properties. Our results demonstrated that MERTK expression in human DCs is regulated by GCs and described a new function of this receptor in directly regulating T-cell response.

MERTK is a tyrosine kinase that belongs to the TAM family and it is involved in limiting the magnitude of DC activation linked to ACs clearance. Interestingly, our findings showed that neutralization of MERTK with monoclonal antibodies in allogeneic MLR cultures leads to increased T-cell proliferation and IFN-γ production. The direct regulation of T-cell response was confirmed by the use of recombinant MERTK-Fc protein (Mer-Fc), used to mimic MERTK on DCs. Our results remarkably showed that Mer-Fc suppresses naïve and antigen-specific memory T-cell proliferation and activation. These findings identified a new non-cell autonomous regulatory function of MERTK expressed on DCs. Additionally, we described that this regulation is mediated by the neutralization of MERTK soluble ligand PROS1. We also found that MERTK is expressed on T-cell surface and that PROS1 drives an autocrine pro-proliferative effect on these cells.

In summary, the results of this work demonstrated that MERTK on DCs regulates T-cell activation and expansion through the competition for PROS1 interaction with MERTK in the T-cells. We showed that MERTK expression in human DCs has a key role in instructing adaptive immunity and identified MERTK as a potent suppressor of T-cell response. Therefore targeting MERTK may provide an interesting approach to effectively increase or suppress tolerance for the purpose of immunotherapy.

Discussion

Discussion

DCs are considered to be the most efficient APCs and master regulators of immunity. They can induce protective immune responses against harmful pathogens, but also contribute to the maintenance of tolerance [54]. Although our knowledge of their biology and function is not yet complete, our increased understanding of DC biology and the possibility to obtain large numbers of DCs *in vitro* from isolated monocytes has boosted the use of DCs in immunotherapy.

Data presented in this thesis focus on establishing a feasible protocol to generate clinical-grade tol-DCs to treat Crohn's disease. Importantly, we have characterized the functional properties and stability of tol-DCs to assure the applicability of these cells in clinical studies (**Results I**). We translated our findings into a phase I clinical trial using autologous monocyte-derived tol-DCs for the treatment of refractory Crohn's disease patients. This thesis further shows that MERTK is a potential biomarker for tol-DCs and demonstrates the involvement of this receptor in tolerance induction (**Results II**).

Generation of clinical-grade tol-DCs to treat Crohn's disease

For decades, most therapies to treat autoimmune diseases have been largely symptomatic and non-disease specific. Although many years of vigorous research have accumulated valuable knowledge on the pathogenic and immunological mechanisms of many immune-based disorders, their direct clinical applications have been sparse. The generation of reproducible and stable clinical-grade tol-DCs is a critical step towards developing therapeutic trials for the treatment of human disorders such as allergies, autoimmune diseases, chronic inflammation, and transplant rejection [85, 166]. Crohn's disease is one of two major IBD and is characterized by a chronic inflammation of the gastro-intestinal tract. In most patients with active disease, remissions can be induced using conventional treatments such as corticosteroids or other strategies such as anti-TNF monoclonal antibodies. However, these therapies fail in inducing or maintaining remission in about 60% of patients. These patients suffer from a poor quality of life due to disease relapse and repeated surgeries because no alternative treatments are available nowadays. In

addition, sometimes the treatments have side effects and cannot be maintained for a long time.

Thereby, research on the development of innovative therapeutic alternatives for the treatment of Crohn's disease is still needed. During the *in vitro* generation of DCs, the functional properties of these cells can be modified by the addition of immunosuppressive agents [56, 167]. Throughout the years, several agents have been used to render DCs resistant to maturation, the so-called tol-DCs [104]. Tol-DCs potential to shape the immune response relies on their ability to suppress effector T-cells, induce T-cell anergy [168] and promote the generation of T-regs [83, 169].

In this thesis, DCs generated by the addition of dexamethasone in combination with a cocktail of pro-inflammatory cytokines yielded clinical-grade DCs with tolerogenic properties. As shown in **Results I**, we performed a detailed characterization of tol-DCs properties and stability compared to iDC and mDCs from healthy donors and Crohn's disease patients (**Figure 13**). The success of DC-based immunotherapy in inducing cellular immune responses is highly dependent on accurate delivery and trafficking of the DC to T-cell-rich areas of secondary lymphoid tissues. As described by Anderson *et al*. [170] the activation of tol-DCs with TLR ligands is one strategy to improve tol-DC migratory properties and antigen presentation capabilities. Based on these findings, we combined dexamethasone with a cocktail of pro-inflammatory cytokines in order to enhance the tolerogenic and migratory properties of these cells.

In order to ascertain the nature of tol-DCs not only phenotypic description is needed but also functional studies are required. During DC-T-cell interactions, the cytokine balance determines the type of T-cell effector response. Over the last few years, increasing evidence suggests that mDCs that lack the ability to deliver signal 3 preferentially promote the differentiation of $CD4^+$ T-cells into IL-10 producing T-cells (reviewed by Joffre *et al.* [171]). Besides, the ability to induce tolerance is indeed one fundamental requisite to consider tol-DCs suitable for the treatment of autoimmune diseases. All these aspects are crucial

when designing DC-based immunotherapy protocols, and also the stability of the final product is of utmost importance.

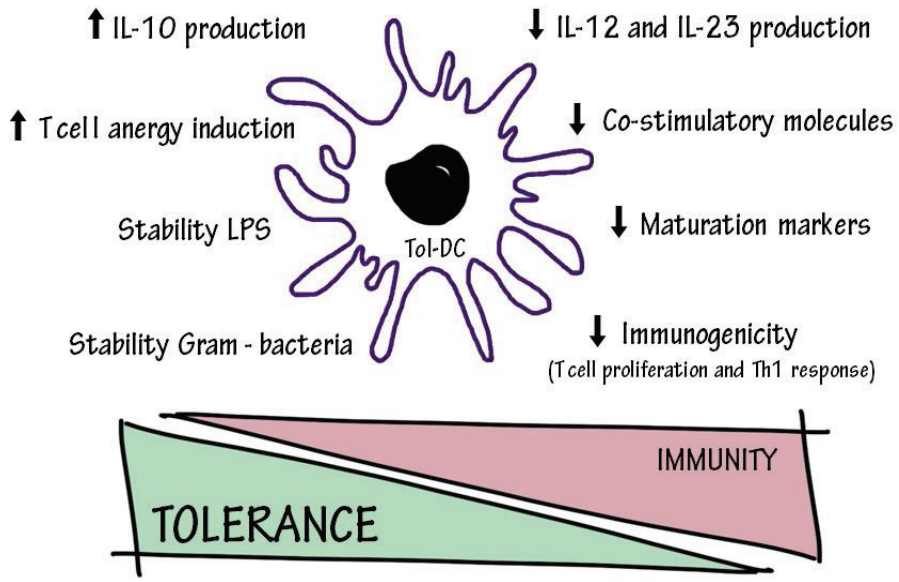


Figure 13. Characterization of human tol-DCs: functional properties and stability. Figure summarizing Results I section [172].

Although the etiological factors involved in the perpetuation of IBD remain uncertain, development of various animal models have provided insights to unveil the onset and the progression of IBD [173]. In the field of DC-based therapy, experimental data generated in murine models of colitis are highly promising especially relating to the ability of tol-DCs to prevent, reverse or ameliorate established colitis [174-176]. In a model of TNBS-induced colitis, which closely parallels the immune activation in Crohn's disease, injection of tol-DCs treated with Vasoactive Intestinal Peptide (VIP) [177] significantly ameliorated the clinical and histopathology severity of colitis in mice. An important aspect of this study was the route of administration of the DCs; the authors clearly show that by intraperitoneal administration DCs gain access to mesenteric lymph nodes where the most important antigen presentation and activation of Th1/Th17 cells takes place [178, 179]. Although several animal models have revealed the therapeutic role of tol-DCs in preventing and ameliorating IBD, the current challenge is to bring tol-DCs therapy to clinical trials. Several issues must be overcome such as the difference between IBD-induced animal models (reviewed by Neurath *et al.* [180]) and the human

disease or the functional differences between mouse and human DCs. Nowadays, these promising results in rodents need to be translated into human application, but despite the difficulties, we are on the right track.

It is well known that, in the gut, interactions between the host and the microbiota play a crucial role in mucosal immune homeostasis. Interestingly, the results of this thesis showed that tol-DCs not only were stable upon stimulation with gram-negative enterobacteria but also some of their tolerogenic properties were even enhanced, such as IL-10 production. In the context of a DC-based therapy, the interaction between enterobacteria and tol-DCs would take place in the inflamed lamina propria of Crohn's disease patients. Interestingly, notwithstanding isolated monocytes from Crohn's disease patients are in an enhanced pro-inflammatory environment [181], we showed that moDCs from Crohn's disease patients can be educated towards tolerogenic phenotype. These findings were crucial for considering tol-DCs as a feasible treatment for Crohn's disease, because it might have occurred that biology of DCs have been altered by genetic variants conferring susceptibility for Crohn's disease. Indeed, Lee *et al.* recently studied the effects of genetic variation on the induction of pathogen-responsive genes in human DCs. Interestingly, the authors described common alleles that may explain inter-individual variation in pathogen sensing and provided functional description for genetic variants that alter susceptibility to inflammatory diseases [182].

The results of this thesis are in line with other preclinical studies that aimed to develop feasible protocols to generate tol-DCs for the treatment of immune-based diseases such as RA or MS [183, 184]. However, only two clinical trials have taken advantage of their specific tolerogenic properties to treat Type 1 Diabetes [89] and RA [90] patients to date. In 2011, we started a phase Ib clinical trial to evaluate the safety and tolerability of tol-DCs immunotherapy in Crohn's disease patients refractory to other treatments. In this study, we hypothesize that the intraperitoneal injection of autologous tol-DCs will facilitate the arrival of DCs to the mesenteric lymph nodes and therefore the inflamed lamina propria of patients. These studies are currently pioneering tol-DCs therapy for the treatment of autoimmune diseases in Europe. Even though

these trials were not designed to prove efficacy, they have all been safe and well tolerated in patients, representing an important step forward in the field.

Challenges for Crohn's disease DC-based therapy

DC-based therapies are generally envisaged to inhibit **antigen-specific** T-cell responses and the appropriated antigen selection to load DCs is under intensive research. In regard with designing efficient tol-DCs therapy for autoimmune diseases, it is crucial to know which immunodominant self-Ags are driving autoimmune responses. The treatment of autoimmune diseases in an Ag-specific manner is important to avoid both systemic immunosuppression and the adverse effects of steroids [185, 186]. While humoral response against antigens derived from microbiota has already been described in Crohn's disease patients, no T lymphocyte Crohn's disease-specific antigen has been properly identified. As reviewed by Alexander *et al.* [187], Crohn's disease patients can be classified based on seroreactivity to different microbial antigens:

a) oligomannan, anti-*Saccharomyces cerevisiae* antibodies (**ASCA**) [188]

b) **anti-I2** (Crohn's disease-related protein from *Pseudomonas fluorescens antibodies*) [189] and **anti-OmpC** (*E.coli* outer membrane porine C antibodies) [190]

c) **anti-pANCA** (perinuclear anti-neutrophil cytoplasmic antibodies) [190]

d) non-responders to any microbial antigens

Although **anti-CBir1** flagellin-positive patients [191] are not included in any of the above groups, CBir and other bacterial flagellins have been largely described as dominant humoral antigens in Crohn's disease [192].

Despite the association of Crohn's disease with a high inflammatory component, mainly corresponding to Th1 and Th17 T-cells [177, 179], the antigenic specificity of these cells remains still unclear. Interestingly, commensal-specific T-cell responses are detected during mouse model of intestinal inflammation with *Toxoplasma gondii* infection [193, 194]. However, whether commensal specific T-cells may represent an important component of

IBD pathogenesis remains to be understood. In animal models, Yamanishi *et al.* [176, 195] identified a specific protein, carbonic anhydrase I (CA I), specifically involved in the IBD pathogenesis. Interestingly the authors demonstrate the role of CA I loaded tol-DCs in preventing the induction of colitis via T-regs. Pedersen *et al.*, administered DCs pulsed with enterobacterial extract to suppress development of colitis [175]. In contrast, other authors have also demonstrated tolerance induction in colitis model without using any specific antigens [174, 177]. This mechanism would involve the production of regulatory cytokines by DCs (TGF-β and IL-10) and the expression of inhibitory receptors that might overcome the requirement of a known antigen. This "trans-tolerance" may result in the generation of specific regulatory responses that may help restoring the mucosal homeostasis.

Nevertheless, the proper selection of the **administration route** will determine the future location of the DCs *in vivo* and represents a crucial point for the therapeutic efficacy in cell therapies. So far, it is not established yet which route of administration is best suited and where the cells need to migrate in order to trigger suitable immune responses to successfully change the course of the inflammatory response. In physiologic conditions, activated DCs migrate from peripheral tissues to the draining lymph nodes. Throughout the years, four main vaccination routes have been used in DC-based therapy:

a) Intravenous (iv)

b) Intradermal/subcutaneous (id/sc)

c) Intraganglionar (ig)

d) Intralesional (il)

I.v administration was the first vaccination route used in DC-based clinical trials, and several studies have reported no apparent adverse effects. One advantage is that it allows systemic distribution of the cells although there are evidences that many cells end up in the spleen, lungs and liver. This systemic distribution would allow a more specific tolerance induction [196]. On the other hand, i.d or s.c administration represents a more physiological route for the function of DCs because it locates the cells in peripheral tissues. Although it is easier to

achieve, it has been demonstrated that low percentage of injected cells reach the draining lymph nodes and that may limit its clinical efficacy [117]. Finally, the i.g route consists of a direct injection of DCs into the lymph node and it is technically more complex. This strategy assures the interaction of DCs with T lymphocytes; however it may affect the lymph node structure and reduce the expected efficacy.

Another possibility, a lot less explored due to the complexity of the technique, is the intralesional administration of DCs. So far studied in tumors, this strategy allows DCs to uptake a broad spectrum of specific antigens presents in the tumor and triggers a more generalized immune response against the tumor. It is tempting to speculate that this route of administration could be suitable for the treatment of Crohn's disease patients due to the direct interaction of tol-DCs with the inflammatory compounds of the mucosa. In fact, tol-DCs would be exposed to a broad-spectrum of possible antigens involved in the inflammatory processes of the disease and therefore generate a local antigen-specific immunosuppression. However, to date comparative studies measuring efficacy of intralesional injection versus other routes of administration are missing. Recently, one study showed that local administration of mesenquimal stem cells in patients with fistulizing Crohn's disease had considerable therapeutic effects compared to systemic administration [197]. In the case of IBD patients, no studies have compared or established the most suitable route of administration for DC-based vaccines.

After administration of DCs, the parameters for monitoring clinical effectiveness also need to be established and are likely to vary depending on the DC-vaccine design. In general, antigen-specific therapies consider the frequency of antigen-specific lymphocytes in peripheral blood an important end-point of the patient's response. It can often be complemented by the evaluation of humoral responses in serum. As mentioned above, lack of Crohn's disease associated antigen makes more complicate the establishment of a protocol to monitor the immunologic outcome in treated patients. Moreover, monitoring a multifactorial disease such as Crohn's disease that involves several factors and immune cells is a challenge that may be helpful to better understand the pathogenesis of the disease.

Mechanisms underlying tol-DCs function

This thesis further aimed at revealing what molecules and mechanisms are implicated in the tolerogenic function of tol-DCs *in vitro.* As described in **Results II**, we identified MERTK as a specific marker for our tol-DCs and we focused on investigating the function of this receptor in regulating human immune response. Members of the TAM family brought our attention because deficiencies in TAM signaling result in human retinal dystrophies and may contribute to lupus and other human autoimmune diseases [155]. In particular, the role of MERTK and its ligands has been well characterized during the last decade, but most of the studies have been performed in genetically modified mice [131, 136]. Therefore, the knowledge of the regulation of these receptors in humans is scarce. The results of this thesis described a new function of human MERTK receptor in regulating adaptive immune response. MERTK expression on human DCs contributes to the suppression of T-cell activation. In contrast to mice system, we demonstrated that human T-cells also express MERTK and it has a relevant role in controlling T-cell proliferation through the binding to its ligand PROS1 (**Figure 14**). Although further research is required to provide evidence of the mechanisms that mediate this regulation *in vivo*, TAM receptors have long been associated with anti-inflammatory responses. Thus, the interest in targeting these molecules for the purpose of autoimmune diseases or cancer therapy has increased in the last years.

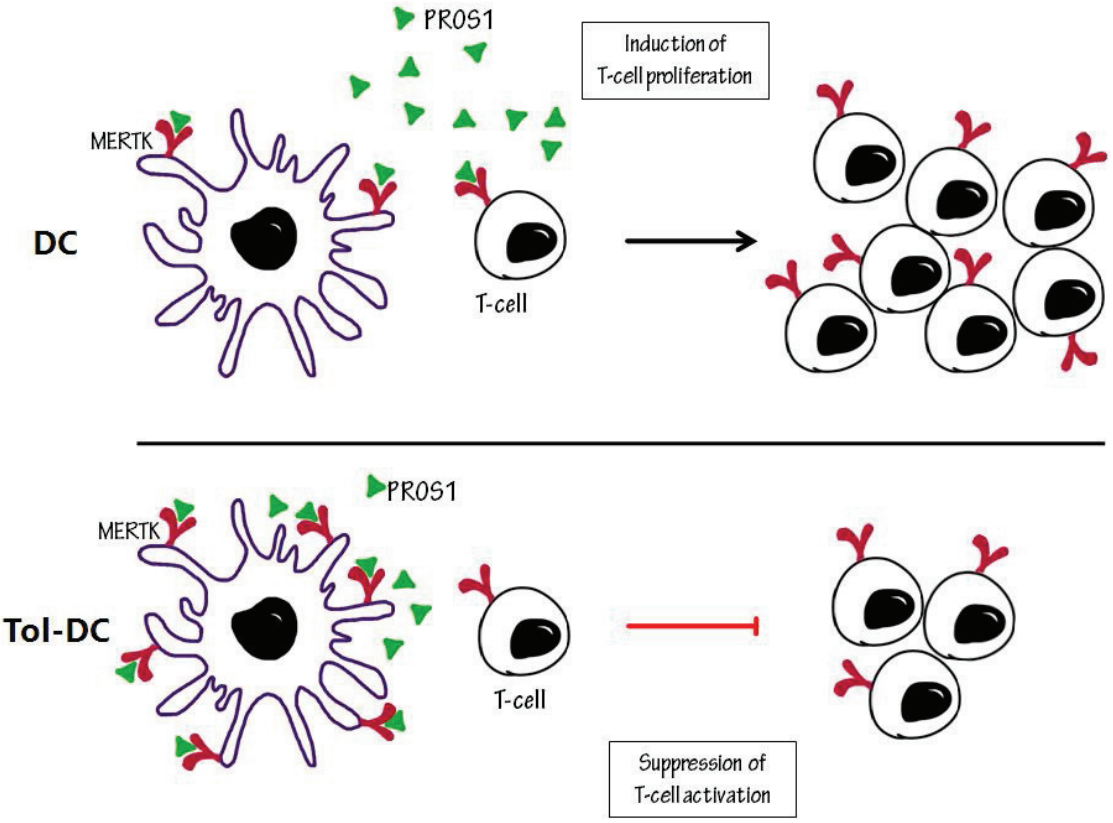


Figure 14. Modulation of T cell proliferation by MERTK and its ligand PROS1. Figure summarizing Results II section [198].

For several years, the study of these receptors have been performed using double or TKO mice that are susceptible to several autoimmune diseases including colitis [199] and retinitis [200]. Recently, Zagórska *et al.* published an interesting study on the diversification of TAM receptor tyrosine kinase function [201]. The authors showed differences in their ligand specificities, receptor shedding upon activation, and divergent roles between AXL and MERTK as phagocytic receptors. These findings suggest that modulation of individual TAM receptors might be relevant for the treatment of human diseases.

The over-expression of the three members of the TAM family has been reported in a variety of human cancers [202]. In tumors, the presence of TAM receptors triggers the engulfment of apoptotic material and subsequent anti-inflammatory macrophage polarization, inducing an immunosuppressive microenvironment that favors tumor cell survival and proliferation. In particular, MERTK has been shown to be over-expressed in patients with B- and T-Acute Lymphocytic Leukaemia [151, 152], acute myeloid leukaemia [203], multiple myeloma [204],

melanoma [153] and glioblastoma [205]. The identification of MERTK in human cancer correlates with poor prognosis of the disease and some *in vitro* studies already aimed at inhibiting MERTK as a novel therapeutic approach for the treatment of cancer [206, 207].

In the opposite context, mouse studies clearly demonstrated that defects in TAM signaling are associated with autoimmunity, but are these defects also evident in human autoimmune diseases? Some literature points to a clear association between reduced PROS1 levels in the circulation of SLE and IBD patients [156, 208], however, none of the known PROS1 deficiencies to date have been classified based on the functional properties of PROS1. Moreover, in 2011, a large genome-wide association study of MS identified an association between polymorphisms in *MERTK* and susceptibility to this disease [209]. Interestingly, a recent study associated the presence of the risk alleles in *MERTK* for MS and a reduced expression of MERTK in peripheral blood human monocytes [210]. Besides, a role for MERTK signaling has also been proposed in Retinitis Pigmentosa [211] and SLE, in both cases directly associated with an impaired clearance of ACs. It has been recently shown that circulating macrophages that express MERTK are significantly increased in SLE and positively correlate with disease activity and severity [212]. Even though these are controversial results, authors pointed out that cell debris probably sustains type I IFN activation in SLE and that would consequently up-regulate the expression of MERTK to mediate immune-suppressive signaling. Interestingly, this study also showed that anti-inflammatory MERTK expressing macrophages (M2c) were reduced in SLE patients compare to healthy controls.

So far, there is no evidence associating genetic abnormalities in TAM expression with Crohn's disease pathogenesis. However, TAM receptors have recently been described as critical for down-regulating pro-inflammatory cytokines under the chronic, but not acute, NOD2 stimulation in the intestinal environment [213]. This interesting study also investigated chronic NOD2 stimulation in human monocyte-derived macrophages and revealed that PROS1 mediates the down-regulation of pro-inflammatory cytokines. Remarkably, they also found that human intestinal macrophages expressed higher TAM levels than peripheral macrophages. These findings are in line with the conclusions of

this thesis; it is tempting to speculate that tolerogenic DC-based therapy for Crohn's disease patients could dampen chronic inflammation in the intestine. The tolerogenic properties of these cells together with MERTK expression confer a potent immunosuppressive capacity that may serve to reverse the exacerbated intestinal inflammatory response of these patients. Although an intensive research regarding TAM signaling in different diseases has improved the understanding of these receptors during the last years, there are still several challenges in the field awaiting clarification. In conclusion, therapeutic modulators of TAM receptors are currently very attractive for the treatment of autoimmunity or cancer.


Conclusions

Conclusions

The conclusions derived from the studies performed during this thesis can be summarized as follows:

1. Clinical-grade tol-DCs present tolerogenic properties and stable phenotype.

    1.1. The combination of dexamethasone with a specific cytokine cocktail yields clinical-grade tol-DCs with semi-mature phenotype, a pronounced shift towards anti-inflammatory versus inflammatory cytokine production, reduced T-cell stimulatory properties and ability to induce T-cell anergy.

    1.2. The tolerogenic properties of dexamethasone-conditioned DCs are stable upon re-stimulation with LPS, CD40L or Gram-negative bacteria.

2. MERTK is a potential biomarker for tol-DCs and contributes to their tolerogenic function by regulating the adaptive immune response.

    2.1. MERTK is expressed on human DCs and up-regulated by glucocorticoids at RNA and protein level.

    2.2. Neutralization of MERTK in allogenic MLR as well as autologous DC-T-cell cultures leads to increased T-cell proliferation and IFN-γ production.

    2.3. MERTK on DCs suppresses human naïve and memory T-cell activation and proliferation through the neutralization of its soluble ligand PROS1.

    2.4. MERTK is also expressed on human T-cells and its interaction with PROS1 has a pro-proliferative effect.

    2.5. Our results suggest that MERTK on DCs controls T-cell activation and expansion through the competition for PROS1 interaction with MERTK in the T-cells.

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