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*This work is dedicated to all patient who have participated in presented studies  
and to my family*

## List of abbreviations

Ab	Antibody
Ag	Antigen
APC	Antigen presenting cell
APRIL	A proliferation-inducing ligand
AZA	Azathioprine
BAFF	B cell activating factor
BCR	B cell receptor
BDCA	Blood dendritic cell antigen
Blys	B lymphocyte Stimulator
BrdU	Bromodeoxiuridine
Btk	Bruton's tyrosine kinase
CARD	Caspase recruitment domain
CD	Cluster of differentiation
cDC	Conventional dendritic cell
CLA	Cutaneous lymphocyte antigen
CMKLR1	Chemokine like receptor 1
CRD	Carbohydrate recognition domain
CsA	Cyclosporine A
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic lymphocyte antigen-4
DC	Dendritic cell
dsRNA	Double stranded ribonucleic acid
EDA-ID	Anhidrotic ectodermal dysplasia with immunodeficiency
ER	Endoplasmic reticulum
Fc	Fragment crystalizable
Fc R	Fc gamma receptor
GC	Glucocorticoids
GM-CSF	Granulocyte macrophage colony stimulating factor
GVHD	Graft versus host disease
HLA	Human leukocyte antigen
HSCT	Hematopoietic stem cell transplantation
ICOS-L	Inducible co-stimulator ligand

IDO	Indoleamine 2,3-dioxygenase enzyme
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IRAK	IL-1 receptor associated kinase
ISRE	Interferon stimulated response elements
ITAM	Immunoreceptor tyrosine based activation motif
ITIM	Immunoreceptor tyrosine based inhibition motif
	Kappa
LC	Langerhans cell
LFA	Leukocyte-function-associated antigen
LPS	Lipopolysaccharide
LRR	Leucine rich repeat
LTA	Lipoteichoic acid
mDC	Myeloid dendritic cell
MDP	Muramyl dipeptide
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
MMF	Mycophenolate mofetil
MyD88	Myeloid differentiation primary response protein
NEMO	NF- B essential modulator
NF	Nuclear factor
NK	Natural killer cell
NKT	Natural killer T cell
NLR	Nod-like receptor
NOD	Nucleotide oligomerization domain
PAMP	Pathogen-associated molecular pattern
pDC	Plasmacytoid dendritic cell
PID	Primary immunodeficiencies
PRR	Pattern associated molecular pattern
RANK	Receptor activator of NF- B
RIG	Retinoic acid inducible gene 1
SLC	Secondary lymphoid tissue chemokine
SMAC	Supramolecular activation cluster

SR	Scavenger receptor
ssRNA	Single stranded ribonucleic acid
TA	Tumor antigen
TAP	Transporter associated with antigen processing
TCR	T cell receptor
TGF-	Transforming growth factor-
Th	T helper cell
TIL	Tumor infiltrating lymphocyte
TIR	Toll-IL-1 receptor
TLRs	Toll like receptors
TNF	Tumor necrosis factor
TRAIL	TNF related apoptosis inducing ligand
TRANCE	TNF-related activation induced cytokine
Treg	T regulatory cell
WBC	White blood cell
XLA	X-linked agammaglobulinemia

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## 1 Introduction

During the past decades several spectacular findings have been made in the field of immunology. Elucidating the functions of the antigen presenting cells (APCs) belong to the most important. Dendritic cells (DCs) represent a specific group of APCs with a unique ability to initiate primary immune responses. Despite the fact that, *in vivo*, they are very rare and difficult to isolate, DCs came very fast into the focus of scientific interests. Development of novel laboratory techniques facilitated a robust expansion of their research. With time it has been proven that DCs play a pivotal role in initiation, maintenance and control of the immune responses. The extraordinary features of DCs were soon investigated in human clinical trials, where DCs have been particularly used as vectors for vaccination protocols, especially in the treatment of tumors.

However, DCs capability to polarize the outcome of immune response and the potential to induce or suppress immunity under specific circumstances led to the idea that they might be also used in the treatment of autoimmune and allergic diseases or in transplantation medicine as well.

There is a need to stress that most of the knowledge has been obtained from the *in vitro* generated DCs, but advanced technological methods bring us the opportunity to study DCs directly *in vivo*. Multiparametric flow cytometry, two-photon microscopy, confocal microscopy and others shed some light on the DCs *in vivo* identification, quantification, *in vivo* trafficking and complex systemic interactions. Data acquired by *in vivo* monitoring reflect normal and various pathological conditions and they might be used as prospective diagnostic tools in medicine.

## 2 The immune system

The immune system works as a highly sophisticated network of protein, cells, tissues and organs, that provides rapid, nonspecific and specific protective immune responses for body against attacks by “foreign” invaders or potentially its own altered cells. In jawed vertebrates the immune system can be divided into two functionally distinct parts: innate and adaptive. These two groups are not isolated but have complex interactions constantly going on between them. The innate immune system senses pathogens through pattern-recognition receptors (PRR), which trigger the activation of antimicrobial defenses and stimulate the adaptive immune responses<sup>1</sup>. The adaptive immune system, in turn, activates innate effectors mechanisms in an antigen-specific manner. However the innate and adaptive immunity deals with the molecular diversity of pathogens in fundamentally different ways, the narrow cooperation between the two components is essential for the efficient host protection. This linkage is mainly mediated through the antigen presenting cells.

### 2.1 Antigen presenting cells

Dendritic cells, macrophages and B-lymphocytes are the three main APC subtypes. Due to the contribution to the process of naïve T-lymphocyte priming DCs stand for the privileged position. However their morphology is sufficiently distinctive, and in fact the term “dendritic cell” has arisen from their characteristic shape (first applied by Steinman and Cohn in 1973 who discovered these cells in mouse spleen), the uniqueness among the other APCs is mainly determined by their functions<sup>2</sup>. Recent findings support that DCs are quite phenotypically heterogeneous, and their only common feature is the ability to migrate and effectively stimulate primary immune responses<sup>3</sup>. In comparison to other APCs, the stimulatory capacity of DCs is highlighting more folds efficacious. It has been shown that exclusively DCs are capable to stimulate naïve, undifferentiated T-lymphocytes<sup>4</sup>. On the other hand, cells already activated during the primary immune responses can be re-stimulated in the presence of another APCs<sup>5</sup>. These facts led to the hypothesis, that DCs initiate the immune responses *de novo*, whereas other types of APCs participate in their amplification.

### 3 Dendritic cells

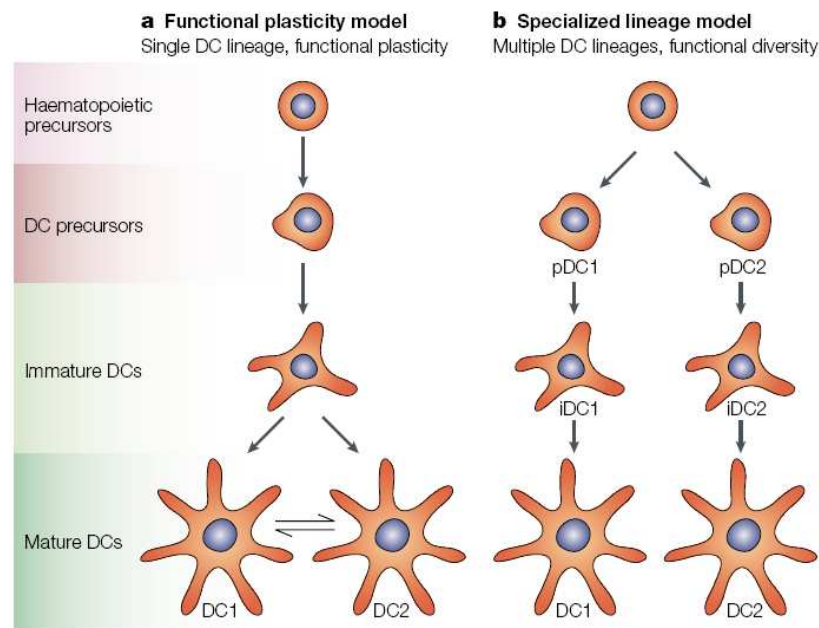
#### 3.1 Biological features

DCs act as the sentinels of immune system residing in most peripheral tissues, particularly at sites of interface with the environment<sup>6, 7</sup>. In the absence of ongoing inflammatory and immune responses they constitutively patrol through the blood, peripheral tissues, lymph and secondary lymphoid organs. In peripheral tissues they take up self and non-self antigens, which are then processed into proteolytic peptides and loaded onto MHC class I and II molecules in process called antigen presentation. Constitutively, however, peripheral DCs present antigens quite inefficiently until a signal from pathogens, often referred to as a “danger signal”, induces DCs to enter a developmental program called maturation, which transforms dendritic cells into efficient APCs and T cell activators. Notably, soon after encountering a danger signal the efficiency of antigen uptake, intracellular transport, degradation and the intracellular traffic of MHC molecules are rapidly modified<sup>7</sup>. Peptide loading as well as the half-life and delivery to the cell surface of MHC molecules increases and the surface expression of T cell costimulatory molecules rises. Concomitant with the modifications of the antigen presentation abilities, maturation also induces massive migration of DCs out of peripheral tissues towards secondary lymphoid organs, thus allowing the interaction with T cells<sup>6</sup>. This is accompanied by the modifications in the expression of chemokine receptors, adhesion molecules, as well as profound changes in the cytoskeleton organization of DCs. In addition to antigen presentation, DCs also influence the outcome of immune responses, because different DCs subsets at different maturation stages express distinct surface molecules and secrete varying cytokines, thus determining selectively the type of induced immune response.

#### 3.2 Subsets of dendritic cells

It has been presumed that several and often opposing roles of DCs cannot all be carried out at once by the same cell and different sets of DCs performing different functions should exist. Such specialized DC subtypes might represent different activation states of a single lineage with functional differences depending entirely on local environmental signals (Fig.1a), or alternatively the specialized DCs subtypes could be the products of entirely

separate developmental lineages. In this case the signals that determine lineage segregation would act earlier and the immediate precursors of the DC would already be separate and functionally committed (Fig.1b). So far it is not clearly known which model is the more probable, however it seems that the reality consists of a combination of both models with a large degree of DCs functional plasticity as the general feature.



**Figure 1 | Alternative models for the generation of functionally distinct dendritic cells subtypes. a |** The functional plasticity model proposes that all DCs belong to a single haematopoietic lineage, the different subtypes of DCs being generated by local environmental influences on a relatively mature but plastic end-product cell. **b |** The specialized lineage model proposes that the different subtypes of DCs derive from early divergences in the developmental pathway, producing several distinct haematopoietic sublineages.

iDC, immature DC; pDC, precursor of DC.

**Adapted from Nat Rev Immunology (Shortman, Liu, 2002)**

Distinct subtypes were initially more evident among mouse than human DCs, because of the availability of different murine lymphoid tissues and the expression on mouse DC of markers not present on human DCs. So far five murine DC subtypes have been identified and characterized by the combination of surface expression of CD8, CD4, CD205 and CD11b<sup>8-10</sup>. In terms of the mentioned development models it was important to know whether these DC subtypes were products of separate developmental lineages or different activation states of a single lineage. Past studies on the mouse DCs focused on the possible origin from separate haematopoietic precursors. The hypothesis was based on the findings that although most DC were thought to be of myeloid origin<sup>11</sup>, there was evidence that some DCs shared early

development steps with B cells and that the thymus DCs shared early steps of development with T cells<sup>12-14</sup>. Thus all CD8<sup>+</sup> mouse DCs were thought to derive from lymphoid-restricted precursors, and all CD8<sup>-</sup> DC to derive from myeloid-restricted precursors, leading to the terms 'lymphoid' and 'myeloid' DCs. Later on it has been shown that this concept was not fully correct, because certain mice knockouts (*c-kit*<sup>-/-</sup> and conditional *Notch1*), which could not form T cells, still produced thymic CD8<sup>+</sup> DC<sup>15, 16</sup>. After myeloid and lymphoid restricted precursors were isolated from the bone marrow, it became evident that both precursors could produce all mature splenic and thymic DCs subtypes, albeit with some bias in the subset balance<sup>17, 18</sup>. Recent data with bromodeoxyuridine (BrdU) labeling studies showed that a degree of DCs sublineage commitment must occur downstream of the early haematopoietic precursor<sup>19</sup>.

Searching for the human homology is complicated by several facts. First of all, there are relatively few studies performed on freshly isolated tissue mature DCs. Second, blood is the only readily available source and finally human DCs lack the expression of CD8, thus the equivalent of mature mouse CD8<sup>+</sup> DCs remains elusive. The only one comparable subset remained the human Langerhans-cell DCs characterized by the expression of CD1a, langerin and the presence of Birberck granules.

However, in a few studies a straight isolation of immature DCs from lymphoid tissues was made and direct comparison with mouse DC subtypes could be performed. Splenic and tonsillar DCs isolated this way showed heterogeneity in the expression of CD4, CD11b and CD11c resembling the complexity of mouse splenic DCs. Recent studies on human thymic DCs make a stronger case for subset segregation, similar to that in the mouse<sup>20, 21</sup>. Most human thymic DCs are CD11c<sup>+</sup>CD11b<sup>-</sup>CD45RO<sup>lo</sup> and lack myeloid markers, so resemble mouse thymic CD8<sup>+</sup> DCs. Many different studies support that DC populations identified in distinct human organs are quite heterogeneous in their cell surface markers expression. Heterogeneity probably reflects the different activation and differentiation stages of DCs<sup>22</sup>, but the specific phenotype is also affected by their anatomic localization. Human DCs have been identified in skin epidermis (Langerhans cells), in dermis (interstitial DCs), in spleen (in marginal zone and T-zones), in germinal centers, thymus, liver and also in peripheral blood.

Despite this heterogeneity, so far there are two major accomplished DC subsets in human with clearly different functional and phenotypic properties. These two subsets are the plasmacytoid (pDC) and myeloid dendritic cells (mDC) (recently renamed as conventional dendritic cells (cDC)). Both populations can be identified in peripheral blood where they represent approximately 1% of the white blood cells (WBC).

- Human plasmacytoid DCs

In 1958 Lennert and Remmele discovered a cell type similar to plasma cells residing in the T cell zones of human lymphoid tissues. These cells lacked typical B and plasma cell markers<sup>23</sup>. Subsequently phenotypic identical cells producing large amount of IFN- $\alpha$  were identified in peripheral blood<sup>24</sup>. Later on it has been shown that these are identical cell populations of plasmacytoid DCs<sup>25</sup>. The circulating precursors of pDCs lack the myeloid marker CD11c but express CD123, the receptor for IL-3 (IL-3R). IL-3 together with other maturation signals like CD40 ligand can induce the differentiation of the progenitors to phenotypic mature DCs<sup>25</sup>. Recently other specific surface markers of peripheral pDC have been identified. These were the BDCA-2 and BDCA-4 molecules and the chemokine-like receptor 1 (CMKLR1)<sup>26-28</sup>. Currently an enormous effort is concentrated on the pDCs research. The main reason for that is the evidence that pDC preferentially induce Th2 responses and might play a role in certain immuno-pathological conditions<sup>29</sup>.

- Human myeloid DCs

MDC are heterogeneous cell population phenotypically different from pDCs. MDC are derived from the myeloid peripheral blood precursors expressing HLA II-class molecules and lacking lineage markers characteristic for other hematopoietic cell lines (so-called lineage negativity). In contrast to pDC, mDC express high amounts of CD11c and lack the expression of CD123. As the mDC are also called the in vitro generated DC from myeloid precursors (monocytes).

### 3.3 Differentiation of DCs in vitro

Most of the knowledge about the biology and developmental origin of human DCs has come from the experiments with in vitro generated immature or precursor DCs. These studies led to the concept of distinct developmental pathways, although the correlation between the naturally occurring DCs and their in vitro generated counterparts is still not clear. There are three basic approaches which have been used to generate human DCs in vitro, however, the most commonly used precursors are the blood monocytes. In the presence of macrophage colony-stimulating factor (M-CSF) they differentiate in macrophages, but in the presence of GM-CSF and IL-4 they turn into DCs<sup>30-32</sup>. The final maturation to CD14<sup>-</sup> CD38<sup>+</sup> CD86<sup>+</sup> - surface MHC-IIhi DCs is then achieved by stimulating with maturation signals such as TNF- $\alpha$

or LPS.

Notably, there is also another model of human monocyte-to-DCs transformation which (although still involves cell culture) mimics better the events *in vivo*. This model uses a layer of endothelial cells over a collagen matrix with the monocytes migrating across this barrier into the matrix thus resembling the entry into tissues<sup>33</sup>. Many of the monocytes remain and become the equivalent of tissue macrophages. However, some reverse direction and migrate back across the endothelial barrier, mimicking the transit from tissues to lymph; these transmigrating monocytes become DCs.

To summarize all of the so far known facts, there are two hypothesized models of DCs subsets development. The older model presumes that the definitive phenotype is already predetermined in the very early stages of differentiation and the diversity of DCs subsets is a result of different development lineages. Contrary to this is the second model which takes in account the fact, that in some studies all DC subtypes could be generated independently to the origin of their precursor<sup>18</sup>. The second model is currently preferred and the idea of “functional plasticity” is more presumable. This model supports the hypothesis that all DCs differentiate from the same haematopoietic precursor and that under certain circumstances an already well defined subtype of DCs can transform into another.

### 3.4 Antigen uptake and processing

Dendritic cells were long believed to display low endo- and phagocytic activities. The primary observations pointed out their inability to take up antigens and despite the high MHC class II expression DCs were not considered as APCs. This statement lasted until the identification of phagocytic Langherans cells (LCs) as the precursors of some DCs in lymphoid organs. Beyond this, some of the bone marrow-derived DCs (at their early stage of development) were shown to internalize particulate antigens. These observations led to the theory that the maturation state of DCs is crucial in the process of antigen uptake<sup>34</sup>.

Immature DCs have several features that allow them to capture antigens. DCs mainly use receptor mediated endocytosis, phagocytosis or macropinocytosis.

Endocytosis is mediated through a variety of receptors. The most important are the Fc receptors (CD64 and CD32), C-type lectins (mannose receptor, DEC-205) and the scavengers. The receptor-mediated endocytosis allows the uptake of macromolecules through the specialized regions in the plasma cell membrane, termed coated pits. The process is initiated

by the signal in the cytoplasmic tail of the endocytic receptor. This part is recognized by the family of adaptor proteins responsible for the recruitment of clathrin lattices and further formation of clathrin-coated endocytic vesicles.

Particulate and soluble antigens are efficiently internalized by phagocytosis and macropinocytosis, respectively. Both processes are actin dependent, require membrane ruffling and result in the formation of large intracellular vacuoles. Phagocytosis is generally receptor mediated, whereas macropinocytosis is rather a cytoskeleton dependent type of fluid-phase endocytosis. DCs can phagocytose apoptotic or necrotic fragments with the involvement of CD36,  $\alpha V\beta 3$  and  $\alpha V\beta 5$  integrins<sup>35, 36</sup>. Important are also the complement receptors, since DCs are able to capture apoptotic particles coated with the iC3b fragments. Notably, opsonisation with iC3b was declared to inhibit the maturation of DCs, thus contributing to the maintenance of self-tissues tolerance<sup>37</sup>.

Macropinocytosis represents the critical antigen uptake pathway of DCs. It's an intensive process which in vitro occurs spontaneously and permanently and all of this even without the addition of stimulating factors (TNF, LPS, IL-1) otherwise necessary for other cell types<sup>38</sup>. Immature DCs constitutively take up antigens in peripheral tissues until they obtain the maturation signal. After that macropinocytosis stops and DCs migrate towards secondary lymphoid tissues to present the engulfed antigens<sup>39</sup>.

Exogenous antigens captured by DCs enter the endocytic pathway of the cell. The internalized antigens are degraded by lysosomal proteases and presented to T cells on MHC class II molecules. Comparing to macrophages, DCs are protease-poor, resulting in a limited capacity of lysosomal degradation. This seems to be very important, because it allows the generation of longer peptide fragments which fit better into MHC class II molecules and the prolonged degradation enables an extended antigen presentation<sup>40</sup>.

In addition to the basic concept of MHC-class I or II restricted antigen presentation, DCs are capable to present exogenous antigens onto MHC class I molecules by a phenomenon called cross presentation<sup>35</sup>. This process leads to an efficient priming of cytotoxic CD8 T lymphocytes and acknowledges the key role of DCs in the defense against viruses or other intracellular pathogens<sup>41</sup>.

## 4 Maturation of DCs

A major process in the life cycle of DCs is termed maturation. Once started, it is irreversible and leads to the transformation of DCs into potent stimulators of T-cells. The pioneering studies on Langerhans cells revealed the changes in antigen presenting characteristics of the cells during in vitro cultivation<sup>42, 43</sup>. This and other similar observations led to the hypothesis that DCs exist in two major functional stages – immature and mature.

### 4.1 Maturation signals

DCs maturation can be induced by a variety of so called “danger signals”. These can be divided into three major groups. In the first group are the common structural motifs of pathogens – pathogen associated molecular patterns, second group contains the “danger signals” produced by the host and the last one is characterized by the signals mediated by activated lymphocytes. Despite the signals diversity, the purpose is always the same - helping the immune system to recognize pathogens or upcoming infection.

The concept of the “danger signals” theory, declaring that their presence is requested for the efficient DCs activation and T cells priming, has been initially postulated by professor Matzinger in 1994<sup>44</sup>. Danger signals can be detected with a variety of receptors where the important families will be described below.

#### 4.1.1 Toll-like receptors

The concept of the innate immune recognition is based on the detection of molecular structures unique to microorganisms via pattern recognition receptors. This theory was proposed by Professor Janeway several years ago<sup>45</sup>, however the direct evidence came with the discovery of toll-like receptors (TLRs) by Medzhitov in 1997<sup>46</sup>. This crucial finding transformed the view on the system of innate immunity and its role in the host protection against pathogens. The discovery of TLRs has been preceded by the identification of the protein called Toll in the fruit fly *Drosophila*. Toll was described as the essential molecule in embryonal patterning<sup>47</sup> and later on shown to be the key component in antifungal immunity<sup>48</sup>. Subsequently a homologous family of Toll receptors has been identified in mammals<sup>46</sup>.

So far eleven TLRs were described. The first characterized mammalian TLR was the human TLR4 that functions as the signal-transducing receptor for LPS<sup>49</sup>. This remarkable observation encouraged an intensive research and identification of further TLRs.

The TLRs are transmembrane proteins, which comprise of an N-terminal leucine-rich repeats (LRRs) and a cytoplasmic Toll-IL-1 receptor (TIR) domain. The extracellular sequences of TLRs create specific structures that might be directly involved in the recognition and binding of the appropriate ligands.

The vertebrate TLRs can be grouped into six major families based on their sequence similarity. Members within each family recognize certain class of PAMPs. The most important agonists are summarized in tab.1. Briefly, the TLR2 subfamily (which includes TLR1 and 6) recognizes lipopeptides, TLR3 dsRNA, TLR4 lipopolysaccharide (LPS), TLR5 flagellin, and the TLR9 subfamily nucleic acids (TLR7/8: ssRNA, TLR9: unmethylated CpG). Murine TLR11 was shown to recognize protease-sensitive molecules from uropathogenic bacteria and a protozoan profilin-like protein from *Toxoplasma gondii*, suggesting that this TLR family recognizes a protein ligand, like TLR5, and provides protection against protozoan pathogens. It has been shown, that TLRs can be also activated by host factors – TLR2 and TL4 senses heat-shock proteins, fibrinogen, components of extracellular matrix<sup>50, 51</sup>.

Important point is the subcellular localization of TLRs which correlates with the nature of ligands rather than their sequence similarity. While TLR1, 2, 4, 5, and 6, located on the plasma cell membrane, recognize bacterial components, antiviral TLR3, 7, 8, and 9 are expressed in the intracellular compartments<sup>52</sup>. The intracellular expression of viral TLRs most likely limits reactivity to self-ligands<sup>53</sup>. Experiments defining various expression patterns of TLRs shed some light on both the functions of individual receptors and the functions of specialized cell types as well. For example, the plasmacytoid DCs express TLR7 and 9<sup>54</sup> correlating with their ability to produce large amounts of type I interferons during viral infections.

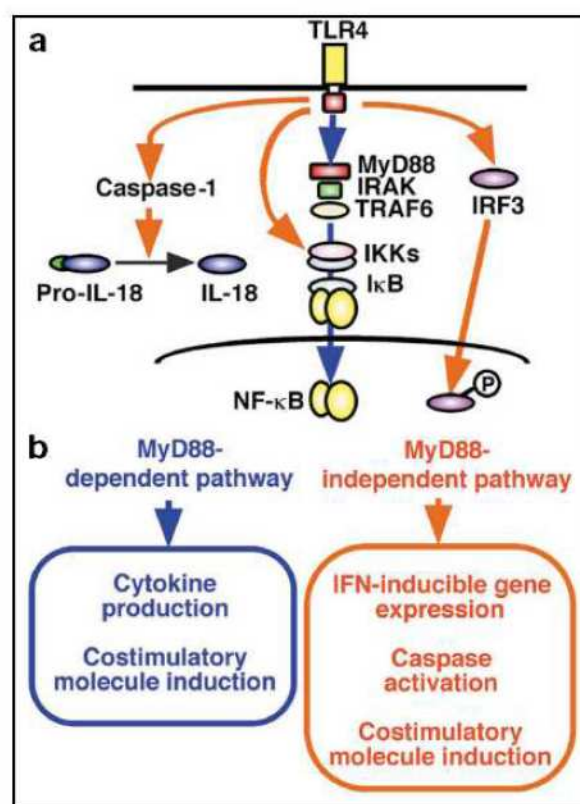
Receptor	Ligand	Origin of ligand	References
TLR1	Triacyl lipopeptides	Bacteria and mycobacteria	112
	Soluble factors	<i>Neisseria meningitidis</i>	113
TLR2	Lipoprotein/lipopeptides	Various pathogens	114
	Peptidoglycan	Gram-positive bacteria	115,116
	Lipoteichoic acid	Gram-positive bacteria	116
	Lipoarabinomannan	Mycobacteria	117
	Phenol-soluble modulin	<i>Staphylococcus epidermidis</i>	118
	Glycoinositolphospholipids	<i>Trypanosoma cruzi</i>	119
	Glycolipids	<i>Treponema maltophilum</i>	120
	Porins	<i>Neisseria</i>	121
	Atypical lipopolysaccharide	<i>Leptospira interrogans</i>	122
	Atypical lipopolysaccharide	<i>Porphyromonas gingivalis</i>	123
	Zymosan	Fungi	124
Heat-shock protein 70*	Host	125	
TLR3	Double-stranded RNA	Viruses	52
TLR4	Lipopolysaccharide	Gram-negative bacteria	9
	Taxol	Plants	126
	Fusion protein	Respiratory syncytial virus	127
	Envelope protein	Mouse mammary-tumour virus	128
	Heat-shock protein 60*	<i>Chlamydia pneumoniae</i>	129,130
	Heat-shock protein 70*	Host	131
	Type III repeat extra domain A of fibronectin*	Host	132
	Oligosaccharides of hyaluronic acid*	Host	133
	Polysaccharide fragments of heparan sulphate*	Host	134
	Fibrinogen*	Host	135
TLR5	Flagellin	Bacteria	136
TLR6	Diacyl lipopeptides	<i>Mycoplasma</i>	137
	Lipoteichoic acid	Gram-positive bacteria	116
	Zymosan	Fungi	138
TLR7	Imidazoquinoline	Synthetic compounds	139
	Loxoribine	Synthetic compounds	12
	Eropirimine	Synthetic compounds	12
	Single-stranded RNA	Viruses	140,141
TLR8	Imidazoquinoline	Synthetic compounds	142
	Single-stranded RNA	Viruses	140
TLR9	CpG-containing DNA	Bacteria and viruses	143
TLR10	N.D.	N.D.	-
TLR11	N.D.	Uropathogenic bacteria	144

**Table 1 | Toll-like receptors and their ligands**

Adapted from *Nat Rev Immunology* (Akira, 2004)

The extensive research of TLRs led to the detailed analysis of their extremely complex intracellular signaling transduction pathways. Briefly, after the ligand binding comes to homo- or heterodimerisation of the receptors resulting into conformational changes, which allow the binding of certain adaptor proteins. The first identified adaptor protein was MyD88 (myeloid differentiation primary response protein)<sup>55</sup>. MyD88 is utilized by all TLRs, with the exception of TLR3. The association of TLRs and MyD88 recruits members of the IL-1 receptor-associated kinase (IRAK) family. Two members, IRAK4 and IRAK1, are sequentially phosphorylated, causing them to dissociate from the receptor complex, and then associate with TRAF6. TRAF6 forms a complex with ubiquitin-conjugating enzymes to activate the kinase TAK1, which in turn activates transcription factors - nuclear factor (NF)- $\kappa$ B and activator protein-1 through the canonical I $\kappa$ B kinase (IKK) complex and the mitogen-

activated protein kinase pathway, respectively. NF- $\kappa$ B activation by ‘MyD88-dependent pathway’ results in the expression of inflammatory cytokine genes, including TNF- $\alpha$ , IL-6, IL-1 $\beta$ , and IL-12. Experiments on the MyD88 $^{-/-}$  deficient mice revealed that there must be also another – so called MyD88 independent pathway. In this pathway the adaptor protein was identified as TRIF and is required for the production of type I interferons and type I interferon-dependent genes downstream of these receptors<sup>56, 57</sup>. All of this data suggests that individual TLRs can mediate distinctive responses by association with a different combination of the adapter proteins<sup>52</sup>. The importance of TLRs signaling is supported by the observations where impaired TLRs or their signaling pathways led to various pathological conditions as will be described in the following chapters.



**Figure 2 | The MyD88-dependent and independent pathways in TLR4 signaling.** (a) Schematic representation and (b) biological outcome of TLR4 signaling pathway. TLR4 can activate the MyD88-dependent pathway (blue arrows), which can also be stimulated by IL-1 and IL-18 and ligands for other TLR family members. TLR4 also activates MyD88-independent pathways (orange arrows). For example, NF- $\kappa$ B activation can be induced, with delayed kinetics, in the absence of MyD88 and leads to induction of costimulatory molecules. Phosphorylation and nuclear translocation of IRF3 can occur in a MyD88-independent manner and is involved in IFN-inducible gene expression. In addition, in Kupffer cells, caspase-1 activation can be induced independently of MyD88 and results in mature IL-18 production.

**Adapted from Nat Immunology (Akira, 2001)**

#### 4.1.2 Other membrane receptors recognizing pathogen structures

Another group of PRRs are the C-type lectins. C-type lectins are transmembrane receptors that bind carbohydrates through one or more carbohydrate recognition domains (CRD). CRDs have specificity for mannose, galactose or fucose structures, but recognition also depends on the carbohydrate branching and spacing. C-type lectins are highly expressed on immature DCs, however their expression decreases following the DCs maturation. This reflects their role in the antigen capture and presentation. Signaling from C-type lectins can induce different downstream reactions depending on whether they are activated in combination with TLRs or not.

The scavengers' receptor family SR-A (SR-A I and II, macrophage scavenger receptors - MSR) have been defined as another PRRs. They bind a broad spectrum of polyanionic ligands like ds-RNA, LPS or LTA. Their importance in immunity was confirmed by mice knockouts, where higher susceptibility to certain pathogens as *Listeria monocytogenes* could be determined<sup>58</sup>.

#### 4.1.3 Cytoplasmic receptors recognizing pathogenic structures

TLRs and extracellular PRRs are poised to detect pathogens that reside in the extracellular space, or that gain access to endosomal/lysosomal compartments inside the cell. However many pathogens, most notably viruses, invade the cytosol, seemingly out of range of TLRs. Recognition of these pathogens is mediated by a variety of intracellular PRRs.

- Caterpillar (NLRs) – NOD and NALP

In addition to TLRs, a protein family called Caterpillar has emerged as another class of intracellular pathogen receptors. All NLRs contain a nucleotide-binding oligomerization domain (NOD) followed by a leucine-rich-repeat domain at the carboxy terminus. At the amino terminus, NLRs have one of the three basic domains and are thereby categorized into three subfamilies: a caspase-recruitment domain (CARD), present in proteins in the NOD subfamily; a pyrin domain, in the NALP subfamily; or a BIR domain (baculoviral inhibitor-of-apoptosis-protein repeat-containing domain), in the NAIP subfamily. The N-terminal effector domain (CARD, Pyrin) allows the interaction with RIP2 protein kinase, which subsequently activates NF- $\kappa$ B and MAPK signaling pathways. The middle domain (NACHT,

NOD) plays the critical role in the regulation of protein oligomerization and the C-terminal domain comprises LRRs, which similarly as in the TLRs interact with the ligand. The Caterpillar family comprises two major groups of protein families – NOD and NALP.

The well-known members of the NOD family are Nod1 and Nod2 which have been shown to recognize meso-diaminopimelic acid (meso-DAP)-containing molecules and muramyl dipeptide (MDP) respectively, both components of peptidoglycan. In addition to their functions in the host protection, NOD2 mutation has been associated with several autoimmune diseases, like Crohn's disease or Blau's syndrome<sup>59-61</sup>.

The NALP proteins are involved in the induction of the inflammatory response mainly mediated by the IL-1 family of cytokines, which are synthesized as inactive precursors and need to be cleaved by the pro-inflammatory caspases. The caspases are activated in a multisubunit complex called the inflammasome, which is categorized according their composition and the involvement of a particular NALP or NAIP. Interestingly, NALP3 inflammasome can be in addition activated by host signals, like low intracellular potassium concentration as a result of stress or bacterial toxins<sup>62</sup>. Necrotic host cells which indicate tissue damage caused by infection or other stimuli also belong to the danger signals capable to mature DCs.

- Helicases similar to RIG (retinoic acid inducible gene 1)

In addition to TLR 3, intracellular recognition of viruses can be mediated by different two types of viral nucleic-acid sensors. The RNA helicase-family proteins RIG-I and MDA5 can detect viral RNA in the cytosol, whereas the recently identified DAI27 protein detects viral DNA. RIG-I and MDA5 recognize different types of viral RNA: single-stranded RNA containing 5'triphosphate and double-stranded RNA, respectively. These structural features are absent from cellular (host) RNAs, which contain either short hairpin structures, in the case of transfer RNAs and ribosomal RNAs, or a 5'-cap structure, in the case of messenger RNA. These structural differences allow discrimination between viral and self-RNAs. Activation of RIG-I or MDA5 results in the production of type I interferons (IFNs; IFN- $\alpha$  and IFN- $\beta$ ) and thereby the induction of antiviral immunity<sup>63, 64</sup>.

## 4.2 Indirect mechanisms of danger signals sensing

DCs are capable to sense danger signals by several indirect mechanisms. An example of this is the detection of pro-inflammatory cytokines produced by various activated cells (macrophages, NK cells, NKT cells, mast and endothelial cells) during the presence of pathogens. Some of the phenotypic changes are due to auto- or paracrine functions of pro-inflammatory cytokines and not as the result of direct TLRs activity. Pro-inflammatory cytokines produced by DCs transmit the information about pathogens to cells that do not have the appropriate PRRs<sup>65</sup>. TLRs activation can be further supported by the synthesis of other mediators like chemokines, prostaglandins, leukotriens and membrane-bounded molecules. Their activity can potentially lead to DCs maturation. However, recent data show that DCs activated by inflammatory cytokines are not fully mature because they are unable to perform the requested third polarization signal (described later). The best evidence is the IL-12p70 a crucial differentiating factor of Th1 cells. It has been shown, that its production can be generated only by PAMPs and never by cytokine action only<sup>65</sup>.

## 4.3 Signals mediated by activated lymphocytes

Activated T lymphocytes support DCs with several signals. CD40L on CD4+ T cells binding its receptor on DCs (CD40) triggers their maturation including the production of IL-12p70. DCs activated this way are licensed for further CD8+ T cells priming<sup>66</sup>. Activated CD8+ T cells produce large amount of IFN- $\gamma$  which together with CD40L optimize further DCs activation. This supports the Th1 and CTL responses against intracellular pathogens<sup>67</sup>. Activated T cells up-regulate the expression of TNF-related activation induced cytokine (TRANCE) which binds to its ligand RANK on DCs and initiates the production of Th1 cytokines and anti-apoptotic bcl-XL protein<sup>68-70</sup>.

NK, NKT and some  $\gamma\delta$ T cells have also the potential to induce the DCs maturation by cytokine production or CD40L binding<sup>71, 72</sup>.

#### 4.4 Maturation process – features of immature and mature DCs

Maturation of DCs leads to complex changes that culminate into complete transformation from antigen capturing to antigen presenting cells. Related to this the most important are:

- Morphological changes
- Chemokine production and different chemokine receptors expression profiles
- Loss of endocytic capacity and changed antigen presentation abilities
- Up-regulation of co-stimulatory molecules expression
- Cytokine and pro-inflammatory factors production

##### 4.4.1 Morphological changes of DCs

Morphological changes accompanying DCs maturation include the loss of adhesive structures, cytoskeleton reorganization and acquisition of high cellular motility<sup>73</sup>. During the maturation DCs become a characteristic shape with long dendrites. An important controller of cytoskeleton remodeling is the actin-bundling protein p55 fascin<sup>74</sup>, highly expressed in blood DCs and interdigitating DCs located in the T cell areas of lymph nodes<sup>75, 76</sup>.

##### 4.4.2 Migration of DCs

Migration of maturing DCs is based on a coordinated action of several chemokines and chemokine receptors. After the antigen uptake, inflammatory stimuli turn off the DCs response to MIP-3 $\alpha$  (and other chemokines specific for immature DCs) through the receptor downregulation or desensitization. Consequently, maturing DCs escape from the local gradient of MIP-3 $\alpha$ . On the other hand, upon maturation DCs upregulate a single known chemokine receptor - CCR7, and accordingly acquire responsiveness to MIP-3 $\beta$  (CCL19) and secondary lymphoid-tissue chemokine (SLC, CCL21)<sup>77</sup>. As a consequence of this DCs leave the inflamed tissues and enter the lymph stream, potentially directed by CCL21 expressed on lymphatic vessels. Mature DCs entering the draining lymph nodes, will be driven into the paracortical area in response to the production of CCL19 and/or CCL21 by cells spread over

the T cell zone. The arriving DCs might themselves become a source of these cytokines allowing amplification and/or a persistence of the chemotactic signal. Because these two chemokines can attract mature DCs and naive T lymphocytes, they are likely to play a key role in helping the Ag-bearing DCs to encounter specific T cells.

There is a need to stress that the classical concept of mature DCs migration is in contrast to the recent observations, where immature DCs bearing apoptotic fragments have been shown to migrate towards secondary lymphoid tissues, thus playing a possible role in the peripheral tolerance maintaining<sup>78</sup>. In addition to these observations, a quite high percentage of DCs encountered from secondary lymphoid tissues were shown to be derived from the circulating blood progenitors and not from peripheral tissues<sup>79</sup>.

#### 4.4.3 Antigen processing

Characteristic for DCs during the maturation process is their loss of antigen capturing features<sup>38</sup>. However, recent observations indicate that the antigen uptake and processing of immature DCs are initially transiently increased by maturation signals<sup>39</sup>.

Mature and immature DCs differ in the expression of MHC-peptide complexes. During the maturation process antigen presentation on MHC class I and II complexes increases rapidly. In immature DCs the newly synthesized MHC class II complexes are localized in MIIC compartments (MHC class II-rich compartments). Upon stimulation these complexes are generated more effectively<sup>80</sup> and it comes to their translocation towards cell surface<sup>81</sup>. It is presumed that all of these processes are trying to ensure the preferential presentation of peptides derived from the antigens, which activated the PRRs. While immature DCs internalize the MHC class II molecules promptly, mature DCs express stable complexes for several days thus allowing an optimal interaction with T cells. Some of the TLR ligands have the ability to increase the cross presentation of exogenous peptides on MHC class I molecules, so a specific activation of CD8+ T lymphocytes is possible<sup>82</sup>.

#### 4.4.4 Co-stimulatory and adhesive molecules expression

Mature DCs in contrast to their immature counterparts express high levels of co-stimulatory molecules like CD80, 86, 83 and CD40<sup>6</sup>. TLR ligands or other PAMPs are strong stimulators of such expression<sup>83</sup>. Co-stimulatory molecules are also up-regulated by pro-inflammatory factors or various T cell signals. In addition, mature DCs express higher amounts of adhesive molecules involved in the DC-T cell interaction. The most important are the CD2, CD11a, ICAM-1, LFA-3 and  $\beta$ 1 and  $\beta$ 2 integrins<sup>22</sup>.

#### 4.4.5 Cytokine production

Maturation of DCs triggers the production of several groups of pro-inflammatory (TNF- $\alpha$ , IL-1, IL-6) or immuno-regulatory (IL-12, IL-18, IFN- $\alpha$ , IL-6, IL-10) cytokines. The cytokine storm is very complex and supports the induction of pro-inflammatory responses or helps to negotiate the negative effects of regulatory T cells<sup>84</sup>. Notably, different TLR ligands trigger the production of divergent cytokines thus affecting the final phenotype of immune response. This emphasizes the critical role of PAMPs recognized by DCs.

### 5 Dendritic cells - T cells interaction

#### 5.1 Immunological synapse

DCs initiate or “prime” T cell responses in secondary lymphoid organs such as lymph nodes, spleen, or mucosal lymphoid tissues. Effective priming of naive T cells is manifested by their clonal expansion and terminal differentiation into effector CD4+ helper or cytotoxic CD8+ T lymphocytes. After the antigen elimination a significant decrease of antigen specific T cells can be marked, however some of them might persist and differentiate into memory cells<sup>85</sup>. The strength of the T-cell response is dependent on many factors, including the concentration of the presented antigen on DCs, the affinity of the T-cell receptor for the corresponding MHC-protein complex, the maturation state of DCs, as well as the type of maturation stimulus. While T-cell stimulation by immature DCs leads to initial T-cell proliferation but only short-term survival (“abortive proliferation”), stimulation by mature DCs results in long-term T-cell survival and differentiation into memory and effector T cells.

## 5.2 Signals delivered by DCs to T cells

T cells activation by DCs is a step-fold process. DCs provide the T cells with three basic signals.

### 5.2.1 Signal 1: antigen specific signal

Signal 1 is the antigen-specific signal that is mediated through T-cell receptor (TCR). If performed alone it is thought to inactivate the naïve T cells by anergy, deletion or co-option into a regulatory cell fate (T regulatory cells - Tregs). In addition to this, it is known that the antigen concentration affects the final phenotype of the response. While higher antigen levels lead preferentially to Th1 response, lower concentrations mostly prime the Th2 responses<sup>86</sup>. The avidity of TCR-MHC-peptide interaction seems to be important as well, because if too strong, the internalization cycle of TCR decreases, new TCR complexes arise slowly and the DCs stimulatory capacity reduces<sup>87, 88</sup>.

### 5.2.2 Signal 2: co-stimulation

#### 5.2.2.1 Co-stimulatory receptors of CD28 family

Initiation of protective immunity requires T-cell co-stimulation, because if absent, T helper cells might become anergic and induce tolerance. The most important co-stimulatory molecules are CD80 (B7.1) and CD86 (B7.2) expressed on DCs which interact with CD28 on T cells. Co-stimulation through CD28 amplifies and stabilizes the phosphorylation of tyrosine motifs and attracts other proteins and kinases to the signalization complex<sup>89</sup>. Initially it has been proposed that the second signal was exclusively performed by CD80/86 binding the CD28, however, recent data show that the situation is proving to be much more intricate since other new B7 family members have been identified. The second signal is thus created by the summation of all positive and negative signals from various co-stimulatory molecules and their corresponding receptors.

### 5.2.2.2 TNF family members

TNF family members - 4-1BBL (CD127L) and OX40L are expressed on maturing DCs. 4-1BBL has been described as the crucial player of the long-term expansion and persistence of CD8<sup>+</sup> memory T cells. OX40L stimulates helper T cells to migrate towards B cell follicles during the T-dependent antibody responses. On the other hand, BAFF, Blys, and APRIL are TNF-like molecules that allow DCs to stimulate T-cell independent antibody responses.

### 5.2.3 Signal 3: cytokines, polarizing factors

Signal 3 is the polarizing signal that is mediated by various soluble or membrane-bound factors, such as interleukin-12 (IL-12) and CC-chemokine ligand 2 (CCL2), that promote the development of Th1 or Th2 cells, respectively. The nature of signal 3 depends on the activation of particular PRRs by PAMPs or tissue factors (TFs). Type 1 and type 2 PAMPs and TFs can be defined as those that selectively prime DCs for the production of high levels of Th1-cell-polarizing or Th2-cell-polarizing factors. Whereas, the profile of T-cell-polarizing factors is primed by recognition of PAMPs, optimal expression of this profile often requires feedback stimulation by CD40L expressed by T cells after activation by signals 1 and 2. It is now clear that cytokines such as interleukin-12, IL-18 and IFN $\gamma$  which are produced by DCs, can bias CD4<sup>+</sup> T-cell priming towards a pro-inflammatory T helper 1-cell fate. These cytokines can act directly on newly activated T cells and, indeed, their production by DCs is often amplified by positive-feedback signals that are provided by the differentiating T cells. These cytokines can also activate natural killer (NK) cells, which produce IFN $\gamma$  and indirectly promote the same type of immunity. It is also thought that DCs can promote Th2-cell responses, perhaps by selectively expressing members of the Jagged family of Notch ligands<sup>90, 91</sup>. In addition, DCs have been implicated in the induction of CD4<sup>+</sup> T-cell differentiation into alternative cell fates, including regulatory cells or the newly discovered IL-17-producing CD4<sup>+</sup> T cells.

### 5.3 Impact of DC maturation status on T cells responses

T cell response characteristics to a particular antigen are affected by the group of factors acting at the time point of the antigen recognition by DCs. The final T cells fate then depends on the quality and quantity of the signal delivered from the DCs together with the co-stimulatory molecules and cytokines produced by DCs or other cells as well. All these factors generate a specific microenvironment around the immunological synapse (described previously). So far most of the researchers accept a slightly modified form of the original DCs maturation model in which mature immunogenic DCs can induce Th1-cell differentiation, Th2-cell differentiation and/or CTL priming, depending on the nature of the maturation signal they received, as well as the constraints imposed by ontogeny and/or environmental modifiers.

## 6 Dendritic cells and peripheral tolerance

There is now ample experimental evidence that DCs in the steady state — that is, in the absence of deliberate exposure to maturation signals — can tolerize peripheral CD4+ and CD8+ T cells by inducing deletion, anergy or T regulatory cells generation<sup>92</sup>. It is thought that this represents an important physiological process designed to purge the peripheral T-cell repertoire of those autoreactive T cells that escaped thymic deletion and that might otherwise be activated by immunogenic mature DCs co-presenting self and foreign antigens during an infection<sup>93</sup>. However, it should also be noted that antigen presentation by steady-state DCs need not result in T-cell inactivation and, in some instances, can result in immunity. Nevertheless, it is clear that negative selection in the thymus is not sufficient to eliminate all potentially pathogenic autoreactive T cells, because mice in which the development or the action of regulatory T cells is compromised often develop autoimmunity.

The exact pathways how the “tolerogenic” DC induce the generation of Tregs cells are not fully understood. One of the most recent discussed mechanisms is the local concentration decrease of amino acid tryptophan mediated by IDO (indoleamine 2,3-dioxygenase enzyme) expressed on tolerogenic DC. T cells, especially the activated ones are sensitive to tryptophane absence and under these conditions they die or preferentially differentiate into T regulatory cells. In addition, Tregs through CTLA-4:CD80/CD86 interaction can induce further tolerogenic DCs, thus amplifying the reaction<sup>94, 95</sup>. Another mechanism is the IL-10

production of tolerogenic DC or the production of other suppressive factors which promote the differentiation to Tregs, however, many others still need to be revealed.

## 7 Extending dendritic cells biology into medicine

Given the central role of DCs in controlling adaptive immunity, they are logical targets for many clinical situations that involve T cells: transplantation, allergy, autoimmune diseases, resistance to infections and to tumors, immunodeficiency and vaccines. From many areas of interest, this review focuses only to those that are connected to experimental studies presented in this thesis.

### 7.1 Dendritic cells in vivo as potential diagnostic biomarker

DCs have been proved to play an important role in various diseases including cancer, autoimmunity, allergy, or infection<sup>96-98</sup>. Their in vivo monitoring during the steady state and pathological conditions represents a potential direction in diagnostic medicine. From the technical and ethical point of view, blood is the only readily source for such monitoring. Novel laboratory techniques allowed DCs identifying in peripheral blood where two major DCs subsets could be found. These are the plasmacytoid and myeloid DCs (described in previous chapters) characterized as CD45+/lineage-/HLA-DR+/CD123+ and CD45+/lineage-/HLA-DR+/CD11c+, respectively. In addition to the identification and quantification of circulating DCs subsets, multi-color flow cytometry permits a simultaneous analysis of further surface or intracellular markers; like chemokines, co-stimulatory molecules, cytokines, etc., thus allowing a more detailed analysis. Data acquired by in vivo monitoring revealed different numbers of circulating DCs subset under various pathological conditions. Several diseases including cancer were associated with decreased numbers of circulating DCs, where some of them even corresponded with the disease stage<sup>99</sup>. It has been shown, that treatment modalities, especially the immunosuppressive agents have a profound impact on the circulating subsets. The decreased circulating DCs numbers have been show to correlate with the devolvement of severe post-transplant complications like acute graft versus host disease (aGVHD)<sup>100, 101</sup>. Taken together, although at their early stages, these studies show that an

important part of diagnostic medicine is developing and further research in this field needs to be performed.

## 7.2 Dendritic cells in organ and bone marrow transplantation

DCs play an important role in the outcome of solid organs or haematopoietic stem cell transplantation. It is important to note that in stem cell transplantation, the major alloreactivity is mediated by the graft immune system, with both donor and recipient DCs playing a role in antigen presentation, however, recipient DCs were defined as the key initiators of T-cell-induced GvH reactions<sup>102</sup>. By contrast, in solid transplantation, the major alloreactivity is mediated by the host immune system, with donor DCs or ‘passenger APCs’ playing a transient, however not negligible role in antigen presentation. Immunosuppressive drugs in current clinical use have been shown to act on the rejection-inducing DCs as well as the rejecting T cells, however their limitations and side effects are well known. The complete mechanisms have yet to be pinpointed to explain the maturation and migration of DCs that accompany transplantation. From the clinical point of view, it seems reasonable to propose that strategies to block some of the DCs functions during transplantation will promote acceptance. Recent discoveries showed that DCs in grafted tissues could regenerate locally, thus providing a long-term source of antigen to stimulate rejection<sup>103</sup>. In this light, alternative pathways to use DCs to induce transplantation tolerance are being assessed. So far, several approaches have been tested to promote graft survival or specifically suppress the unfavorable immune reactions. One of them involves the activation of recipient natural killer cells, which reject donor DCs in tissue culture models and in vivo. Another pathway is to induce DCs to become tolerogenic, for example, to express the tolerogenic ILT3/ LILRB4 molecule, or to induce graft-specific FoxP3 Tregs to suppress graft-rejecting T cells<sup>104</sup>. In haematopoietic stem cell transplantation, regimens that lead to recipient DCs depletion are currently being tested in the clinic.

## 7.3 Dendritic cells and TLRs signaling in primary immunodeficiencies

TLRs mediated antigen recognition has been shown to play a critical role in protective immunity in vivo. DCs equipped with a broad spectrum of TLRs thus represent the key components of such sensing. The essential importance of functional TLRs has been already

shown in mice knockouts where diverse infectious phenotypes, depending on the TLR-pathogen combination could be observed. Based on this, human analogues with impaired TLRs sensing or transduction were searched. Anhidrotic ectodermal dysplasia with immunodeficiency (EDA-ID) belongs to the primary immunodeficiencies (PID) where association with impaired TLRs signaling was clearly defined<sup>105, 106</sup>. The infectious phenotype of these patients is characterized mostly by infections with encapsulated pyogenic bacteria, such as *Haemophilus influenzae*, *Streptococcus pneumoniae*, or *S. aureus*. The described genetic cause associated with the X-linked dominance was identified in the NF- $\kappa$ B essential modulator (NEMO, also known as IKK- $\gamma$ ) gene. Important was that identification of NEMO defined a completely new group of PIDs, marked by impaired NF- $\kappa$ B activation. Further investigation revealed a hypermorphic mutation in the I $\kappa$ B subunit associated with the autosomal dominant form of the disease<sup>107</sup>.

In 2003, Casanova and his colleagues first reported three children with autosomal recessive amorphic mutations in interleukin-1 receptor-associated kinase 4 (IRAK4)<sup>108</sup>. IRAK-4 plays a crucial role downstream of individual TLRs and IL-1R receptors and upstream of TNF receptor-associated factor-6 (TRAF-6). Comparing to the EDA-ID, IRAK4 deficient patients are characterized with a purely immunological syndrome. The susceptibility to a narrow range of pyogenic bacterial infections (mostly caused by *Streptococcus pneumoniae* and *S. aureus*) become increasingly rare with age resembling a level of redundancy in TLR-mediated immunity.

Casanova's group also described a mutation in the TLR3 and UNC-93B protein, both manifested with herpes simplex encephalitis (HSE)<sup>109, 110</sup>. Children who lacked functional UNC-93B (an endoplasmic reticulum protein required for TLR signaling) fail to signal through TLR7, TLR8, and TLR9 and displayed impaired TLR3-dependent interferon  $\alpha$ ,  $\beta$ , and  $\gamma$  production as well. In the case of TLR3 mutation the defect has been defined in the region thought to be critical for dsRNA binding.

X-linked agammaglobulinemia (XLA) is a primary immunodeficiency caused by loss-of-function in Bruton's tyrosine kinase (Btk). Btk signaling has been shown to be crucial in the development, activation, and survival of B cells; however, several pieces of evidence indicated that Btk is a component of Toll-like receptor (TLR) signaling pathways. The recent studies provided by our group revealed an impaired TLR8 signaling upon stimulation with ssRNA, thus providing an explanation for the susceptibility to often fatal enteroviral infections in XLA patients<sup>111</sup>.

## 7.4 Dendritic cells and immunosuppressive agents

Immunosuppressive agents are well known for their capacities to inhibit lymphocyte activation and proliferation. In recent years, several studies have proved that these otherwise diversely acting agents also affect the development and functional immunobiology of DCs, in vitro and in vivo. Given the central role of DCs in immunity and tolerance, it is important to know these effects and to use the possible advantages of their pharmacological modulation.

From the group of anti-proliferative drugs (purine nucleotide biosynthesis inhibitors) azathioprine (AZA) and mycophenolate mofetil (MMF) are the mostly used. AZA and MMF have been shown to have immunosuppressive effects on T cell allostimulatory capacity of human epidermal LCs and bone marrow (BM)-derived mouse DCs, respectively<sup>112, 113</sup>. In addition, Mehling and his group demonstrated the impairment of the antigen presenting capacities of LCs following chronic MMF treatment in vivo.

The widely used group of calcineurin inhibitors has been also shown to have profound effects on DCs functions. Cyclosporin A (CsA) impairs the allostimulatory capacity of in vitro generated mouse BM-derived DCs by downregulation of their surface costimulatory molecule expression. Recently, Matsue and his colleagues reported a bi-directional DC-T cell interaction blockage in TCR transgenic mice following antigen presentation<sup>114</sup>. With regard to mouse LCs, CsA negatively affects their antigen presenting capacity in vitro and the capacity to induce contact hypersensitivity reactions in vivo as well. Upon maturation with TNF or LPS, human DCs treated by CsA showed an inhibition of co-stimulatory molecules expression and T cell allostimulatory capacity. Tacrolimus (FK506), another drug from the calcineurin inhibitor group directly affects the DCs functions. FK506 suppresses GM-CSF-stimulated growth of mice bone marrow mDCs without affecting the MHC class II or co-stimulatory molecules expression. In addition, tacrolimus blocks all changes that result from the mouse bi-directional DC-T cell interaction. The effects on human monocyte-derived DCs have been showing some discrepancies. Most of the studies do not show any effect of FK506 when added to cultures during maturation, however, some experiments proved reduced expression of CD83, decreased T cell stimulatory capacity and cytokine production. Tacrolimus is often used as topical agent. It has been shown that FK506 has effects on epidermal DCs. In these cases a decreased expression of IgE receptors on both LCs and DCs was observed<sup>115</sup>. Rapamycin (RAPA), structurally resembling FK506 suppresses the functional activation and endocytosis of BM-derived DCs in vitro and in vivo. In reports by Chiang, RAPA-treated BM-derived DCs had markedly impaired ability to induce antigen-

specific cytotoxic T cell activities and exhibited decreased IFN- expression through the inhibition of Stat4 activation pathway. In human DCs, RAPA induces apoptosis, impairs the receptor-mediated endocytosis, co-stimulatory molecules expression and the allostimulatory capacity.

Glucocorticoids (GC) are the mostly used immunosuppressives. So far, there are more than 50 reports to date regarding the influence of GC on DCs, however new discoveries are still appearing. It was shown that the presence of GC in cell cultures strongly affected the phenotype of monocytes-derived DCs<sup>116</sup>. Controversial reports were published regarding the endocytic capacity of GC-treated DCs and their ability to induce T cell stimulation. Several of them showed an unchanged stimulatory capacity in contrast with other authors. The main reason for these conflicting data are the different maturation stages of DCs, drug concentrations and exposure times used in the experiments<sup>117</sup>.

In addition, to the previously described mostly used agents, a variety of other drugs have been shown to inhibit DCs maturation and function. Among them are aspirin, vitamin D and N-acetyl-L-cysteine.

The aim of this thesis was to gain insight into the biology of human dendritic cells in the context of clinical immunology. The results of the thesis are presented in the form of three original articles studying the role of DCs in clinical medicine from different aspects.

In the first study we analyzed the pattern of DCs reconstitution after allo BMT and characterized the impairments of DCs homeostasis in the case of acute GVHD.

Second study identifies a defect in the function of myeloid DCs in patients with X-linked agammaglobulinemia, a primary immunodeficiency.

Last study describes the modification of DCs function by the most frequently used group of immunosuppressive drugs, glucocorticoids. In this study, we identify DCs as important targets of glucocorticoids in vivo and characterize the functional consequences of glucocorticoids treatment on DCs both in vitro and in vivo.

Results of the thesis have been summarized in three manuscripts that are shown in their original form, as published in the international scientific journals. Preceding comments resume and discuss the main points of the work and explain their importance.

9.1 Kinetics of dendritic cells reconstitution and costimulatory molecules expression after myeloablative allogeneic haematopoetic stem cell transplantation: implications for the development of acute graft-versus host

Allogeneic hematopoetic stem cell transplantation (HSCT) with myeloablative conditioning represents a unique opportunity to monitor the kinetics of reconstitution of DCs and their dynamics in distinct pathologies. In this study we extensively analyzed kinetics and pattern of circulating DCs subsets reconstitution after myeloablative HSCT. As DCs play a major role in the pathogenesis of acute graft versus host disease (GVHD), we separately analyzed patients who developed acute GVHD and compared this cohort to group with uncomplicated posttransplant course. In our study, peripheral blood DCs were monitored from the earliest phase of hematopoetic reconstitution until day 365 after HSCT. Our results showed that both myeloid DCs and plasmacytoid DCs appeared at earliest stages after engraftment and relative numbers within white blood cells compartment peaked between days 19-25 after HSCT. Their proportion then gradually declined and absolute numbers of both DC subsets remained lower for the whole follow-up period when compared to healthy age matched controls. Expression of costimulatory molecules, especially CD83 and CD86 transiently increased between days 15 and 35 and then went back to low steady state levels. Interestingly, patients who developed acute graft-versus-host disease had significantly lower numbers of circulating DCs whereas the decrease in DC counts preceded the onset of clinical symptoms by at least 24h and was independent of corticosteroids administration. Together with recently published studies, this study provides further insight into the biology of DCs in the settings of allogeneic HSCT and reveals quantification of plasmacytoid and myeloid DCs as a potential biomarker for the prediction of acute GVHD development.



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# Kinetics of dendritic cells reconstitution and costimulatory molecules expression after myeloablative allogeneic haematopoietic stem cell transplantation: Implications for the development of acute graft-versus host disease

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**Abstract** Allogeneic hematopoietic stem cell transplantation (HSCT) represents a unique opportunity to monitor the kinetics of reconstitution of dendritic cells (DCs) and their dynamics in distinct pathologies. We analyzed DCs reconstitution after myeloablative HSCT. We separately analyzed patients with acute GVHD. DCs were monitored from the earliest phase of hematopoietic reconstitution until day +365. Both myeloid DCs and plasmacytoid DCs appeared at earliest stages after engraftment and relative numbers within white blood cells compartment peaked between days 19–25 after HSCT. Their proportion then gradually declined and absolute numbers of both DC subsets remained lower than in controls during the whole follow-up. Patients with acute GVHD had significantly lower numbers of circulating DCs. Decrease in DC counts preceded onset of clinical symptoms by at least 24 h and was independent of corticosteroids administration. This study reveals quantification of plasmacytoid and myeloid DCs as a potential biomarker for the prediction of acute GVHD development.

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## Introduction

Dendritic cells (DCs) are highly specialized antigen presenting cells that initiate and regulate immune responses. They play a critical role in coordinating innate and adaptive components

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of the immune systems [1]. Human DCs are a rare, phenotypically and functionally diverse group of bone marrow-derived antigen presenting cells found in tissues throughout the body, including peripheral blood, tissues and secondary lymphoid organs. In steady state, immature (non-activated) DCs avidly take up and process antigens. Upon encounter with pathogens or other danger-associated stimuli such as cytokines, products of damage tissues or activated innate lymphocytes, DCs undergo a process of activation (maturation) wherein they acquire the capacity to activate immune response [2]. DCs maturation includes increased rate of MHC translocation to the cell surface, increased expression of costimulatory molecules and migration to the secondary lymphoid organs where they initiate immunity against presented antigens [3,4]. Depending on the nature of activation stimulus DCs activate various effector mechanisms. For example, after interaction with pathogen activated DCs, T cells can be polarized to make interferon  $\gamma$  and activate macrophages (Th1 cells), or IL-4, IL-5, IL-13 (Th2) cells. DCs activated by specific stimuli can also induce Th17 cells that produce IL-17 for resistance to extracellular infections [5]. Alternatively, immature DCs or DCs activated in the presence of IL-10 or TGF $\beta$  guide T cells to become immunosuppressive by making IL-10 or by differentiating into FoxP3+ cells [6,7].

In contrast to mice, there is only limited data on *in vivo* DCs in humans as their frequency is very low and they are not accessible for direct studies. Most of the studies on human DCs have been performed on *in vitro* generated monocyte-derived DCs [8,9]. Peripheral blood DCs represent the only DCs compartment, in humans, available for direct studies and recent technological advances enabled their identification and detailed characterization. In humans, there are two DCs subsets with distinct markers and functions. Plasmacytoid DCs with typical plasma cell-like morphology selectively express Toll like receptors 7 and 9, can be identified as CD45RA+, HLA class II+, CD123+ and CD11c neg. Upon activation, plasmacytoid DCs produce high levels of type I interferons and play an important role in defense against viruses [10,11]. Myeloid DCs are CD45RA+, HLA type II positive, CD11c+ and CD123 negative. These cells mature in response to a variety of stimuli and produce IL-12 primarily in response to Toll-like receptors stimulation [12]. They express a complementary set of Toll-like receptors to plasmacytoid DCs [13].

Peripheral blood DCs compartment is thought to continuously replenish tissue-resident DCs, although experimental evidence in humans is limited [14]. Circulating DCs are in the state of dynamic balance with resident DCs and disturbances of DCs homeostasis during various immunopathologies are likely to be reflected by changes in circulating DCs [15].

It's thus important to obtain comprehensive information about quantity and function of circulating DCs during steady state and in pathology [16]. Allogeneic hematopoietic stem cell transplantation (HSCT) with myeloablative conditioning represents a unique opportunity to monitor the kinetics of reconstitution of hematopoietic cells. In this study we extensively analyzed kinetics and pattern of circulating DCs subsets reconstitution after myeloablative HSCT from unrelated donor. As DCs play a major role in the pathogenesis of acute graft versus host disease (GVHD), we separately analyzed patients who developed acute GVHD and compared this cohort to group with uncomplicated post-transplant course.

## Materials and methods

### Patients

In the period between January 2006 and December 2007 13 patients (age range 3–20, median 11 years) and 10 healthy age-matched controls were enrolled in the study. Blood samples of all subjects were collected after obtaining informed consent from parents, and the study was approved by the institutional review board. Four males and nine females with various hematological and hematological diseases underwent allogeneic HSCT with complete myeloablative regimen. In eight cases the donor was unrelated, and these patients were additionally treated with anti-thymocyte globulin.

Presence of acute GVHD was classified daily according to the Seattle criteria in a 5-point clinical staging system according to the three main organs involved: skin, gastrointestinal tract, and liver [17,18]. Based on these organ scores, an overall clinical grading was defined from 0 to IV.

### Monoclonal antibodies and flow cytometry

For *in vivo* DC quantification, peripheral blood was stained by FITC-conjugated lineage cocktail of monoclonal antibodies (CD3, CD14, CD16, CD19 and CD56) Becton Dickinson, Heidelberg, Germany), PE-CD123– (eBioscience, San Diego, CA), PE-Alexa 750-HLA-DR (Exbio, Prague, Czech Republic), Pacific Blue-CD45 (Dako, Glostrup, Denmark) and APC-CD11c (Invitrogen, Paisley, UK). Whole blood was stained with the mixture of monoclonal antibodies and incubated for 15 min at room temperature. Red blood cells were then lysed and cells washed twice with PBS. Co-stimulatory molecules expression was analyzed on Ficoll separated PBMCs and Alexa680-CD80 (Exbio), PC5-CD83 (Becton Dickinson) and PC5-CD86 (Becton Dickinson) were used in addition to monoclonal antibodies used for DCs subsets identification. Samples were analyzed on FACS Aria (Becton Dickinson). At least 200.000 events were acquired and subsequently analyzed in FlowJo (TreeStar, Ashland, OR). All samples were processed and analyzed immediately after blood sampling.

### Circulating DCs identification and quantification

Myeloid DCs and plasmacytoid DCs were defined as CD45+/lineage neg./HLA-DR+/CD11c+ and CD45+/lineage neg./HLA-DR+/CD123+ cells, respectively. Absolute number of myeloid DCs and plasmacytoid DCs was calculated from the white blood count multiplied by the proportion of each subset within the population of white blood cells [19].

### Statistics

Mann–Whitney test was used for statistical analysis and  $p < 0.05$  was considered to be significant.

## Results

### Patients and study design

We analyzed detailed kinetics of DCs subsets reconstitution in thirteen pediatric patients who underwent allogeneic

HSCT. All patients were treated with myeloablative conditioning and received acute GVHD prophylaxis based on institutional guidelines (Table 1). Graft source and engraftment characteristics are summarized in Suppl. Table 1. All patients were monitored for the presence of acute GVHD on the daily basis. Eight patients developed acute GVHD, five patients had no signs of acute GVHD in post-transplant period, as shown in Table 2.

In order to get a detailed view on the characteristics of DCs reconstitution following allogeneic HSCT, we started monitoring plasmacytoid and myeloid DCs subsets when WBC count reached  $0.3 \times 10^9/L$  and both subsets were quantified every third day until D+30 post HSCT. All samples were processed and analyzed immediately after blood sampling. We then continued to monitor DCs numbers once a week from D+30 to D+60 post HSCT and three more samples were collected at D+100, D+180 and D+365 (Fig. 1A). Myeloid DCs and plasmacytoid were defined as CD45+/lineage neg./HLA-DR+/CD11c+ and CD45+/lineage neg./HLA-DR+/CD123+ cells, respectively (Fig. 1B).

### Pattern of DCs reconstitution in patients without acute GVHD

We monitored reconstitution kinetics in patients after HSCT and to understand the normal reconstitution pattern of DCs, we first analyzed cohort of patients without acute GVHD. Both plasmacytoid and myeloid DCs were detectable at earliest stages after engraftment and relative numbers within white blood cells compartment peaked between days 19 and 25 after HSCT (Fig. 2A). Frequency of myeloid

**Table 2** Outcome of HSCT

Patient	UPN	Chimerism	aGVHD grade	cGVHD grade	Outcome
1	267	CC D+21	O	no	Alive/well
2	268	CC D+28	III	no	Alive/well
3	269	CC D+21	I	no	Alive/well
4	271	CC D+21	II	extensive	Alive/well
5	270	CC D+21	O-I	no	Alive/well
6	272	CC+D28, D+42 5% autologous	O	no	Alive/well
7	274	CC+D28	II	yes	Alive/well
8	275	D+28 6% autologous	O	no	Alive/well
9	6092	CC D+21	II	extensive	Alive
10	278	CC D+14	III-IV	extensive	Died D+156
11	6058	CC D+14	O	no	Alive/well
12	288	CC D+21	II	extensive	Alive/well
13	291	CC D+21	II	no	Alive/well

Summary of data on peripheral blood chimerism, presence of acute and/or chronic GVHD and clinical outcome for patients included in the study.

CC: complete chimerism, UPN: unique patient number.

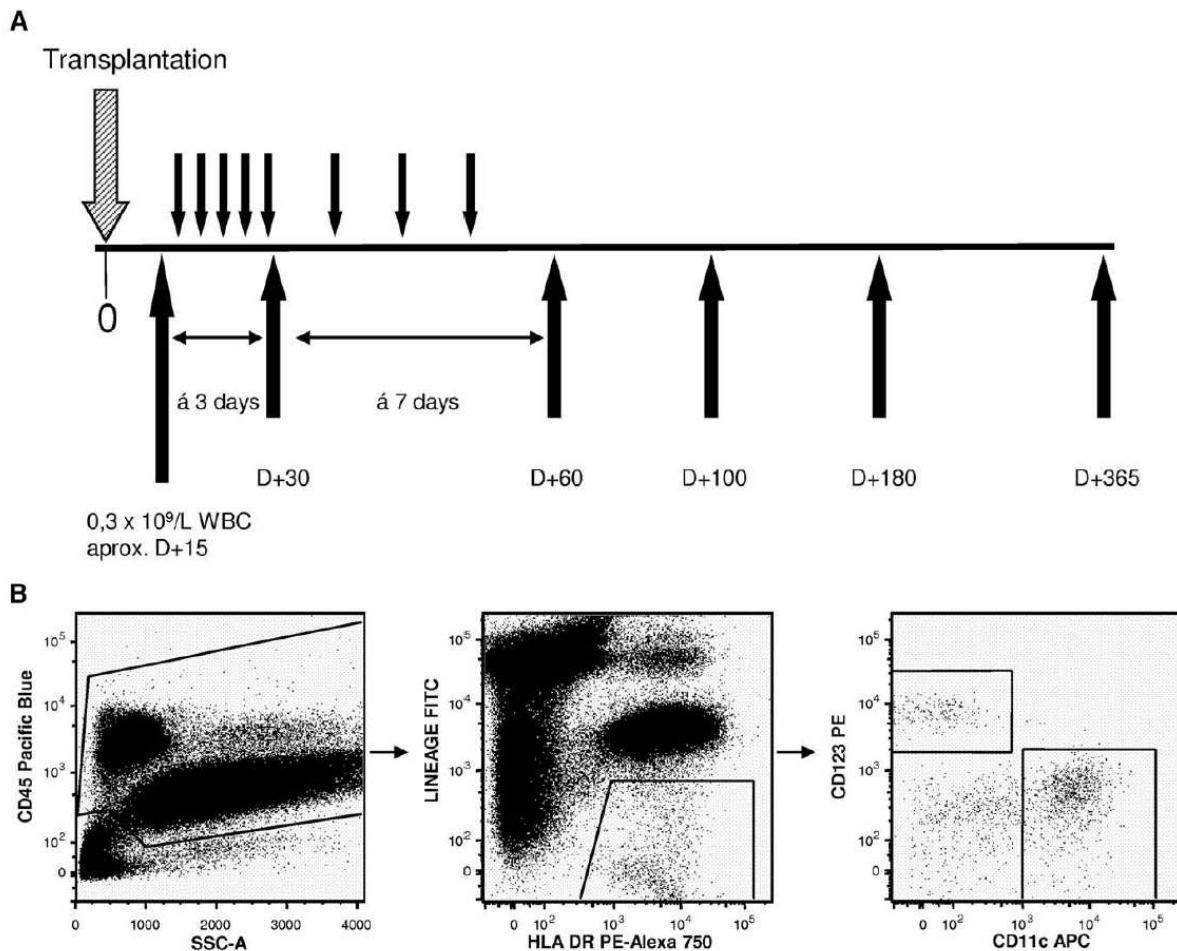
DCs during this period was even higher than in the group of healthy controls. Frequency of plasmacytoid DCs was also relatively higher immediately after engraftment but the difference was not statistically significant. Relative number of plasmacytoid DCs then declined and remained significantly lower than in healthy controls throughout the whole

**Table 1** Patients characteristics

Patient	UPN	Age	Sex	Dg at HSCT	Stage	Donor	HLA allele match	Conditioning regimen	Serotherapy	GvHD prophylaxis
1	267	20	F	sALL	CR1	UD	9/10	TBI 12 Gy, Etoposide	ATG	CSA, MTX
2	268	16	M	AHL	CR1	UD	8/10	TBI 12 Gy, Etoposide	ATG	CSA, MTX
3	269	7	F	SAA		UD	10/10	TBI 6 Gy, Cyclophosphamide	ATG	CSA, MTX
4	271	10	F	AML	CR2	UD	9/10	Busulfan, Cyclophosphamide, Melphalan	ATG	CSA, MTX
5	270	11	F	MDS	RAEB	UD	9/10	Busulfan, Cyclophosphamide, Melphalan	ATG	CSA, MTX
6	272	15	F	SAA		MSD(F)	10/10	Fludarabine, Cyclophosphamide, Campath	O	CSA, MTX,
7	274	19	M	ALL	CR3	MSD(M)	9/10	TBI 12 Gy, Etoposide	O	CSA, MTX
8	275	3	F	SAA		MSD(M)	10/10	Cyclophosphamide, ALG	O	CSA, MTX
9	6092	10	F	AML	CR3	UD	9/10	TBI 12 Gy, Melphalan	ATG	CSA, MTX
10	278	9	F	sMDS	RAEB	MMFD(F)	5/10	Fludarabine, Thiotepa, Melphalan	O	OKT3, MP
11	6058	18	M	HD	CR2	MSD(M)	10/10	Fludarabine, Melphalan	O	CSA, MTX
12	288	16	F	AML	CR2	UD	5/6	Busulfan, Cyclophosphamide, Melphalan	ATG	CSA, MP
13	291	8	M	ALL	CR2	UD	9/10	TBI 12 Gy, Etoposide	ATG	CSA, MTX

Details on indications for HCT, type of donor, HLA match, conditioning regimen and GVHD prophylaxis for thirteen patients included in the study are shown.

UPN: unique patient number, UD: unrelated donor, MSD: matched sibling donor, ALL: acute lymphoblastic leukemia, sALL: secondary acute lymphoblastic leukemia, SAA: severe aplastic anemia, CR: complete remission, MDS: myelodysplastic syndrome, AML: acute myeloid leukemia, ATG: anti-thymocyte globulin, MMFD: mismatched family donor, CSA: cyclosporine A, MTX: methotrexate, MP – methylprednisolone, TBI – total body irradiation, and ALG: anti-lymphocyte globulin.



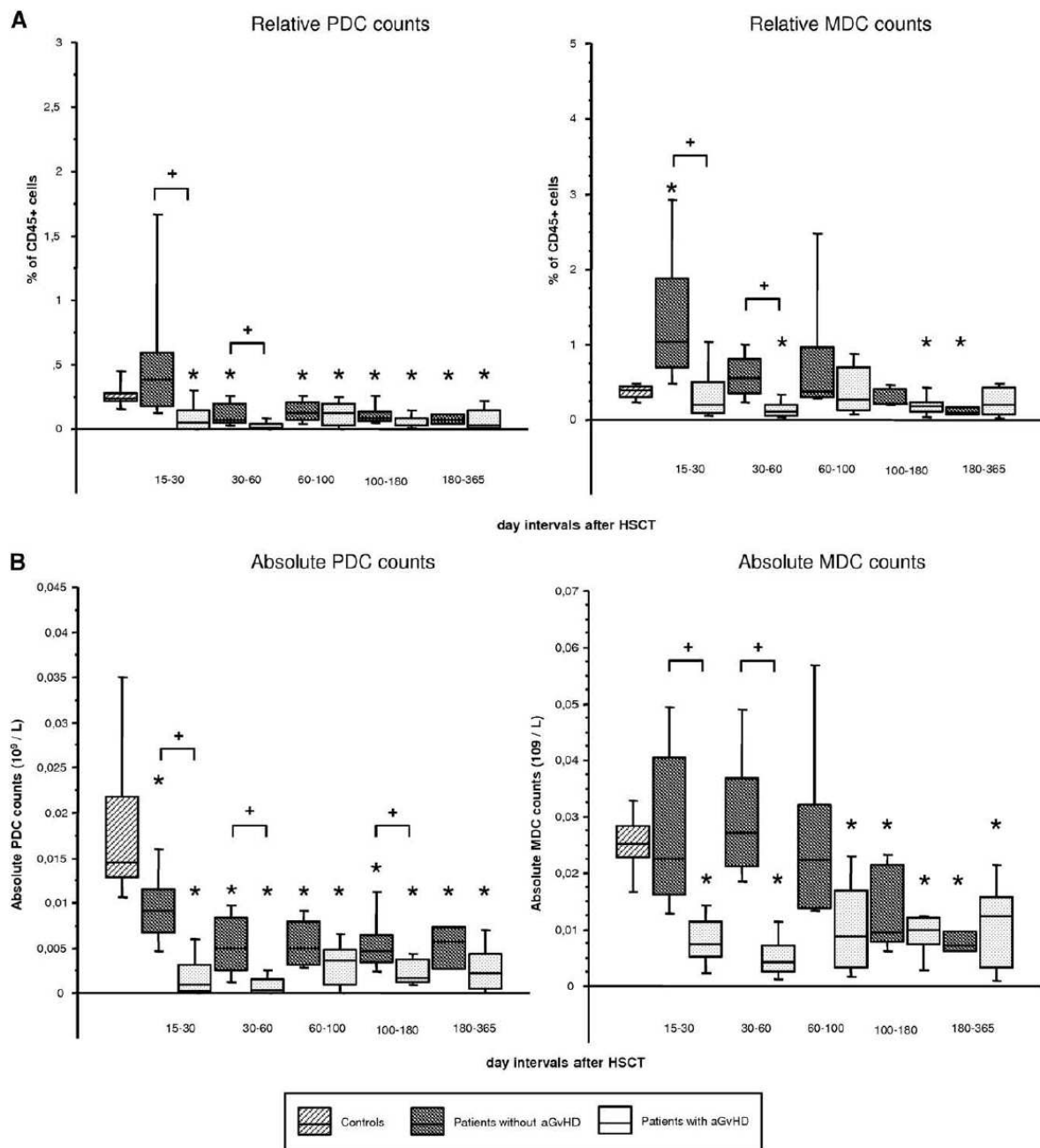
**Figure 1** Study design (A) and strategy of peripheral blood plasmacytoid and myeloid DCs identification by flow cytometry (B). A. DCs numbers and subset distribution were analyzed before HSCT and in regular intervals after patients underwent HSCT. Blood samples for DCs analysis were collected every three days after total WBC reached  $0.3 \times 10^9/L$  until day +30 post HSCT. DCs were then quantified every week until day +60 and then on day +100, day +180 and on day +365 after HSCT. B. For in vivo DC quantification, peripheral blood was stained by FITC-conjugated lineage cocktail mAbs (CD3, CD14, CD16, CD19 and CD56), PE-CD123, PE-Alexa 750-HLA-DR, Pacific Blue-CD45 and APC-CD11c mAbs. Myeloid DCs and plasmacytoid were defined as CD45+/lineage-/HLA-DR+/CD11c+ and CD45+/lineage neg./HLA-DR+/CD123+ cells, respectively. Absolute number of mDCs and pDCs was calculated from the white blood count multiplied by the proportion of each subset within the population of white blood cells. Co-stimulatory molecules expression was analyzed on Ficoll separated PBMCs and Alexa680-CD80, PC5-CD80 and PC5-CD83 mAbs were used in addition to mAbs used for DCs subsets identification. Samples were analyzed on FACS Aria. Mann-Whitney test was used for statistical analysis and  $p < 0.05$  was considered to be significant.

post-transplant period. Proportion of myeloid DCs also declined after D+30 but their frequency remained comparable to values obtained for the cohort of healthy donors until D+365 when we detected significantly lower DC counts in patients than in healthy controls.

Absolute numbers of plasmacytoid DCs were significantly lower during whole post HSCT period and never reached steady-state DCs levels measurable in healthy controls (Fig. 2B). Myeloid DCs were detectable in comparable numbers as in controls until D+100 and their number then declined to significantly lower levels between days D+180 till D+365.

### Significantly lower DCs counts in patients with acute GVHD

We then separately analyzed eight patients who developed acute GVHD. Interestingly, patients in acute GVHD cohort had almost undetectable levels of plasmacytoid DCs and dramatically reduced numbers of myeloid DCs subset, in all analyzed time intervals (Fig. 2, grey bars). Acute GVHD is routinely treated by administration of corticosteroids and as we and others reported, corticosteroids administration leads to severe decline in circulating DCs [20]. We thus analyzed DCs reconstitution in all patients with acute GVHD



**Figure 2** Peripheral blood dendritic cell subsets after SCT. Relative (A) and absolute (B) DCs numbers in the posttransplant course in patients without (black bars) or with (grey bars) acute GVHD. Data shown are mean/SD of DC counts of all patients analyzed within indicated time frame. \* $p$  value < 0.05 for comparison with peripheral blood DCs numbers in healthy controls. + $p$  value < 0.05 for comparison between DCs numbers between patients without and with acute GVHD.

in detail to dissect whether reduced DCs counts correspond to the period of corticosteroids administration or if DCs levels are reduced even prior GVHD onset and treatment. Figure 3 shows detailed kinetics of DCs reconstitution for both subsets. In all patients, onset of clinical symptoms of acute GVHD was preceded by a significant reduction of

absolute DCs numbers. Depending on the time of blood sampling, we detected distinct patterns. In patients 2, 10, 13, we detected rapid decline in DCs numbers immediately prior to onset of acute GVHD after a period of typical DCs reconstitution and DC counts within normal range. In patients 3, 4 and 7, DCs levels prior acute GVHD

were significantly lower when compared to DC counts in patients without acute GVHD. Decline in DCs counts preceded development of clinical symptoms in all patients by at least 24 h. Both patterns were similar for plasmacytoid and myeloid DCs subsets. Initiation of acute GVHD treatment by glucocorticoids led to the abrogation of circulating plasmacytoid DCs and induced further decrease of myeloid DCs within 24 h similarly to the findings in systemic autoimmune diseases.

### Expression of costimulatory molecules on DCs subsets during reconstitution of hematopoiesis

Expression of costimulatory molecules upon activation of DCs represents a critical event in the initiation of an immune response. There is limited data on costimulatory molecules expression on circulating DCs during steady state or in different pathological states. We thus analyzed expression of CD80, CD83 and CD86 on DCs subsets simultaneously with their quantification by multiparametric flow cytometry. In healthy controls, costimulatory molecules were expressed at very low levels (Fig. 4). In contrast to the situation in controls, plasmacytoid DCs in patients post allogeneic HSCT expressed significantly higher levels of CD80 and CD83 during first 60 days post HSCT. Myeloid DCs expressed higher levels of all costimulatory molecules tested, i.e. CD80, CD83 and CD86 until D+100. Expression of costimulatory molecules for both plasmacytoid and myeloid DCs normalized later in the post-transplant course, i.e. after D+100.

We did not see any significant differences in the expression of costimulatory molecules between patients without and with acute GVHD (data not shown).

### Discussion

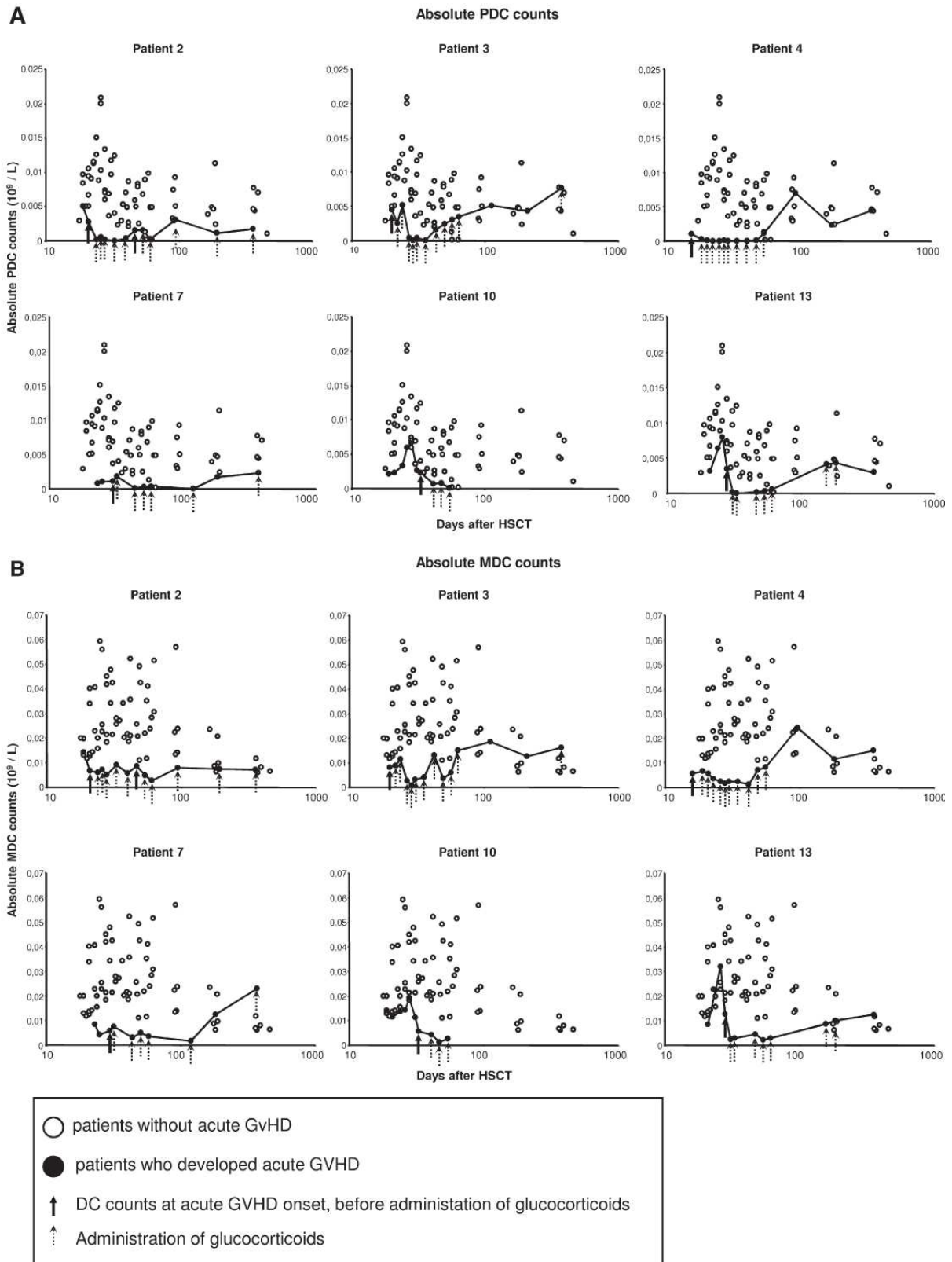
DCs activation status is a critical event in the activation of adaptive immunity. Immature or incompletely mature DCs induce regulatory T cells and participate in the maintenance of peripheral tolerance while activated DCs induce immunity [21,22].

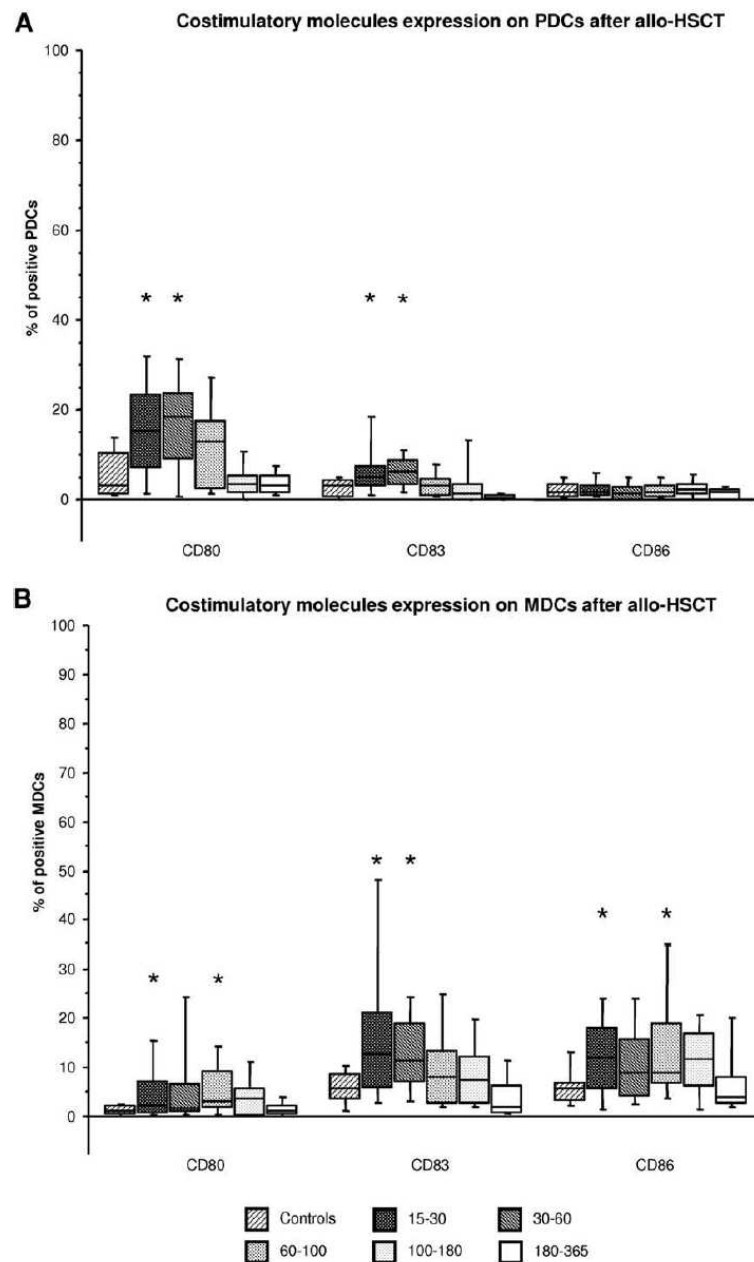
Most of the available data on DC subsets characteristics in homeostasis and in immunopathology comes from mice studies. However, there are considerable differences in DC biology in mice and humans. Peripheral blood represents the only compartment readily accessible for studies of DCs biology in humans and peripheral blood DCs counts or activation status are likely to be impaired early in immune pathologies.

In this study we monitored the kinetics of DCs reconstitution in patients after allogeneic HSCT. In addition to common combination for detection of circulating DCs, we used pan-leukocyte marker CD45 to better quantify absolute DCs numbers. Both myeloid and plasmacytoid DCs engrafted rapidly during the earliest periods of donor immune system reconstitution. Absolute numbers of plasmacytoid DCs were significantly lower than in healthy controls during the follow up period. Circulating myeloid DCs were comparable to healthy controls during the first 100 days after BMT and their number then declined between days +180 to +365.

We then separately analyzed DCs reconstitution in patients who developed acute GVHD. There is compelling

evidence from animal studies that DCs contribute to the development of GVHD [23]. Several elegant studies in mice showed the central role of both persisting host DCs and donor derived DCs in the initiation of murine GVHD [24,25]. Peripheral blood DCs engraft promptly and chimerism studies confirm their donor origin [26]. Skin resident Langerhans cells are, however, replaced by donor cells after prolonged periods of time [27–29]. We show that all patients who developed acute GVHD had significant reduction in DC subsets, especially during the first 60 days post HSCT when compared to both healthy controls and patients without GVHD. GVHD is routinely treated by corticosteroids and their administration leads to rapid decline in circulating DCs [20]. It's thus important to distinguish treatment-related decline in DCs from possible GVHD-associated decrease. This has been a limit of previously performed studies that correlated presence of acute GVHD and DC numbers. Design of our study allowed to analyze DCs reconstitution immediately prior clinical onset of acute GVHD and after the initiation of corticosteroids treatment. We show that patients with acute GVHD have lower numbers of both DCs subsets even before onset of clinical symptoms. We either detected a severe decline in circulating DCs immediately prior acute GVHD after a period when patients had DCs within the normal range. Alternatively, patients who developed acute GVHD had lower DC levels during the whole pre-GVHD period. Circulating DCs remained low after the initiation of corticosteroids treatment. Recently, several studies, with different experimental designs, demonstrated that patients with acute GVHD have overall lower numbers of circulating DCs [30,31]. In these studies, DC numbers were only analyzed at limited time points which did not allow to distinguish between acute GVHD- or corticosteroid-related decline in DCs. Reddy et al. showed that patients with low DC count around the time of engraftment had an increased incidence of acute GVHD [32]. Similarly, a very recent study evaluated D+28 DC counts and reported association of low DCs with the development of acute GVHD [33]. Our study complements this data as we analyzed DC counts at multiple time points (rather than one time snapshot) and show detailed kinetics of DC reconstitution prior acute GVHD onset. It's interesting to note that in half of the patients with acute GVHD in this study, we detected circulating DCs within the normal range and acute GVHD onset was preceded by their rapid decline. It's thus important to determine whether one time analysis of DC counts at the time of engraftment reliably predicts acute GVHD or if it's better to monitor DC reconstitution more closely. Identification of mechanisms responsible for decreased DC counts in acute GVHD requires further investigation. Most likely the mechanism for acute GVHD-related decrease differs from corticosteroids induced circulating DC depletion. Corticosteroids are known to induce DC apoptosis [20,34,35]. Based on findings in murine models, we could speculate that the decrease prior acute GVHD could be caused by homing of blood DCs into inflamed tissues to replenish tissue resident DC compartment [36,37]. Other possible mechanisms include decreased hematopoiesis or killing of DCs presenting recipient antigens by alloreactive donor T cells [38]. Technical and ethical issues complicate design of studies that would contribute to the elucidation of acute GVHD related DC decrease in humans. For example, analysis of DCs infiltration in the skin biopsies from patients with acute GVHD could be of great interest [37,39,40].





**Figure 4** Expression of costimulatory molecules on peripheral blood plasmacytoid (A) and myeloid (B) DCs after SCT. Data shown are mean/SD of costimulatory molecules expression in all patients at indicated time intervals post HSCT. \* $p < 0.05$  value for comparison with peripheral blood DC numbers in healthy controls.

DC maturation reflected by an increase in costimulatory molecules expression is a prerequisite for activation of immunity and analysis of DC activation status could also yield

relevant biomarker. We thus analyzed expression of CD80, CD83 and CD86 costimulatory molecules on both DC subsets. As expected, expression of these molecules on DCs in healthy

**Figure 3** Detailed kinetics of DCs subsets reconstitution in patients with acute GVHD treated with corticosteroids. Each subpanel represents detailed data on DCs reconstitution and corticosteroids treatment for one patient with acute GVHD (black circles, black line). For comparison, DCs counts detected in non-acute GVHD patients are shown as empty circles. Absolute plasmacytoid (A) and myeloid (B) DCs numbers in patients with acute GVHD are shown as black dots. Number of peripheral blood DCs at the time of clinical manifestation of acute GVHD is indicated by thick black arrow. DCs numbers in samples collected after the initiation of acute GVHD treatment by corticosteroids are indicated by dotted black arrows.

donors was very low, almost undetectable as steady state DC precursors in peripheral blood are in immature state. Interestingly, we detected a significant expression of CD80 and CD83 on plasmacytoid DCs and expression of CD80, CD83 and CD86 on myeloid DCs within first 60 to 100 days post transplant. DC activation is likely caused by cytokine storm after HSCT and perpetuated by interaction of donor derived DCs with host tissues expressing major and minor histocompatibility antigens [41]. However, expression of none of the costimulatory molecules analyzed in our study differed between patients with or without acute GVHD. Interesting recent report by Lau et al. showed that early activation marker CMRF-44 expression on myeloid DCs predicted the development of acute GVHD [42,43]. It's thus possible that other than the "classical" costimulatory molecules could serve as markers predicting the risk of acute GVHD.

Overall, in this study we present detailed data on the kinetics of circulating DC subsets reconstitution in patients after allogeneic HSCT. We show that patients with acute GVHD have significantly lower DC counts prior to the onset of clinical symptoms. The decrease in DC counts preceded onset of clinical symptoms by at least 24 h and was independent of corticosteroids administration. Together with recently published studies, this study provides further insight into the biology of DCs in the settings of allogeneic HSCT and reveals quantification of plasmacytoid and myeloid DCs as a potential biomarker for the prediction of acute GVHD development. Given the key role of activated DCs in inducing GVHD, targeting of DC compartment or modulation of their activation status might represent a novel strategy in the management of acute GVHD. However further studies are needed to gain more insight into the role of DC in the pathogenesis of GVHD in humans.

### Authors' contributions

R.H. performed research, analyzed data and wrote the paper, V.B. performed research, analyzed data, J.K. performed research, T.K. conceptualized the work, performed research, R.F. enrolled and treated patients on research protocols, J.S. designed research, analyzed data and wrote the paper, J.B. conceptualized the work, designed research, P.S. conceptualized the work, enrolled and treated patients on research protocols, R.S. conceptualized the work, designed research, analyzed data and wrote the paper. All authors declare no competing financial interests.

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### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.clim.2008.10.009.

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## 9.2 Impaired Toll-like receptor 8-mediated IL-6 and TNF- $\alpha$ production in antigen-presenting cells from patients with X-linked agammaglobulinemia

The critical role of Bruton tyrosine kinase (Btk) in B cells has been documented by the block of B-cell development in X-linked agammaglobulinemia (XLA). However, less is known about Btk function in myeloid cells. Several pieces of evidence indicate that Btk is a component of Toll-like receptor (TLR) signaling. We analyzed whether Btk deficiency in XLA is associated with an impaired dendritic cell compartment or defective TLR signaling. We analyzed the expression of TLRs 1 to 9 on myeloid DCs generated from XLA patients and evaluated their response to activation by specific TLR agonists. We show that XLA patients have normal numbers of circulating DCs. Btk-deficient DCs have no defect in response to stimulation of TLRs 1/2, 2/6, 3, 4, and 5 but display a profound impairment of IL-6 and TNF- $\alpha$  production in response to stimulation by TLR-8 cognate agonist, ssRNA. These findings may provide an explanation for the susceptibility to enteroviral infections in XLA patients.

## Brief report

Impaired Toll-like receptor 8-mediated IL-6 and TNF- $\alpha$  production in antigen-presenting cells from patients with X-linked agammaglobulinemiaKlára Sochorová,<sup>1</sup> Rudolf Horváth,<sup>1</sup> Daniela Rožková,<sup>1</sup> Jiří Litzman,<sup>2</sup> Jiřina Bartůňková,<sup>1</sup> Anna Šedivá,<sup>1</sup> and Radek Špířek<sup>1</sup><sup>1</sup>Institute of Immunology, Charles University, 2nd Medical School, Prague, Czech Republic; <sup>2</sup>Department of Clinical Immunology and Allergology, Masaryk University, Brno, Czech Republic

The critical role of Bruton tyrosine kinase (Btk) in B cells has been documented by the block of B-cell development in X-linked agammaglobulinemia (XLA). Less is known about Btk function in myeloid cells. Several pieces of evidence indicate that Btk is a component of Toll-like receptor (TLR) signaling. We analyzed whether Btk deficiency in XLA is associated with an impaired dendritic cell (DC) compart-

ment or defective TLR signaling. We analyzed the expression of TLRs 1 to 9 on myeloid DCs generated from XLA patients and evaluated their response to activation by specific TLR agonists. We show that XLA patients have normal numbers of circulating DCs. Btk-deficient DCs have no defect in response to stimulation of TLRs 1/2, 2/6, 3, 4, and 5 but display a profound impairment of IL-6 and TNF- $\alpha$

production in response to stimulation by TLR-8 cognate agonist, ssRNA. These findings may provide an explanation for the susceptibility to enteroviral infections in XLA patients. (Blood. 2007;109:2553-2556)

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## Introduction

Bruton tyrosine kinase (Btk) is a member of the Tec family of protein tyrosine kinases, expressed in all hematopoietic cells except T cells and natural killer (NK) cells. The critical role of Btk signaling in the development, activation, and survival of B cells has been documented by the block of B-cell development and absence of mature B cells in the peripheral blood of patients with X-linked agammaglobulinemia (XLA), a primary immunodeficiency caused by loss-of-function mutations in Btk.<sup>1</sup>

Less is known about the function of Btk in the myeloid cells. Recent studies have shown multiple defects in the development and function of myeloid cells in *xid* mice, a murine counterpart of XLA.<sup>2</sup> In accordance with these reports, peripheral blood mononuclear cells (PBMCs) from XLA patients were shown to produce reduced amounts of TNF- $\alpha$  in response to LPS stimulation.<sup>3</sup> Several pieces of evidence indicate that Btk is a component of Toll-like receptor (TLR) signaling pathways. Mammalian TLRs are crucial for the recognition of pathogen-associated molecular patterns (PAMPs), structures unique to microorganisms and shared among infectious agents. At least 10 TLRs have been identified in humans and are expressed predominantly on macrophages, dendritic cells (DCs), neutrophils, and monocytes. All TLRs contain extracellular leucine-rich repeat domains and a cytoplasmic signaling domain known as the Toll-interleukin-1 receptor (TIR) domain that mediates signaling through TLRs. Recognition of PAMPs by corresponding TLRs leads to the activation of antigen-presenting cells, mainly DCs, and represents a crucial step in the initiation of innate and adaptive immune responses. TLR-activated immature DCs enhance expression of surface costimulatory and antigen presentation-associated molecules, produce proinflammatory and Th1 polarizing cytokines, migrate to the secondary lymphoid

organs, and activate antigen-specific T cells.<sup>4</sup> Moreover, TLR signaling has recently been shown to be important for DC differentiation. Btk has been shown to interact with TIR domains of TLRs 4, 6, 8, and 9 and was also found to associate with other components of TLR signaling. LPS, TLR-4 agonist, induces phosphorylation of Btk and activates its kinase activity.<sup>5</sup> Given the importance of TLR signaling for the activation of DCs and for the activation of innate and adaptive immune response, we decided to address the issue of whether Btk deficiency in XLA patients is associated with an impaired DC compartment or defective TLR signaling.

## Patients, materials, and methods

Six male patients diagnosed with XLA, according to the World Health Organization (WHO) classification, and 15 healthy controls were included in this study after informed consent was obtained. The study was approved by the investigational review board (IRB) of 2nd Medical School, Charles University, Prague, Czech Republic. Btk mutations were identified in all patients by direct sequencing of cDNA samples as described. All patients were receiving regular intravenous immunoglobulin (IVIG) replacement therapy at 3- to 4-week intervals and were free of any serious infections at the time of blood sampling. Heparinized blood samples were collected prior to the infusion of IVIG. Monocyte-derived DCs from patients' blood and from control groups were generated as described previously.<sup>6,7</sup> Expression of TLRs 1 to 9 on in vitro-generated DCs was analyzed by real-time reverse transcriptase-polymerase chain reaction (RT-PCR) (for details, contact the corresponding author).

Immature DCs were stimulated by TLR-specific agonists, and their phenotypic and functional characteristics were studied as described previously.<sup>8</sup> TLR agonists were used in the concentrations that induced

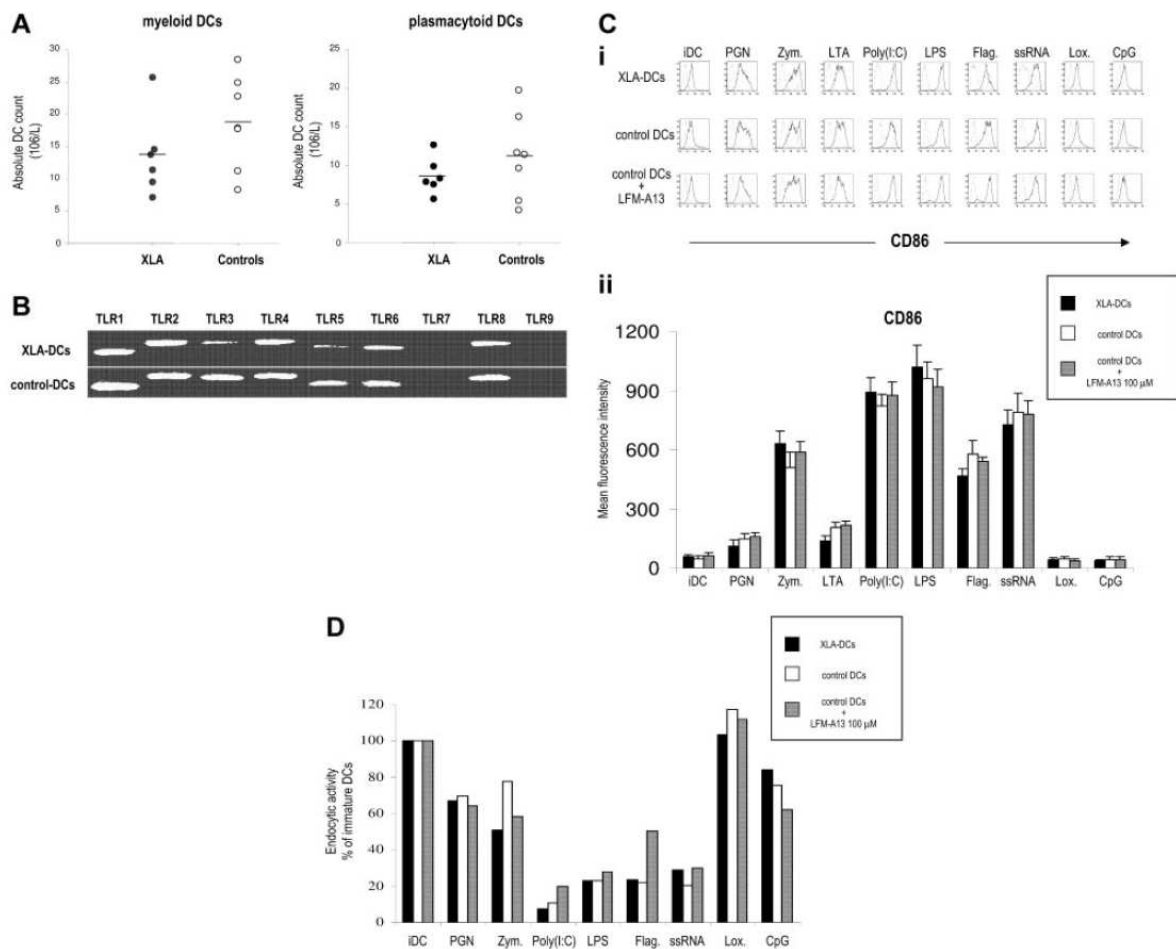
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**Figure 1. Subsets of mDCs and pDCs in XLA patients.** (A) Peripheral blood DC subsets in XLA patients and healthy controls. Myeloid DCs (mDCs) were identified as lineage-negative/HLA-DR<sup>+</sup>/CD11c<sup>+</sup> cells and plasmacytoid DCs (pDCs) as lineage-negative/HLA-DR<sup>+</sup>/CD123<sup>+</sup> cells. Absolute numbers of circulating DC subsets in peripheral blood are shown. Points correspond to individual patients; horizontal bars represent the mean value for the group. (B) Expression of TLRs 1 to 9 on immature DCs from XLA patients and healthy blood donors analyzed by RT-PCR. Both XLA-DC and control DCs expressed TLRs 1 to 6 and 8, a profile characteristic of myeloid DCs. As expected, we did not detect TLR-7 and TLR-9, molecules typically expressed on plasmacytoid DCs. (C) Characteristics of DCs in XLA patients. (i) CD86 expression (thick line) versus isotype control (thin line) is shown. Representative results of 1 of 10 experiments are shown. (ii) Summary of CD86 expression on XLA-DCs, control DCs, and control DCs plus LFM-A13 after activation with TLR agonists. Average of mean fluorescent intensity values plus SDs for 5 different XLA patients and healthy controls is shown. (D) Endocytic activity of XLA-DCs and DCs from healthy donors after stimulation with TLR agonists.

significant cytokine production in control DCs without impairing their viability (Table S1, available on the *Blood* website; see the Supplemental Table link at the top of the online article).

For *in vivo* DC quantification, peripheral blood was stained by FITC-conjugated lineage cocktail monoclonal antibodies (mAbs) (CD3, CD14, CD16, CD19, and CD56), PE-CD123, PC5-HLA-DR, and APC-CD11c mAbs. We defined myeloid DCs (mDCs) and plasmacytoid DCs (pDCs) as lineage-negative/HLA-DR<sup>+</sup>/CD11c<sup>+</sup> and lineage-negative/HLA-DR<sup>+</sup>/CD123<sup>+</sup> cells, respectively. The absolute number of mDCs and pDCs was calculated from the white blood count multiplied by the proportion of each subset within the population of white blood cells. DC subsets were analyzed on FACS Aria (Becton Dickinson, Prague, Czech Republic).

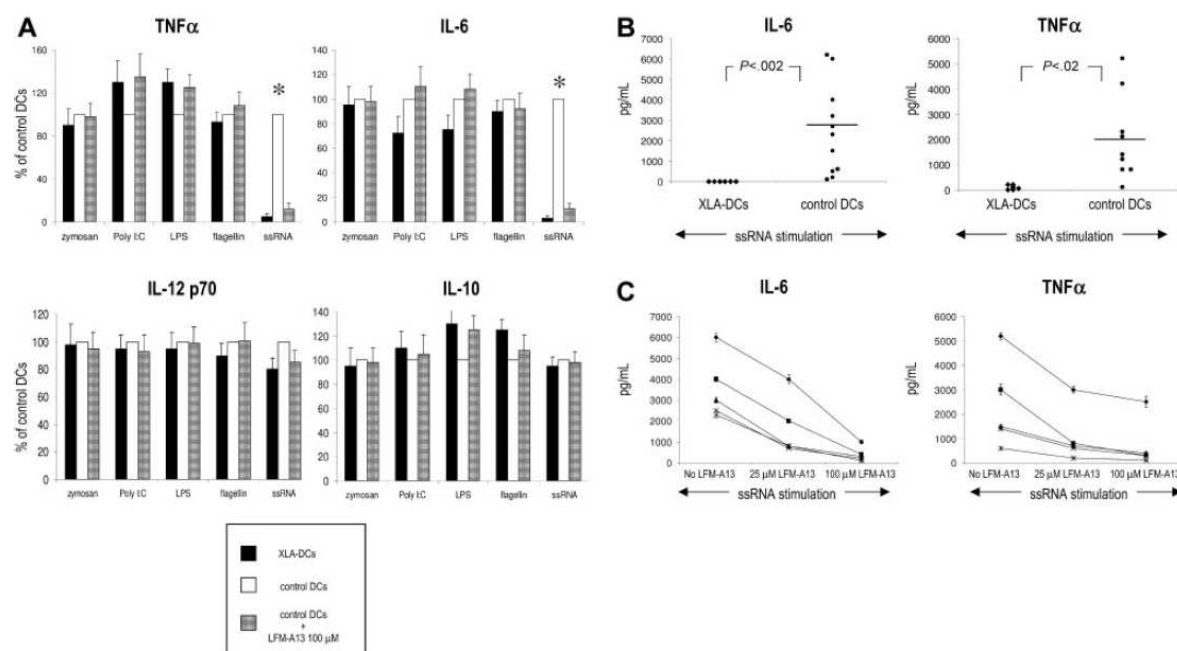
The Mann-Whitney test was used for statistical analysis, and *P* below .05 was considered significant.

## Results and discussion

TLR signaling is important for the differentiation of DCs from their precursors.<sup>9</sup> In the first set of experiments, we analyzed the

frequency and absolute numbers of myeloid and plasmacytoid DCs subsets in peripheral blood of XLA patients. Although there was a tendency toward lower numbers of DCs in XLA patients, the difference was not statistically significant for any subset (Figure 1A).

We then generated immature monocyte-derived DCs from monocytes of XLA patients (XLA-DCs) to evaluate their ability to be activated in response to TLR stimulation. Monocytes from XLA patients differentiated normally in immature DCs, and there was no difference in the yield, morphology, survival, or phenotype between XLA-DCs and DCs from healthy donors (data not shown). Both XLA-DCs and control DCs expressed TLRs 1 to 6 and 8, a profile characteristic of myeloid DCs. As expected, we did not detect TLR-7 and TLR-9, molecules typically expressed on plasmacytoid DCs (Figure 1B). Activation of XLA-DCs, control DCs, or control DCs in the presence of LFM-A13, a Btk inhibitor, with TLR agonists led to similar maturation-related changes in surface molecule expression (ie, up-regulation of CD80, CD83, CD86, and HLA classes I and II) (Figure 1C and data not shown). Decrease of



**Figure 2.** Cytokine production by XLA-DCs and DCs from healthy controls after stimulation with TLR agonists. (A) Relative production of TNF- $\alpha$ , IL-6, IL-12 p70, and IL-10 by XLA-DCs, control DCs, and control DCs preincubated with 100  $\mu$ M LFM-A13 after stimulation with TLR agonists. Data are expressed as relative production as compared with control DCs. Bars show means/SD of 4 different patients and healthy controls. \**P* value for comparison with control DCs; *P* < .05. (B) IL-6 and TNF- $\alpha$  production by XLA-DCs and DCs generated from healthy controls after ssRNA stimulation. Dots represent individual patients; horizontal bars, means. The Mann-Whitney test was used for statistical analysis. (C) The effect of Btk inhibitor LFM-A13 (at 2 different concentrations) on the production of TNF- $\alpha$  and IL-6 by DCs generated from 5 healthy blood donors. Representative results of 5 independent experiments are shown.

endocytosis is another hallmark of DC maturation, and stimulation of TLRs led to the significant decline in the ability of XLA-DCs and control DCs to endocytose FITC-labeled dextran (Figure 1D). Similarly, XLA-DCs and control DCs were comparable in their capacity to induce CD4 T-cell proliferation when used as stimulators in allogeneic mixed leukocyte reactions (data not shown). We next evaluated the production of proinflammatory and Th1 polarizing cytokines after TLR stimulation. Stimulation of TLRs 1 to 6 induced comparable IL-6, TNF- $\alpha$ , IL-10, and IL-12 production by XLA-DCs, control DCs, and DCs preincubated with LFM-A13 (Figure 2A). However, stimulation with TLR-8 agonist, ssRNA, failed to induce any IL-6 and TNF- $\alpha$  production by XLA-DCs, while control DCs produced high levels of both cytokines after ssRNA stimulation (*P* < .002 and *P* < .02 for IL-6 and TNF- $\alpha$ , respectively; Figure 2A-B). To determine whether this dramatic defect in cytokine production could be attributed to Btk deficiency, control DCs were pretreated with LFM-A13 before ssRNA stimulation. Preincubation of control DCs with LFM-A13 significantly decreased IL-6 and TNF- $\alpha$  production in a dose-dependent manner without affecting the viability of DCs (Figure 2C). XLA-DCs thus have impaired capacity to produce IL-6 and TNF- $\alpha$  in response to TLR-8 stimulation.

The role of Btk in TLR signaling pathways has been recently suggested. Btk is phosphorylated in response to the stimulation of TLR-4 by LPS and associates with TIR domains of TLRs 4, 6, 8, and 9.<sup>5</sup> A very recent report has identified Mal as the downstream target of Btk tyrosine kinase function in myeloid cells.<sup>10</sup> Here we show that absence of Btk in XLA has no effect on the frequency and phenotypic characteristics of circulating DCs subsets. Moreover, XLA-DCs have TLR expression patterns similar to control DCs, and their activation by specific agonists for TLRs 1/2, 2/6, 3, 4, and 5 leads to analogous phenotypic and functional changes as in control DCs. In a pioneer study, Gagliardi et al<sup>11</sup> evaluated the

effect of LPS on some aspects on DC maturation and did not detect any differences from control DCs. Absence of any detectable defect in LPS-mediated signaling has also been recently documented in monocytes from XLA patients.<sup>12</sup> However, in contrast with these reports and with our own findings, 2 recent reports showed a slight decrease in IL-1 and TNF- $\alpha$  production by XLA monocytes and macrophages stimulated with LPS and Palm-Cys, TLR-4 and TLR-2 agonists, respectively.<sup>3,13</sup> We did not confirm the impaired DC function after TLR-4 binding. However, we detected a profound defect in IL-6 and TNF- $\alpha$  production by XLA-DCs in response to TLR-8 stimulation by its cognate agonist, ssRNA.<sup>14,15</sup> Selective impairment of IL-6 and TNF- $\alpha$  production suggests existence of different intracellular signaling pathways in response to ssRNA, as recently reported by Kanneganti et al.<sup>16</sup> Finding of an impaired TLR-8 response is interesting with respect to the clinical presentations of XLA patients. Although periodic infusions of IVIG lead to satisfactory levels of circulating antibodies, XLA patients frequently suffer from chronic and potentially very severe and fatal enteroviral infections (ssRNA viruses).<sup>17</sup> Given the crucial importance of pathogen recognition for the homeostasis and survival, mammals have evolved multiple and redundant sensory mechanisms. This redundancy can be reflected at the level of individual TLRs (one TLR can be linked to multiple signaling pathways via different adaptor proteins). More importantly, pathogens are usually recognized by multiple pattern recognition receptors, including TLRs.<sup>18</sup> Such redundancy limits the risk of systemic life-threatening infections by large groups of pathogens. However, it is conceivable that while accessory mechanisms of pathogen recognition provide sufficient protection against most infections, a subtle defect in the signaling pathway can account for an increased frequency of infections caused by a particular pathogen.<sup>19</sup> Enteroviral infections are in fact the major cause of mortality in XLA

patients. Inappropriate and insufficient recognition of viral ssRNA by Btk-deficient DCs might thus contribute to the susceptibility of XLA patients to enteroviral infections.

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## Authorship

Contribution: K.S., R.H., and D.R. performed research; J.L. coordinated patients' samples; J.B. designed research; and A.S. and R.S. designed research, analyzed data, and wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

A.S. and R.S. contributed equally to this study.

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### 9.3 Glucocorticoids severely impair differentiation and antigen presenting function of dendritic cells despite upregulation of Toll-like receptors

In this study, we investigated the effect of clinically widely used glucocorticoids (GC) on the multiple characteristics of DCs. Despite their wide use in the treatment of many autoimmune, inflammatory and allergic diseases, the complex cellular and molecular mechanisms underlying GC effects are not completely understood. Given the crucial role of Toll-like receptor triggering for the initiation of DCs maturation program, we analyzed the expression of TLR 2, 3, 4 by GC-treated DCs. We show that presence of GC leads to the impairment of DC function at two levels: First, GC during DC differentiation skew their development into a qualitatively distinct population incapable of inducing the efficient immune response (high production of IL-10, whereas no production of IL-12 p70 was detected). Second, GC presence during the process of maturation impairs the quantity, that is, extent of this process (significantly reduced DC IL-12 p70, TNF production and T cell stimulatory function). Despite the fact that GC increased expression of TLR2, 3 and 4 on DC, their stimulation with TLR-derived signals did not induce maturation.

To extend our *in vitro* findings, we analyzed the distribution of DC subsets in the blood of patients treated with high-dose corticosteroids. Administration of high-dose GC to the patients with systemic autoimmunity induced a decrease of circulating myeloid DCs and abrogated plasmacytoid DCs. These findings provide further insights into the mechanisms of GC immunosuppressive functions and reveal additional mechanisms of their therapeutic efficiency.



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# Glucocorticoids severely impair differentiation and antigen presenting function of dendritic cells despite upregulation of Toll-like receptors

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## KEYWORDS

Dendritic cell;  
Corticosteroids;  
Immunosuppressive  
therapy;  
High-dose  
corticosteroids;  
Toll-like receptors,  
maturation

**Abstract** Glucocorticoids (GCs) are widely used as anti-inflammatory and immunosuppressive agents. Effects of GC have mainly been attributed to the suppression of T cells. Recently, several studies have indicated the role of dendritic cells (DC) in GC-mediated immunosuppression. We investigated the effect of GC on characteristics of DC. Given the crucial role of Toll-like receptor (TLR) triggering for the initiation of DC maturation program, we analyzed the expression of TLR2, 3, 4 by GC-treated DC. To extend our *in vitro* findings, we analyzed the distribution of DC subsets in the blood of patients treated with high-dose corticosteroids. DC differentiation in presence of GC was skewed to a qualitatively distinct population incapable of inducing an efficient immune response, whereas GC presence during the process of maturation significantly reduced DC IL-12 p70 and TNF production and T cell stimulatory function. Despite the fact that GC increased expression of TLR2, 3 and 4 on DC, their stimulation with TLR-derived signals did not induce maturation. Administration of high-dose GC to the patients with systemic autoimmunity induced a decrease of circulating myeloid DC and abrogated plasmacytoid DC. These findings provide further insights into the mechanisms of GC immunosuppressive functions and reveal additional mechanisms of their therapeutic efficiency.

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*Abbreviations:* DC, dendritic cells; APC, antigen presenting cell; Poly (I:C), Polyriboinosinic polyribocytidylic acid; CD40L, CD40 ligand; PAMPs, pathogen-associated molecular patterns.

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## Introduction

Dendritic cells (DC) are highly specialized antigen presenting cells (APC) with the unique ability to stimulate naive T lymphocytes [1]. In human peripheral blood, two DC subsets can be identified. The first is represented by myeloid DC (mDC), which are CD11c<sup>+</sup>, CD45RO<sup>+</sup> and CD123 negative. These cells mature in response to a variety of stimuli but produce IL-12 primarily in response to LPS or CD40L

stimulation [2]. The second subset is represented by plasmacytoid DC (pDC). These cells are CD11c negative, CD45RA<sup>+</sup> and CD123 high and, upon exposure to viruses, produce very high levels of type I interferon [3].

In vivo DC exist in two functionally distinct states that are commonly termed as immature and mature. Immature DC reside in peripheral tissues and are well equipped for antigen uptake and processing. Recent reports suggest that the primary function of immature DC is to maintain peripheral tolerance [4,5]. In the presence of maturation stimuli such as pathogen-derived structures, inflammatory cytokines or other endogenous indicators of danger (heat shock proteins, uric acid), a maturation program is started via respective receptors and immature DC irreversibly transform to the mature form [6].

Cells of the innate immune system recognize the invading microbial pathogens by a set of germ-line encoded receptors that are referred as pattern-recognition receptors. Toll-like receptors (TLRs) function as the major pattern-recognition receptors in recognizing pathogen-associated molecular patterns. To date, 10 members of the human TLR family have been cloned. Each member of the TLR family specifically recognizes particular pathogen-associated structures [7]. For the TLRs investigated in this study, TLR2 recognizes peptidoglycan and bacterial lipoprotein from Gram-positive bacteria [8], TLR3 mediates responses to double stranded RNA [9] and TLR4 is involved in the recognition of Gram-negative-derived LPS [10]. Myeloid and plasmacytoid DC subsets express complementary sets of TLRs; mDC express all TLRs except TLR7 and TLR9 that are expressed at high levels by pDC [11]. Detection of microbial presence by TLRs initiates a process of DC maturation. The maturation process includes upregulation of MHC molecules and also of B7 family of costimulatory molecules. TLRs are also responsible for induction of cytokine and chemokine production by DC [1,12]. Some cytokines are important for regulating the function of regulatory T cells (IL-6) [13] and others, like IL-12, are responsible for directing the T cell responses. A number of compounds have been shown to interfere with or to modulate DC maturation. The study of such compounds is important, as targeted modification of DC maturation status either in vitro or in vivo has profound implications on the outcome of the immune response and could be used in the targeted immunotherapy of tumors, autoimmune diseases, allergies and other immune dysregulations.

Since glucocorticoids (GC) were first administered to patients suffering from rheumatoid arthritis [14], they have been widely used as anti-inflammatory and immunosuppressive agents. Moreover, GCs are also a major component of the treatment protocols for several lymphoproliferative diseases (acute lymphoblastic leukemia, multiple myeloma) due to their T cell depletory effect. Most of their effects are mediated through their interaction with cytoplasmatic glucocorticoid receptors and subsequent modulation of the gene transcription at several levels [15]. Until now, immunosuppressive effects of corticosteroids have mainly been attributed to the suppression of T cell activation [16]. However, it is now clear that they also affect other cells within the immune system network. Among others, GC downregulate cell surface expression of the adhesion

molecules like ICAM-1 and E-selectin on endothelial cells [17,18]. GC were described to suppress the expression of costimulatory molecules and the production of other immunologically relevant mediators such as lysozyme, prostaglandin and granzyme B [19,20]. Well studied is the inhibition of Th1 cytokines production [21]. On the other hand, enhanced expression of several cytokine receptors was observed [22].

Recently, several studies have indicated the important role of APC in GC-mediated suppression of immunity. It was shown that the presence of GC in cell cultures strongly affected the phenotype of monocytes-derived DC [23–25]. Controversial reports were published regarding the endocytic capacity of GC-treated DC and their ability to induce T cell stimulation. Several of them showed an unchanged stimulatory capacity [26,24] in contrast with other authors [23,25]. This discrepancy may be caused by different experimental designs, by different protocols used for DC generation, by various concentrations of tested glucocorticoids and by differences in activation status of treated DC [27].

In this study, we investigated the phenotypic and functional impact of GC treatment on differentiation and maturation of monocyte-derived DC using a well-described DC generation model [28,29]. Given the crucial role of TLRs triggering for the initiation of the DC maturation program, we analyzed the kinetics of TLR2, 3 and 4 expression on GC-treated DC. To extend our in vitro findings to the clinically relevant settings, we analyzed the distribution of DC subsets in the peripheral blood of patients with systemic autoimmunity treated with high-dose corticosteroids.

## Materials and methods

### Media and cell cultures

Complete culture medium (CM) was used for the culture of lymphocytes and dendritic cells and consisted of RPMI 1640 (Cambrex, Verviers, Belgium) supplemented with 10% heat-inactivated fetal bovine serum (Cambrex), 2 mM L-glutamine (Cambrex) and 1% penicillin/streptomycin (Cambrex). Cells were cultured at 37°C in a 5% CO<sub>2</sub> atmosphere.

The following clinically used preparations of glucocorticoids were used in this study: dexamethasone (Dexamed, Medochemie, Cyprus), methylprednisolone (Solu-Medrol, Pharmacia and Upjohn, Belgium) and prednisone (Prednison, Zentiva, Czech Republic).

### DC generation and treatment with glucocorticoids

Immature monocyte-derived DC were generated as previously described [30]. Briefly, peripheral blood mononuclear cells (PBMCs) were obtained from buffy coats of healthy donors and monocytes were separated by 2 h adhesion in 75 cm<sup>2</sup> culture flasks (Nunc, Roskilde, Denmark). Adherent monocytes were cultured for 5 days in CM in the presence of GM-CSF at 500 IU/ml (Leukine, Berlex, Richmond, CA) and 20 ng/ml of IL-4 (Peprotech, London, UK). For some experiments, DC were generated from monocytes purified from the PBMC by immunomagnetic separation with anti-CD14 microbeads (Miltenyi Biotech, Bergisch Gladbach,

Germany). To test the effect of glucocorticoids on immature DC differentiation, graded concentrations of tested agents were added into the culture medium. On day 5, various immature DC preparations were seeded in 24-well plates (Nunc) at  $5 \times 10^5$  DC/ml and activated by either Poly (I:C) (Sigma, St. Louis, MO) at 50  $\mu\text{g/ml}$ , LPS (Sigma) at 1  $\mu\text{g/ml}$ , peptidoglycan at 5  $\mu\text{g/ml}$  (PGN, Invivogen, CA, USA) or zymosan at 10  $\mu\text{g/ml}$  (Invivogen) in the presence or absence of glucocorticoids. Immature and mature GC-treated DC were evaluated for their morphology, viability and they were phenotypically and functionally characterized.

For functional analysis of TLR, DC were incubated with dexamethasone for 24 h and then extensively washed three times. Pretreated DC were seeded in the 24-well plates and stimulated by TLR ligands for further 24 h. Production of cytokines was measured in collected supernatants.

### Flow cytometry

FITC or PE-conjugated monoclonal antibodies (mAbs) against the following molecules were used: CD80-FITC, CD83-FITC, CD86-PE, CD14-PE (Immunotech, Marseille, France), CD11c-PE, HLA-DR (BD Biosciences, San Jose, CA), TLR2-PE, TLR3-PE and TLR4-PE (eBioscience San Diego, CA). DC were stained for 30 min at 4°C, washed twice in PBS + 0.1% BSA and analyzed on FACSCalibur (BD Biosciences) using Cell Quest software. DC were gated according to their FSC and SSC properties, and dead cells were excluded from the analysis. Appropriate isotype controls were included and 5000 viable DC were acquired for each experiment.

### FITC-dextran endocytosis

DC ( $1 \times 10^5$ ) in 100  $\mu\text{l}$  of complete medium were incubated with 200  $\mu\text{g/ml}$  of FITC-dextran (40,000 m.w., Sigma). After 1.5 h, cold medium was added to stop the experiment. Cells were washed three times with ice-cold PBS and analyzed by FACSCalibur. DC cultured on ice served as a negative control.

### Cytokine detection

Culture supernatants from DC stimulated at various conditions were collected after 24 h and analyzed for the production of cytokines by Luminex (LuminexCorp) using preconfigured 22-plex kit for human cytokines according to the manufacturers instructions (Upstate, NY, USA). IL-12 p70, IL-10 and TNF production was also analyzed by standard ELISA kits (Immunotech; Biosource, Camarillo, CA).

All samples were analyzed in duplicates and the Mann–Whitney test was used to determine the statistical significance. A *P* value  $<0.05$  was considered to be statistically significant.

### Induction of lymphocyte proliferation

CD4<sup>+</sup> T cells were positively selected using CD4 microbeads (Miltenyi Biotech). Purity of CD4<sup>+</sup> population used for further studies always exceeded 95%. CD4<sup>+</sup> T cells were subsequently labeled by 1  $\mu\text{M}$  CFSE (Molecular Probes, Eugene, OR) as described previously [31]. DC ( $1 \times 10^4$ ) were cultured with  $1 \times 10^5$  allogeneic CFSE-labeled CD4<sup>+</sup> T cells in

96-well U-bottomed plates (Nunc) in the final volume of 200  $\mu\text{l}$  of complete medium. After 5 days, cells were harvested and the induction of T cell differentiation was evaluated by gradual CFSE dilution in dividing T cells by flow cytometry.

### RNA isolation, reverse transcription, and real-time PCR analysis of TLR expression

Total RNA was extracted from 0.5 to  $1 \times 10^6$  of DC using a modified method described by Chomczynski and Sacchi [32]. One microgram of RNA was treated by 1 U of DNase (Fermentas, St. Leon-Rot, Germany) 30 min at 37°C. The enzyme was inactivated by heating at 65°C for 10 min in the presence of 2 mM EDTA (Fermentas). RNA was then transcribed into cDNA using M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Presence of DNA contamination and verification of successful RNA isolation was tested by PCR amplification of the *abl* gene with intron spanning primers: forward primer 5' -TTC AGC GGC CAG TAG CAT CTG ACT t-3', reverse primer 5' -TGT GAT TAT AGC CTA AGA CCC GGA GCT TTT-3'. This reaction results in a 764-bp product when DNA is a template and a 201-bp product in RNA.

Real-time PCR reactions were performed in duplicates using the first-strand cDNA and Platinum Taq polymerase (Invitrogen), 200 nM dNTP (Promega, Southampton, UK), 2–4 mM MgCl<sub>2</sub> (Invitrogen), 0.2  $\mu\text{M}$  probe and 0.5–1  $\mu\text{M}$  primers (TIB MOLBIOL, Berlin, Germany). The following primers were used: TLR2: 5' -AGG CGG ACA TCC TGA ACC T-3', 5' -GGC CAG CAA ATT ACC TGT GTG-3', Taqman probe 5' -CTC CAT CCC ATG TGC GTG GCC T-3'; TLR3: 5' -GCC AGT TCA AGA TGC AGT GA-3', 5' -CCA TTA TGC AAA AGA TTC AAG GTA-3', Taqman probe 5' -ACA TTC CTC TTC GCA AAC AGA GTG CA-3'; TLR4: 5' -TAT TCC CGG TGT GGC CAT T-3', 5' -GTG CTG GGA CAC CAC AAC AA-3', Taqman probe 5' -TTC GGC TTT TAT GGA AAC CTT CAT GGA TG TT-3'; TLR9: 5' -TGA AGA CTT CAG GCC CAA CTG-3', 5' -TGC ACG GTC ACC AGG TTG T-3', Taqman probe 5' -AGCACCCTCAACTTCACCTTGGATCTGTCT-3';  $\beta$ -actin: 5' -GCT GAT CCA CAT CTG CTG GAA-3', 5' -ATT GCC GAC AGG ATG CAG AA-3', Taqman probe 5' -CAA GAT CAT TGC TCC TCC TGA GCG CA-3'. Reactions were amplified and quantified using iCycler (Bio-Rad, Hercules, CA). The relative quantity of TLR genes was normalized using  $\beta$ -actin reaction as exogenous control.

### Patient population and identification of peripheral blood DC subsets by flow cytometry

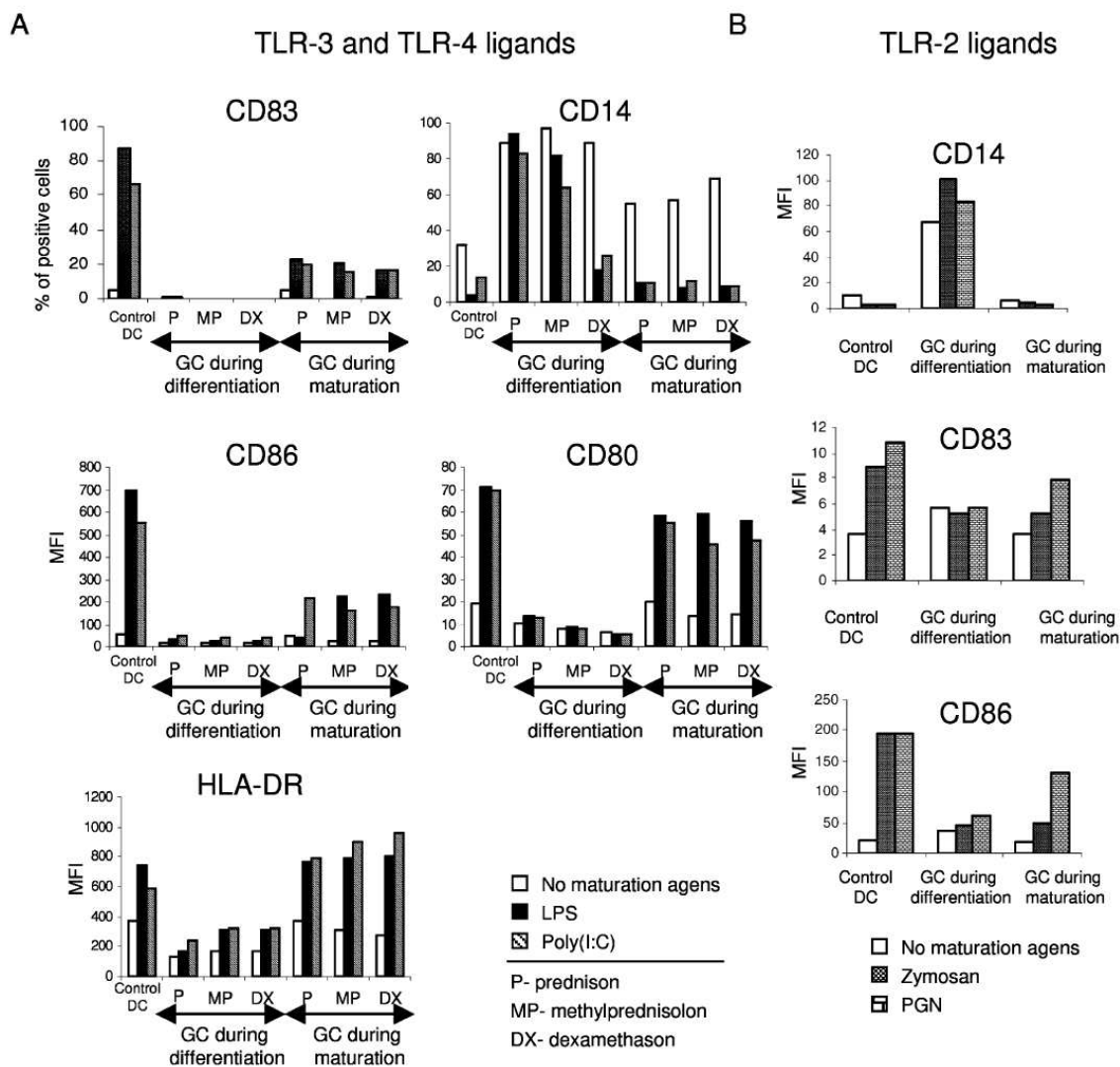
Peripheral blood from 5 patients (2 females, 3 males; average age, 9.4 years) with systemic onset juvenile idiopathic arthritis was collected after obtaining informed consent. One hundred microliters of peripheral blood was labeled by the combination of the following mAbs: FITC-labeled lineage cocktail, CD11c-PE, HLA-DR-PC7 and CD123-APC (BD Biosciences). After 30 min of incubation, red blood cells were lysed, cells washed twice in PBS + 0.1% BSA and analyzed by FACSAria (BD Biosciences). Dead cells were excluded from analysis by DAPI staining. Mann–Whitney test was used for statistical analysis. *P* value  $<0.05$  was considered to be statistically significant.

**Results**

**Phenotypic characteristics of DC generated in the presence of glucocorticoids**

To investigate the effect of clinically used glucocorticoids on DC differentiation and maturation, we used dexamethasone (DX), methylprednisolone (MP) and prednisone (P) at a concentration of  $10^{-6}$  M determined in the preliminary experiments. Concentrations above  $10^{-6}$  M negatively affected DC yield and viability (data not shown). Freshly isolated monocytes cultured for 5 days in the presence of IL-4, GM-CSF and GC differentiated into nonadherent, large cells with distinct dendrites. In contrast to the DC-like morphology, GC-treated cells retained high expression of

CD14 molecule and expressed very low or no levels of CD80, CD86, HLA-DR when compared to immature DC (Fig. 1). In the next step, immature DC or cells differentiated in the presence of GC were washed and activated by LPS or Poly (I:C) in order to induce their maturation. As shown in Fig. 1A, the presence of GC during the differentiation period abrogated their capacity to mature upon relevant stimulation. Presence of GC during the maturation of immature DC decreased their ability to upregulate costimulatory molecules, HLA molecules and express CD83. CD14 expression increased slightly in immature DC exposed to GC; however, increase was suppressed in the presence of maturation stimuli. A similar effect on the expression of maturation-associated markers was seen with agonists of TLR2, zymosan and PGN (Fig. 1B). As maturation-related



**Figure 1** Phenotype analysis of dendritic cells cultured with glucocorticoids. (A)  $10^{-6}$  M of prednisone (P), methylprednisolone (MP) or dexamethasone (DX) were present during DC differentiation or maturation by LPS or Poly (I:C). (B) DC were differentiated in the presence of DX and activated with TLR2 ligands, PGN and zymosan with or without DX. FACS analysis was performed 24 h after the addition of maturation stimuli. Representative data from three experiments are shown.

changes in DC phenotype play an essential role in T cell activation and in eliciting an immune response, these data suggest that DC differentiated in the presence of GC could have profound functional defects. Dexamethasone, methylprednisolone and prednisone had comparable effects on the inhibition of DC function. We thus used dexamethasone as the only compound in some of the subsequent experiments.

### Functional profile of GC-treated DC

In order to assess the function of GC-treated cells, we tested their ability to capture FITC-dextran, their cytokine production and T cell stimulatory potential. DC differentiated in the presence of GC had high endocytic potential, comparable or higher than immature DC. When treated with Poly (I:C) for 24 h, the endocytosis of FITC-dextran significantly decreased. On the other side, endocytic activity of these cells remained higher after the treatment with LPS when compared to the control mature DC. If the presence of corticosteroids was limited to the process of DC maturation, endocytosis was dramatically reduced to the levels observed for mature DC (Fig. 2A). We then screened for the modulation of cytokine production by DC in the presence of GC. The presence of GC during the differentiation period and during DC maturation nearly abolished p70 IL-12 and TNF production in response to TLR2, 3 and 4 stimulation (Fig. 2B). In addition, DC differentiated in the presence of GC did not produce any detectable amounts of p40 IL-12. However, IL-12 p40 production was preserved in DC treated with GC during maturation. On the other hand, DC differentiated in the presence of GC produced significantly higher amounts of IL-10. Modification of cytokine production was mediated by GC because it was inhibited by GC-specific inhibitor RU-846 (Fig. 2C). Finally, DC differentiated in the presence of GC were not able to induce the proliferation of allogeneic CD4 T cells even when activated by maturation agents. The addition of GC to immature DC on day 5, together with LPS or Poly (I:C) had only a marginal effect on their capacity to activate CD4 T cells (Fig. 2D).

### GC treatment leads to the increased expression of TLRs on DC

Dendritic cells express a wide spectrum of Toll-like receptors. Binding of corresponding ligands, specific pathogen-associated molecular patterns (PAMPs), represents the most effective signal for the initiation of DC maturation program. As our experimental model includes PGN, zymosan, Poly (I:C) and LPS, ligands for TLR2, 3 and 4, respectively, we further investigated whether GC treatment of DC modifies the expression of TLR family

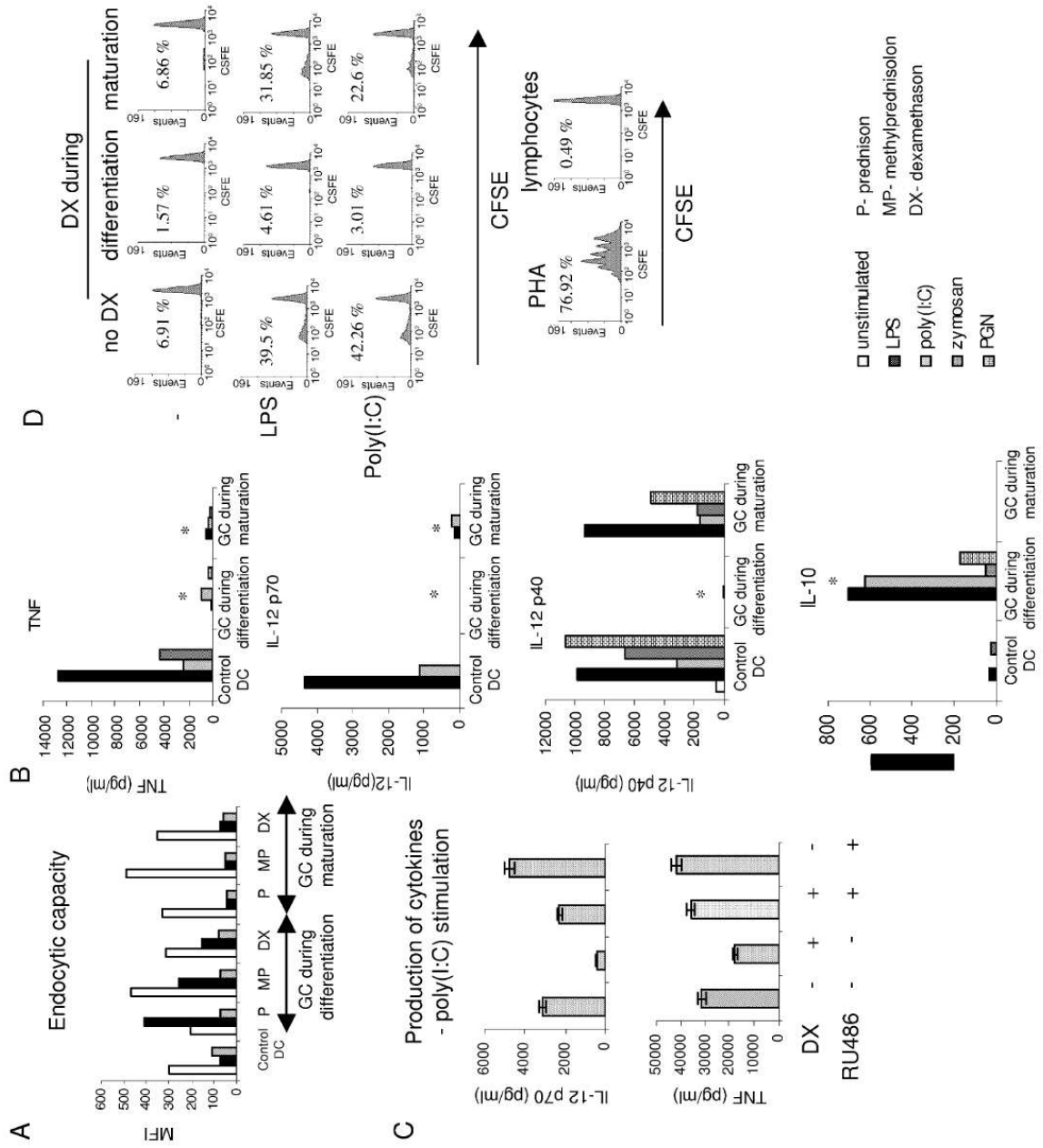
members. Expression of TLR2 and TLR4 in control DC decreased after the maturation was induced by the TLR ligands tested (LPS, Poly (I:C)). TLR3 mRNA levels remained stable after the stimulation by Poly (I:C) or LPS. Surprisingly, GC treatment of both differentiating and immature DC led to the significant increase in the expression of TLR2 and TLR4 and to the slight increase of TLR3 (Fig. 3A). Levels of TLR mRNA were also enhanced in cells that were treated simultaneously with GC and maturation stimuli. Although we did not find significantly increased values of TLR3 expression in DC differentiated in presence of GC, GC together with TLR3 agonist Poly (I:C) synergistically enhanced expression of this gene. The highest relative rise was seen in the expression of TLR2 (when compared to the mature DC). GC-induced increase of TLR expression during DC differentiation and maturation was dose dependent, as shown for TLR2 and TLR4 expression (Figs. 3B and C). Furthermore, Fig. 3C also shows a comparison of TLR2 and TLR4 expression in monocytes and DC differentiated in the presence or absence of GC. In contrast to control DC, where expression of TLR was downregulated during DC differentiation, GC-treated DC maintained high levels of TLR2 and TLR4.

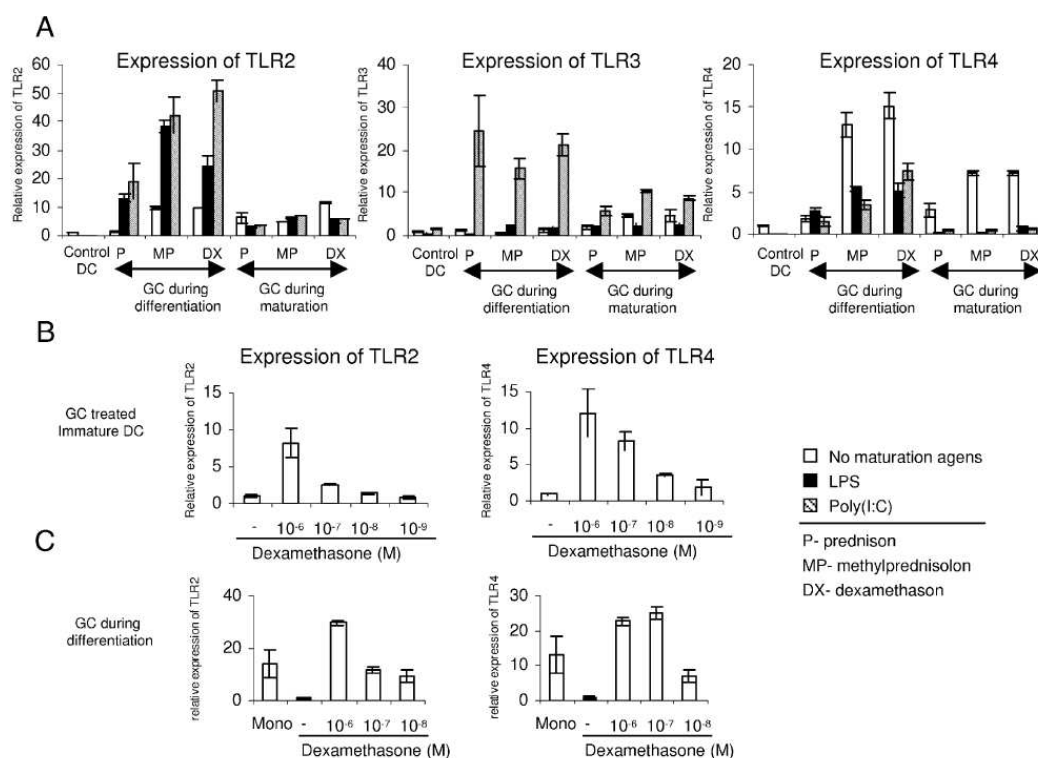
Precise studies of TLR expression at the protein level are hampered by the lack of good mAbs for the flow cytometry. However, using two commercially available mAbs against TLR2 and TLR4, we confirmed the real-time RT-PCR data at the protein level (Fig. 4). DX present during differentiation of DC induced significantly higher surface expression of both TLR2 and TLR4. Although activation by TLR2, 3 and 4 agonists slightly decreased TLR2 and 4 expression levels, they remained higher than those of untreated cells. In accordance with the real-time RT-PCR data, DX induced significantly higher levels of TLR2 and TLR4 even if its presence was restricted to the time of DC maturation.

### Functional relevance of high expression of Toll-like receptors

Because GC treatment led to the increased TLR expression, we next sought to evaluate whether the increased TLR expression provided DC with the increased capacity to respond to the relevant stimuli once they are in the GC-free environment. Immature DC were cultured with  $10^{-6}$  M dexamethasone for 24 h, then the cells were extensively washed and stimulated with TLR2, 3 and 4 agonists. As shown in Fig. 5, GC-pretreated DC that expressed higher levels of TLRs displayed significant alteration in the quantities of produced cytokines. Despite increased levels of TLRs, they produced significantly lower levels of TNF, p70 IL-12 and p40 IL-12. In addition, TLR stimulation of DC pretreated with dexamethasone led to the production of

**Figure 2** Functional characteristics of glucocorticoid-treated cells. (A) Endocytic activity. Endocytic capacity is expressed as mean fluorescence of gated DC 1.5 h after the initiation of the assay measured by flow cytometry. (B) Cytokines production. Quantity of TNF, IL-12 p70, IL-12 p40 and IL-10 was analyzed in culture supernatants by Luminex. Results of one representative experiment are shown. \* $P < 0.05$ . (C) Effect of inhibition of GC receptor by RU486 on IL-12 p70 and TNF production after stimulation by LPS. \* $P < 0.05$ . (D) T cell stimulatory capacity of GC-treated DC. CFSE-labeled CD4 T cells were stimulated for 5 days by DC treated with GC during the differentiation or maturation. Percentage of dividing T cells is shown for each histogram. Representative histograms of one out of five experiments are shown.





**Figure 3** Quantification of TLR2, TLR3 and TLR4 mRNA expression in GC-treated DC. Real-time quantitative PCR of TLR2, TLR3 and TLR4 expression in GC-treated DC. Levels were normalized to the levels of  $\beta$ -actin. Relative expression of immature control DC was set to 1. (A) Immature DC and DC differentiated in presence of  $10^{-6}$  M GC subsequently activated by LPS or Poly (I:C). (B) Immature DC cultured for 24 h with increasing concentration of dexamethasone. (C) Monocytes (Mono) and DC differentiated for 5 days in presence of different doses of dexamethasone.

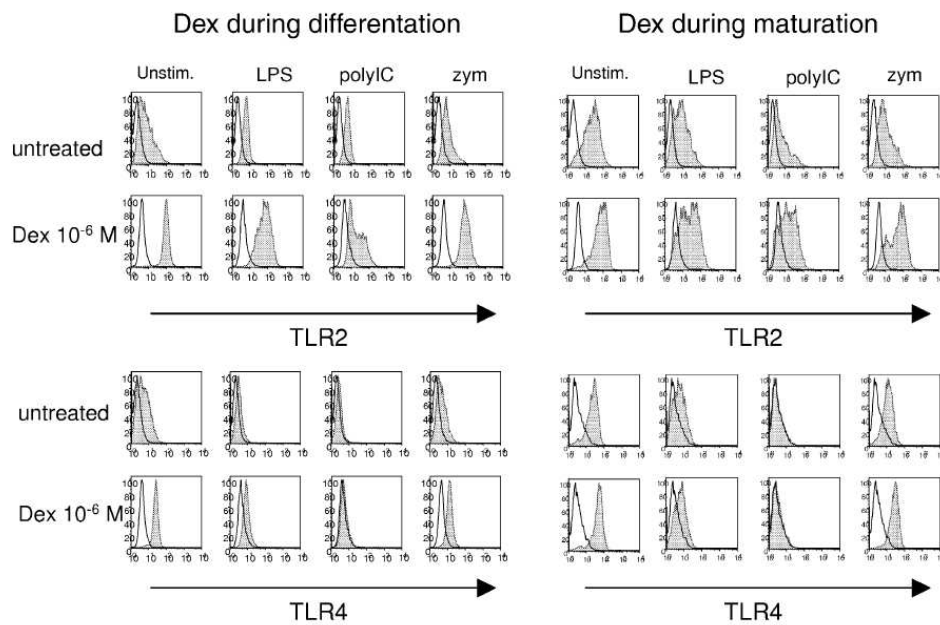
high quantities of IL-10. The observed effect on cytokine production was dose dependent (Fig. 5B).

### DC populations in patients treated with high-dose corticosteroids

The in vitro experiments have shown a severe impairment of DC function by GC exposure. As GCs are widely used for the treatment of allergic, autoimmune and tumor diseases, we next sought whether administration of GC affects circulating peripheral blood DC subsets. We analyzed peripheral blood DC subsets in 5 patients treated for systemic autoimmune disease by the high-dose i.v. administration of methylprednisolone (Solu-Medrol). Methylprednisolone in the final doses of 1000 mg/m<sup>2</sup> was administered for 3 consecutive days and peripheral blood DC subsets were quantified 24 h following the dose. Myeloid DC were identified as lineage-negative/HLA-DR-positive and CD11c-positive, and plasmacytoid DC as lineage-negative/HLA-DR- and CD123-positive cells. Administration of the high-dose methylprednisolone led to a significant decrease in the number of myeloid DC. This effect was even more prominent for plasmacytoid DC, this subset was undetectable in the peripheral blood 24 h after the administration of dexamethasone (Fig. 6). DC numbers before administration of high dose methylprednisolone were comparable to the DC numbers in the peripheral blood of healthy controls (data not shown).

### Discussion

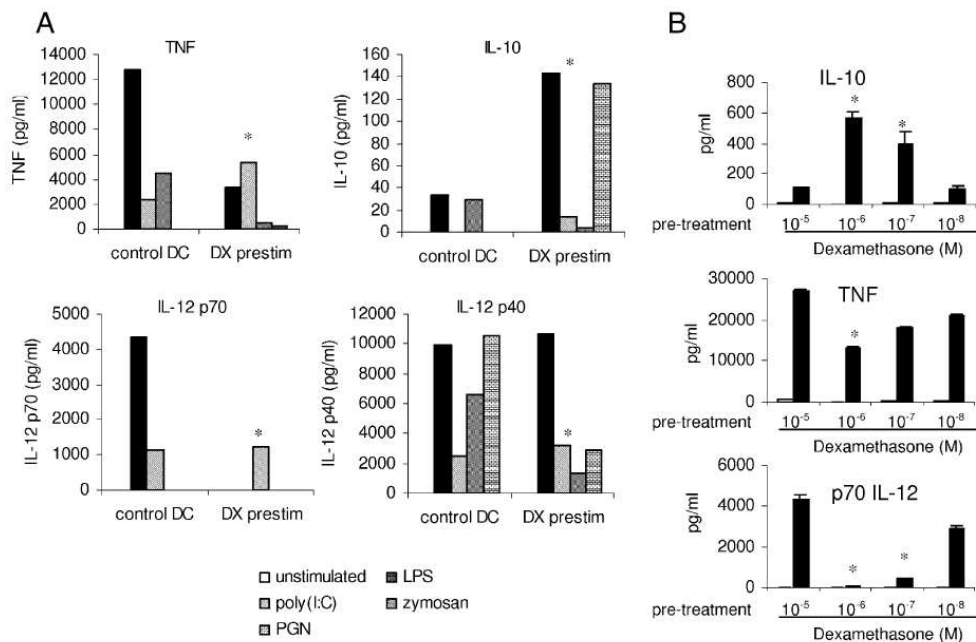
In this study, we investigated the effect of clinically used GC on the multiple characteristics of DC differentiation, maturation and their in vivo homeostasis. Despite their wide use in the control of many autoimmune, inflammatory and allergic diseases, the complex cellular and molecular mechanisms underlying GC effects are not completely understood. To investigate GC effects on DC differentiation and maturation, we used a two-step culture system for monocyte-derived DC generation. As TLR ligation represents the most potent means of initiating DC maturation, we used PGN, zymosan, Poly (I:C) and LPS, specific ligands for TLR2 (PGN and zymosan), TLR3 (Poly (I:C)) and TLR4 (LPS). Previous studies investigating GC effect on DC functions either did not study the maturation-associated defects after GC treatment or used less potent DC activators, such as TNF or CD40L [23,25]. Proinflammatory cytokines produced at the site of inflammation enhance some of DC maturation characteristics, especially expression of costimulatory and adhesion molecules, but do not initiate production of IL-12. The isolated effect of proinflammatory cytokines does not lead to the priming of Th1 immune response [33]. CD40L is an important DC activation signal later in the activation process after the activated DC migrate to the secondary lymphoid organs and interact with activated T cells. Its effect on the activation of GC-treated DC was tested in the



**Figure 4** Surface expression of TLR2 and TLR4 on DC treated with glucocorticoids during differentiation (left) or maturation (right). Surface TLR2 and TLR4 expression (shaded histograms) was analyzed on immature DC and on DC treated with GC during differentiation or maturation by TLR2, 3 and 4 agonists. Staining with isotype control is also shown. Representative results of three experiments are shown.

previous work by Piemonti et al. [23]. It would also be of interest to evaluate some novel modifications of DC maturation protocols, that is, combination of interferon  $\alpha$  and Poly (I:C) for their capacity to overcome GC-induced suppression of DC maturation [34]. Our results show that GC dramatically impair both stages of DC lifecycle. DC differentiation in the presence of GC is skewed to the nonadherent cells with typical DC morphology. However, these cells express very high levels of CD14, a molecule that is absent on DC, and lack costimulatory molecules that are mildly positive on immature DC. When stimulated with TLR-derived signals, these macrophage-like cells showed no changes in phenotype or function. In addition, the expression of maturation-associated molecules remained very low or undetectable, they produced very low amounts of TNF and p70 IL-12 while producing higher amounts of IL-10 and retaining high endocytic activity. These findings are in accordance with previously published study using different DC culture model [26]. Cells differentiated in the presence of GC were incapable of stimulating T cell proliferation. Presence of GC during the process of DC maturation also had an inhibitory effect on their phenotype and function. Although DC matured by TLR ligands in the presence of GC partially retained their ability to undergo maturation, their ability to do so was significantly lower in all aspects (i.e., the expression of maturation-associated markers, cytokine production, T cell stimulatory capacity) compared to control DC. Presence of GC thus leads to the impairment of DC function at two levels: First, GC during DC differentiation skew their development into a qualitatively distinct population incapable of inducing the efficient immune response; and second, GC presence during the process of maturation impairs the quantity, that is, extent of this process.

As mentioned above, TLRs function as the major pattern-recognition receptors in recognizing pathogen-associated molecular patterns. Activation of TLRs by their specific ligands leads to the production of proinflammatory and Th1-polarizing cytokines and induces the expression of costimulatory molecules. This initiates the process of DC maturation and subsequently leads to the efficient adaptive immune response [12]. Given the importance of TLRs for DC activation, we analyzed the expression of TLRs on DC treated with GC. We show that GC increase the mRNA expression of TLR2, 3 and 4 and surface TLR2 and TLR4 expression on DC. This observation is supported by the recently published microarray data revealing upregulation of TLRs in PBMC mononuclear cells after GC challenge [35]. Although TLR expression on the GC-treated DC has not yet been investigated, several reports have recently described the increase of TLR2 expression on the epithelial cell lines treated with dexamethasone [36–39]. These studies concentrated on the molecular mechanisms of this phenomenon. When bound to its ligand, glucocorticoid receptor (GR) binds specific DNA sequences known as glucocorticoids responsive elements (GRE) and promotes or inhibits the transcription of many genes. Increase in TLR2 expression has been attributed to the inhibition of p38 MAP kinase by GC-induced MAPK phosphatase-1 (MKP-1) [36]. However, in the experimental model used by Hermoso et al., increase of TLR2 in epithelial cells was observed after simultaneous treatment by GC and TNF as opposed to the isolated GC effect shown in our study. This suggests that yet another mechanism could be implied in GC-induced TLR expression. It cannot be excluded that the promoter regions of TLRs contain GRE and GC directly induce TLR expression [38]. It was also reported that the increased TLR2 expression on the



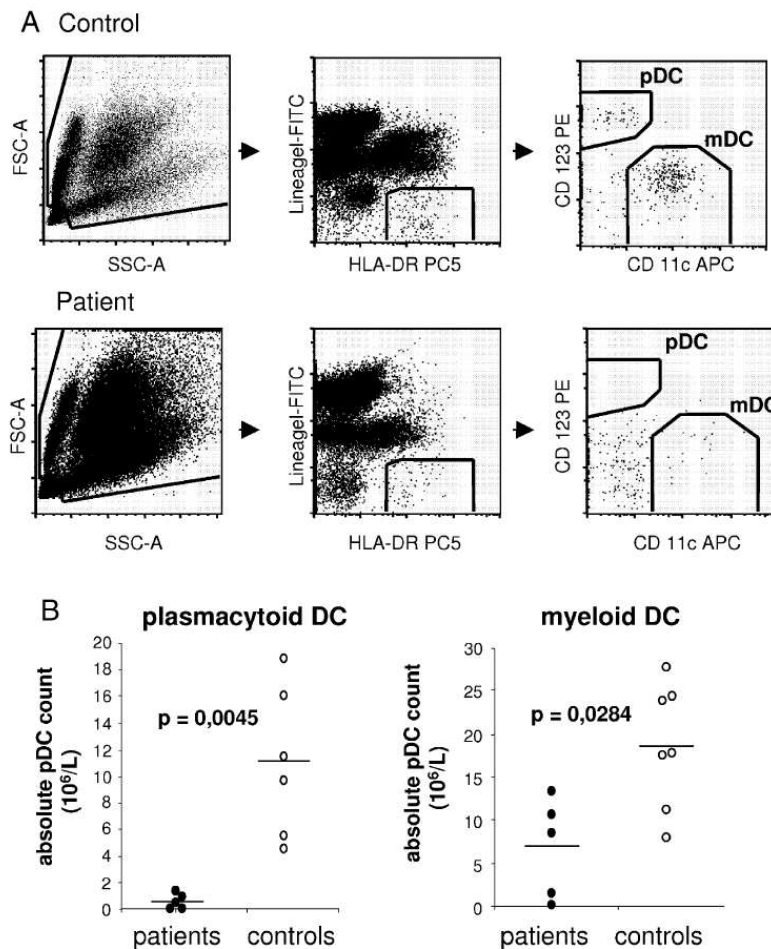
**Figure 5** Production of cytokines by DC pretreated with GC and restimulated by LPS. (A) DC cultured with or without  $10^{-6}$  M dexamethasone for 24 h were extensively washed and stimulated with PGN, zymosan, Poly (I:C) or LPS. 24 h later, supernatants were collected and production of IL-10, TNF, IL-12 p40 and IL-12p70 measured by Luminex. (B) Same setup as in panel A but the dose–response effect is shown for LPS stimulation.

epithelial cells enhanced their anti-microbial functions [40]. Moreover, increased expression of TLR2 was observed in lung DC in mice with sepsis [41]. However, GC-treated DC with increased expression of TLRs did not have higher capacity to respond to the stimulation by TLR2, TLR3 and TLR4 agonists—they produced significantly lower levels of IL-12 and TNF and higher amounts of IL-10. These observations are in accordance with a recent report showing reduced capacity of monocytes from patients with sepsis to respond *ex vivo* to LPS despite observed increased levels of TLR2 and TLR4 protein [42]. TLR activation initiates signaling pathways that are partially shared between all members of the TLR family. Triggering of TLRs leads ultimately to the activation of NF $\kappa$ B transcription factor and also to the activation of MAP kinases. Interestingly, all these molecules were shown to be the targets for GC. GC thus interfere with multiple signaling pathways initiated by TLR activation [43]. Immune response in the absence of TLR signaling showed skewing to the Th2 type of the immune reaction [44]. It is therefore possible that endogenous glucocorticoids, which are recognized as Th2 inducers (when released during systemic infections) may induce increased TLR expression but they block TLR signaling and regulate potentially harmful Th1 response by favoring Th2 immune response. Although in this study we did not analyze the effect of GC-treated DC on the development or function of regulatory T cells, it is important to note emerging evidence pointing to the important role of TLRs for their function. TLR-mediated activation of DC and IL-6 production leads to the blocking of suppression by regulatory T cells and allows activation of pathogen specific adaptive immune response [13,45]. Another study points to the role of TLR2 expressed on

regulatory T cells themselves. TLR2 triggering augmented proliferation of regulatory T cells and resulted in a temporal loss of the suppressive phenotype by directly affecting regulatory T cells [46,47]. GC induced increase of TLR expression and its consequences for the homeostasis of regulatory T cells will have to be analyzed in further studies.

In the case of immature DC, interference with TLR signaling decreases the extent of maturation and impairs the ability of the innate immune system to adequately recognize and fight the microbial infection. TLRs are also expressed by other cells of both the innate and adaptive immune systems and the consequences of GC treatment of these cells remain to be investigated.

In the last part of this study, we extended the relevance of the *in vitro* findings to the common therapeutic situation. GCs are widely administered in autoimmune, inflammatory and allergic diseases. We showed that the administration of high-dose corticosteroids for the treatment of systemic autoimmune disease abolished circulating plasmacytoid DC and significantly decreased the number of myeloid DC. These findings are complementary with previously published studies in patients treated with corticosteroids in various therapeutic settings such as immunosuppressive treatments after bone marrow or renal transplantations or for autoimmunity [48–52]. There are several explanations for the decreased numbers of circulating DC and future studies are needed to evaluate the precise contribution of different mechanisms to this phenomenon. After the administration of high-dose corticosteroid regimens, the *in vivo* GC concentration is likely to exceed  $10^{-6}$  M. This concentration leads to the rapid apoptosis of DC precursors *in vitro*. Lower concentrations could lead to the skewed differentiation of



**Figure 6** Peripheral blood DC subsets after the administration of high-dose corticosteroids. (A) Identification of peripheral blood DC subsets. Myeloid DC (mDC) were identified as lineage-negative/HLA-DR-positive/CD11c-positive cells. Plasmacytoid DC (pDC) as lineage-negative/HLA-DR-positive/CD123-positive cells. Representative dotplots for healthy control (upper row) and patient treated by high-dose Solu-Medrol (lower row) are shown. (B) Absolute numbers of circulating DC subsets in peripheral blood 24 h after the administration of high-dose Solu-Medrol. Points correspond to the individual patients, horizontal bar represents mean value for the group.

DC precursors towards the cells with macrophage-like characteristics. These cells would not be detected as DC because of high expression of CD14. Interestingly, a recent report has showed the importance of TLR signaling for the DC differentiation [53]. Abrogation of TLR signaling by GC could thus also contribute to the impaired differentiation of peripheral blood DC from their precursors. Finally, it is also conceivable that the impaired maturation in response to the appropriate stimuli in the presence of higher concentration of GC fails to upregulate chemokine receptors necessary for the migration of mature DC to the secondary lymphoid organs, and that decreased peripheral blood DC counts are caused by the increased number of DC residing in peripheral tissues. However, this mechanism contrasts with the reports showing the depletion of interstitial and plasmacytoid DC following GC administration in a mouse model [50,54]. At present, little is known about the in vivo present DC. New technologies will enable the studies of peripheral blood DC,

their phenotypic profile and functions in various pathological states and therapeutic protocols.

Taken together, we have addressed present the complex study of the effect of GC on the phenotypic and functional characteristics of DC both in vitro and in vivo. The severe impairment of DC differentiation and maturation after TLR stimulation, together with the impaired numbers of peripheral blood DC subsets, provide further insights into the mechanisms of GC function and reveal additional mechanisms of their therapeutic efficiency.

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The field of dendritic cell biology is robust, with many newly recognized functions in their control of immunity and tolerance. There is also no shortage of mysteries and challenges. DCs own extraordinary features, that allow them either intensify or subdue T-cell responses, depending on whether resistance or tolerance needs to be increased. At the present time, DCs biology provides opportunities to study some of the most challenging areas of medicine. The biology of DCs is ready to be extended to dissect disease pathways and to direct its prevention and treatment. Despite the obstacles to research in patients, now we have the knowledge and tools to think systemically about diseases, plan their therapies, and investigate how humans respond. DCs are an early player in disease development and an unavoidable target in the design of treatments. If we take in account that immunology, including T-cell-mediated immunity, has a central role in understanding how diseases develop, therapies aimed at the upstream events initiated by DCs have their rationale.

In our studies we have been trying to answer some of the questions that might bring new insights into the understanding of DC roles in immunity and diseases. We have translated novel identification strategies into several parts of medicine. Using multicolor flow cytometry we have developed a method for identification of DCs in peripheral blood with the simultaneous monitoring of their co-stimulatory molecules. This method allowed an extensive monitoring and analysis of DCs subsets following the allogeneic-HSCT. Interestingly, DCs have been shown to reconstitute in the early stages after the transplantation, however their decline and low numbers could be observed during the further follow-up period comparing to healthy controls. We have detected two completely distinct DCs reconstitution patterns regarding the development of acute GVHD, where patients with this complication had significantly lower number of both DC subsets and this even prior the development of clinical symptoms and application of high-dose steroid therapy. These results show that monitoring of DC might have predictive value for in the diagnostics of aGVHD.

In our second study we analyzed the critical role of Btk function in myeloid cells in XLA patients, whilst several evidence indicated that Btk might be a component of Toll-like receptor signaling. We have analyzed whether Btk deficiency in XLA could be associated with an impaired dendritic cell compartment or defective TLR signaling. Our data revealed a profound impairment in IL-6 and TNF- $\alpha$  production in response to TLR-8 cognate agonist

ssRNA stimulation. These findings may provide an explanation for the susceptibility to enteroviral infections in XLA patients.

In the third study we have complexly analyzed the effects of widely used GC onto DC functions. We have shown profound impaired T cell stimulatory capacities and abrogated pro-inflammatory cytokine production, despite an increased expression of several TLRs. We have also revealed that DCs treated by GC fail to mature upon TLR-derived stimulation. Our *in vitro* findings have been extended by *in vivo* analysis, where decreased circulating myeloid and abrogated plasmacytoid DCs counts could be measured. These data provide further insights into the mechanisms of GC immunosuppressive functions and reveal additional mechanisms of their therapeutic efficiency.

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13 List of publications

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