PhD program in Molecular and Translational Medicine

DIMET

Human Dendritic Cell subsets: cytokine production and their role in T cell priming

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TABLE OF CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

Research following the discovery of dendritic cells forty years ago is profoundly changing immunology and its many interfaces with medicine. It took until 1973 for Ralph Steinman and Zanvil Cohn to begin the modern era of dendritic cell science by showing that dendritic cells are a new class of white blood cells with a number of distinctive features and functions; the importance of this discovery earned him the Nobel Prize in Physiology or Medicine in 2011.

These previously unknown cells are now recognized as cells that bridge innate and adaptive immunity, displaying unique capacity to prime T cell responses.

1. DISCOVERY OF DENDRITIC CELLS

Dendritic cells were discovered in 1973 by Ralph Steinman and the late Zanvil A. Cohn at the Rockefeller University[1-3]. At the time, Steinman and Cohn were studying spleen cells to understand the induction of immune responses in a major lymphoid organ of the mouse. They were aware from research in other laboratories that the development of immunity by mouse spleen required both lymphocytes and "accessory cells," which were of uncertain identity and function. The accessory cells were thought to be typical macrophages, but despite extensive laboratory experience with macrophages, Steinman and Cohn encountered a population of cells with unusual shapes and movements that had not been seen before. Because the cells had unusual tree-like or "dendritic" processes, Steinman named them "dendritic cells."

The research experience of the Cohn-Steinman laboratory with the cell biology of macrophages enabled the confident identification of dendritic cells as novel cells having distinct properties and, eventually, functions. Dendritic cells are a critical, and previously missing link in the immune system. As sentinels, dendritic cells patrol the body seeking out foreign invaders, whether these are bacteria, viruses, or dangerous toxins. After capturing the invaders, that are rich sources of antigenic proteins, dendritic cells convert them into smaller pieces and display the antigenic fragments on their cell surfaces on dedicated antigen-presenting MHC molecules. The dendritic cells then travel to lymph nodes or the spleen where they stimulate other cells of the immune system, such as T lymphocytes, to mount vigorous immune responses.

By 1979, Steinman had learned to enrich the small population of dendritic cells (normally comprising one percent of the cells in the spleen)[4]. Once a sufficient quantity of cells could be obtained, with a high degree of purity, then functional studies on the new cells could begin. These studies revealed their potent stimulatory role in immune function. Comparable dendritic cells were found in many organs and animal species, including in human blood. Subsets of dendritic cells were identified, each having its own surface markers. Dendritic cells were seen in the T-cell areas of organs of the lymph system, the ideal location for initiating immunity. Laboratories worldwide started to study dendritic cells and demonstrated their potent immune stimulatory functions.

These observations also permitted studies on the origin of dendritic cells from bone marrow, where their development was traced through a pathway shared with macrophages and granulocytes. By 1992, Steinman, together with Maramatsu[5] and Schuler[6], developed methods to generate a large number of dendritic cells from their progenitors.[5] At this point, dendritic cells were readily available for cellular and molecular biologists, and their studies have greatly expanded dendritic cell research.

2. DENDRITIC CELLS: ORIGIN AND DEVELOPMENT

The classification of mononuclear phagocytes as DCs and macrophages is largely based on phenotypic and/or functional criteria. This has led to a substantial confusion[7], especially during infection or inflammation when many of the supposedly defining markers and functions of DCs change profoundly[8]. Gene expression analysis and ontogenetic relationships have been used to refine the definition of DC and macrophages/monocytes; nevertheless, there remains the need for an overarching ontogenetic definition that unifies the DC family and establishes it as an independent leukocyte lineage.

It is well known that, except for Langerhans cells, all other DC populations derive from a hematopoietic stem cell (HSC) that develop in the bone marrow (BM)[9]; in the mouse, a series of progressively restricted progenitor cells have been described, whereas the bone marrow origin of human tissues DCs has been formally proven by hematopoietic stem cell transplantation[10] but the cascade of progenitors is still to be clearly elucidated.

In the mouse system, successive commitment steps in the bone marrow include granulocyte-macrophage precursors (GMPs) and macrophage/DC progenitors (MDPs). Within the bone marrow, MDPs differentiate into monocytes and into the common DC precursor (CDP)[11]. CDPs do not exit the BM but give rise to plasmacytoid DC and to pre-DCs; at steady-state, pre-DCs are found in the blood and spleen[12], they can enter the lymph nodes and integrate in the DC network, giving rise to conventional DC (Figure 1).

A very recent study by Reis e Sousa's group reported that precursors of conventional DC can be defined by expression of the C-type lectin receptor DNGR-1, allowing the definition of the DC lineage by ontogeny.[13]

The equivalents of MDPs and CDPs have not yet been described in humans; MLP (multi-lymphoid progenitor) and GMP (granulocyte-macrophage progenitor) appear to have DC potential, at least by single cell in vitro assays or adoptive transfer into immunodeficient mice.[14] It has been postulated that both are able to give rise to the human equivalent of MDPs, and hence to CDPs and DCs by a common pathway equivalent to the mouse model.

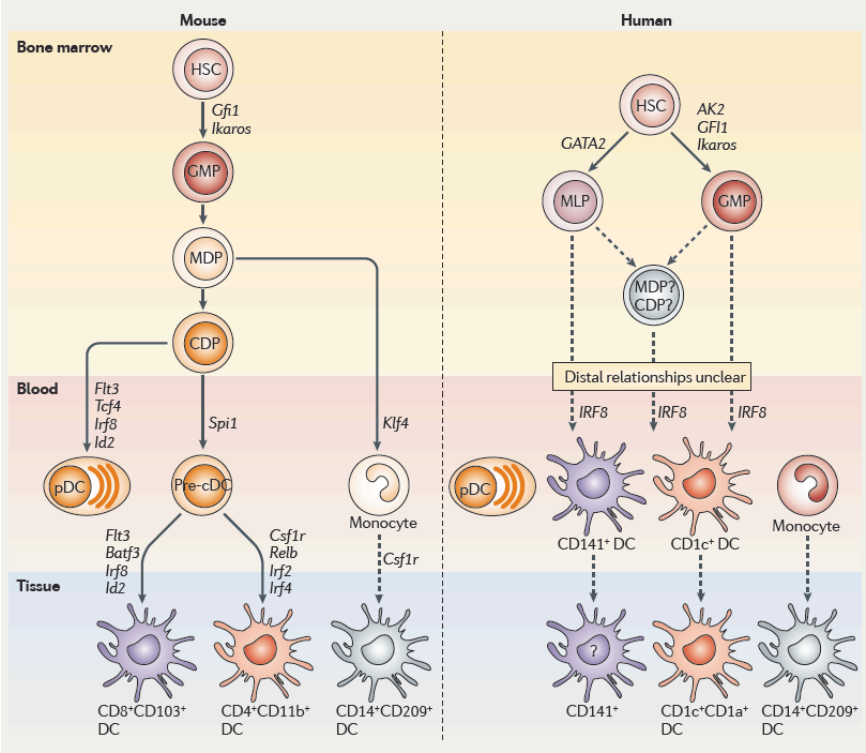

Figure 1. DC ontogeny
Models of the cellular intermediates and genetic requirements for the dendritic cell differentiation in mice and humans.
From Collin M. et al, Nat Rev Immunol. 2011.

Cell fate decision in the hematopoietic system involves the actions of a small number of regulatory transcription factors, which establish specification in pluripotent progenitors and induce commitment and differentiation to the different lineages, as depicted in Figure1.

Particular attention should be pointed on Interferon Regulatory Factor 8 (IRF8), a transcription factor that plays critical roles in the lineage commitment of DC.

IRF8 knock-out mice lack plasmacytoid DCs (pDC) and CD8α$^+$ DCs[15]; however, mice having a point mutation in IRF8 gene (R294C) lack only CD8α$^+$ DCs[16] indicating that distinct IRF8-dependent mechanisms mediate the development of these two DC subsets.

In the human system, IRF8 is controlling both plasmacytoid[17] and myeloid DC development, and studies on IRF8 mutations showed that patients can suffered from life-threatening infections, having a complete impairment of DC development or, in other cases, displaying a milder immunodeficiency with a selective depletion of CD1$^+$ (mDC1) DCs.[18]

In addition to regulating pDC and DC subsets generation in vivo, IRF8 also controls genes that have important roles in DC functional responses, such as IFNα in pDC and IL-12 in mouse CD8α$^+$ and human myeloid DC.

3. DENDRITIC CELL CLASSIFICATION

Arising from all the work done so far on dendritic cell biology, it is clear that DCs are not just one cell type but they comprise different subtypes: DC appear to be distinct entities with different fates. They differ not only in phenotype, but also in their genetic program, anatomic location, expression of receptors for pathogen molecules and abilities to program T-cell responses.

DC can be classified based on different criteria[19]: first either as precursors of DCs (pre-DC) or as DC products themselves; second according to their migration property; third based on their presence or

absence in steady state or inflammation; fourth based upon activation state of the DC and finally DC can be classified according to the different subsets (see Table 1).

Table 1. DC classification

1	**DC stage**	Pre-DC	Developed DC
2	**DC migration**	Migratory	Resident
3	**Host state**	Steady state	Inflammatory
4	**DC activation state**	Unactivated	Activated
5	**DC subtypes**	Different DC	subtypes

Adapted from Naik S. Immunol Cell Biol (2008)

3.1 Pre-DC and Developed DC

The term pre-DC refers to the cells in the immediate previous step of DC development; DC can differentiate by default in the steady state or may require additional signals provided by infection of inflammation.

3.2 Migration

Based of their migratory capacity, DC can be classified as follows: ”migratory DC”, develop from early precursors in the peripheral tissues, where they act as sentinels for pathogens or self-antigens. From the peripheral tissues they migrate via afferent lymphatics to the draining lymphoid organs. During migration, DC acquire a mature phenotype, needed for the optimal presentation of antigen to T cells[20].

There are some DCs that do not migrate, they are the so called “resident DC”, found in all lymphoid organs, as well as gut and dermis.

The other major category of DC is the tissue-resident DC, found in lymph nodes, gut, thymus and spleen; these DC do not traffic from other lymphoid tissues, their residency makes them ideally placed to sense the pathogen that are transported in the blood.

DCs appear as a heterogeneous group of cells and a lot of studies have been done in order to come to an exhaustive classification that combines the above-mentioned criteria and DC ontogeny. The majority of the studies conducted on DC biology were performed in mice, due to the paucity and the difficulty to obtain DC from human tissues.

Nevertheless, human dendritic cells have been classically defined as leukocytes that lack other leukocytes lineage markers (CD3, 14, 15, 19, 20, and 56) and express high levels of MHC class II (HLA-DR) molecules.[21] In the last years, several efforts have been done in order to shed light into the human DC system.

According to the literature, the major categories of *in vivo* DCs are conventional DCs, Langerhans cells, and plasmacytoid DC; the mostly used monocyte-derived DCs are *in vitro* generated DCs, as discussed below.

3.3 Conventional DCs

Mouse conventional DCs are specialized for antigen processing and presentation. They can be grouped into two main classes based on their localization in tissues and their migratory pathways. First category is referred to migratory DCs: these cells traffic continuously from peripheral tissues to the draining lymph nodes charged with tissue antigens; they display a mature state and are found in skin, liver, kidney and lung. Mouse migratory DC can be broadly divided according to the expression of integrins CD11b and CD103 as follows: $CD11b^+$ DCs and $CD103^+$ DCs.

The second major category of conventional DCs is the lymphoid tissue-resident DCs that arise from blood-born precursors[22] and remain in lymphoid organs during their entire life cycle, displaying an immature state. In the mouse, "resident" DC can be classified by their expression of surface molecules CD4 and CD8α into $CD4^+$ DC, $CD8\alpha^+$ DCs and $CD4^-$ $CD8\alpha^-$ DCs.

Conventional DCs do exist also in humans, known as "myeloid" DCs, but less is known about populations of resident and migratory DCs; it is known that in humans there are populations of DC that resemble the murine conventional ones, found in blood, spleen and tonsils and expressing different markers: their classification has been possible thanks to the discovery of BDCA-family antigens (Blood Dendritic Cell Antigen)[23]. Human myeloid cells express CD11c (like mouse DC) and have been grouped into mDC1 ($CD11c^+$ $BDCA1^+$) and mDC2 ($CD11c^+$ $BDCA3^+$).[23,24]

Correlation of the human and mouse DC subsets has been hampered by differences in their defining markers (notably, human DCs do not express CD8α).

Interestingly, computational genome-wide expression profiling clustered human BDCA3$^+$ (also termed CD141$^+$) DC and BDCA1$^+$ (also termed CD1c$^+$)DC with the mouse CD8α$^+$ and CD8α$^-$ conventional DC subsets, respectively (Figure 2).

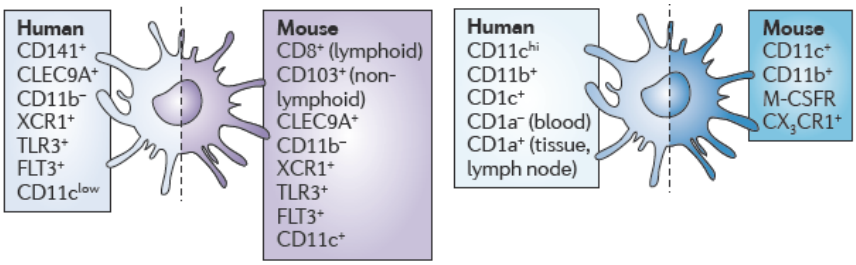

Figure 2. Conventional/Myeloid DC classification in mice and humans
Human "myeloid" dendritic cells (DCs) are equivalent to mouse "classical" DCs. They express different markers, as shown. BST2, bone marrow stromal antigen 2; CX3CR1, CX3-chemokine receptor-1; FLT3, FMS-related tyrosine kinase 3;
Adapted from Collin M. et al, Nat Rev Imm. 2011.

3.4 Plasmacytoid DCs

pDC are quiescent cells that are broadly distributed in the body. They are characterized by their ability to rapidly produce large amounts of type I interferons (IFNs), a critical feature to control viral infections. In the steady state pDC show a plasma cell-like morphology[25,26] and express several characteristic markers that differ from mice and humans: sialic acid-binding immunoglobulin-like lectin H (SIGLEC-H), bone marrow stromal antigen 2 (BST2), CD11c but nor CD11b in mice, blood DC antigen 2-4 (BDCA2-BDCA4), CD123 but not CD11c in humans. CD45RA is an antigen expressed by both human and mice pDC (Figure 3).

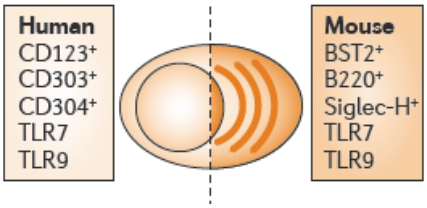

Figure 3. pDC classification in mice and humans
Plasmacytoid DCs are functionally closely equivalent in both species.
Adapted from Collin M. et al, Nat Rev Imm. 2011.

3.5 Langerhans cells

Langerhans cells (LCs) are a specialized subset of DC that populates the epidermal layer of the skin. They account for 3-5 % of all nucleated cells in the epidermis of mice and humans and are arranged in a network that occupies the interstices between neighboring keratinocytes. The skin provides the first line of defense of the organism against a broad range of pathogens and the resident Langerhans cells have a pivotal role in the capture and presentation of antigens. It is known that LCs migrate in the draining lymph nodes in the steady state[27] and their rate of migration is increased upon inflammation[28]. After leaving the epidermis, DC migrate trough the dermal lymphatic vessels and localize in the T cell area of the skin-draining lymph nodes [29], where they can present foreign antigens. A recent work showed that LC pool in the skin is maintained by the presence in the skin of IL-34, a non-redundant cytokine produced by keratinocytes and important for the development of LCs during embryogenesis as well as for their homeostasis in the adult skin.[30]

Mice and human LCs share the expression of the hematopoietic marker CD45 and the expression of specific adhesion molecules,

such as E-cadherin and EpCAM, that anchors them to keratinocytes. In addition, LCs are characterized by the unique expression of Langerin and Birbeck granules.

3.6 Monocyte-derived DCs

Monocytes are circulating cells of the mononuclear phagocyte system, first studied as macrophage precursors[31], and later recognized to have an added potential to develop into DCs: in 1994 Sallusto F. and colleagues showed that monocytes cultured with interleukin -4 (IL-4) and granulocyte macrophage colony-stimulating factor (GM-CSF) acquire a typical dendritic morphology, the so called monocyte-derived DC (moDC)[32]. Several reports documented in mice the differentiation of monocytes into $CD11c^+$ MHC II^+ moDC during different models of infection[33,34]. Big efforts where made by Steinman and colleagues in order to clarify the role of moDCs in the induction of T cell responses in vivo in both human and mouse; they show that DC-SIGN marks fully differentiated moDCs that are recruited from blood to lymph nodes by lipopolysaccharide (LPS) or gram- negative bacteria, where they exert functions similar to conventional/myeloid DCs.[35]

4. DC AND THEIR ROLE IN ADAPTIVE IMMUNE RESPONSE

Dendritic cells (DC) constitute a unique system of cells able to initiate and orient immune responses. As a component of the innate immune system, DC organize and transfer information from the outside environment to the cells of the adaptive immune system; they act as a bridge between innate and adaptive immunity and are able to

modify and adapt the T-cell response to the type of invading pathogen.

Different steps lead to the initiation of a specific adaptive immune response, through the recognition and uptake of the antigen, its processing and presentation to T cells.

The entire process is fine-tuned, with different DC subsets showing different behaviors.

4.1 ANTIGEN RECOGNITION

At the initial stage of a primary infection, DCs constitute an integral part of the innate immune system that recognize pathogens through germline-encoded patter-recognition receptors (PRRs), a set of evolutionary conserved proteins that mediate cell activation.[36] PRRs directly recognize conserved microbial molecules, known as pathogen-associated molecular patterns (PAMPs), many of which are shared by different classes of pathogens. Examples of PAMPs are nucleic acids, component of the cell wall of bacteria and yeasts (LPS, flagellin, ecc).

Let know focus on different PRRs expressed on DC subsets.

4.1.1 PATTERN RECOGNITION RECEPTORS: TOLL-LIKE RECEPTORS

The first group of pattern recognition receptors to be described was the *Toll-like receptors* (TLRs) group. *Toll* is a gene initially described in *Drosophila melanogaster*, where its product plays an important role in establishing the dorsal-ventral axis during embryogenesis.[37]

A decade later, Jules Hoffmann discovered that Toll mediated protection against bacterial and fungal infections[38], introducing the gene into immunology. Soon after, Bruce Beutler assigned the murine Tlr4 gene to be the long searched for receptor responding to the potent bacterial endotoxin lipopolysaccharide (LPS).[39] This was the beginning of a new era in innate immunology, and in the last decade, innate detection of PAMPs has grown to a field in itself.

TLRs are type I trans-membrane proteins with ectodomains containing leucine-rich repeats recognizing their respective ligands and a cytosolic Toll-IL-1 receptor (TIR) domain to further activate intracellular signaling cascades when the receptor is activated. So far, 10 and 12 functional TLRs have been identified in humans and mice, respectively, with TLR 1-9 being conserved in both species. Studies on mice deficient in each TLR have demonstrated that each TLR has a distinct function in terms of PAMPs recognition and immune response.[40]

PAMPs recognized by TLRs include lipids, lipoproteins, proteins and nucleic acid derived from a wide range of microbes such as bacteria, viruses, parasites and fungi.[40] This recognition occurs in different subcellular compartments[41], including the plasma membrane, endosomes and lysosomes. TLR signaling pathways were intensively studied and showed that individual TLRs selectively recruit distinct adaptor molecules downstream, providing specific immunological responses tailored to the infecting microbes.

It has to be underlined that inappropriate TLR responses contribute to acute and chronic inflammation, as well as to systemic autoimmune diseases.

TLRs are largely divided into two subgroups depending on their cellular localization and respective PAMP ligands. One group is composed of TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11, which are expressed on cell surfaces and recognize mainly microbial membrane components such as lipids, lipoproteins and proteins; the other group is composed of TLR3, TLR7, TLR8 and TLR9, which are expressed exclusively in intracellular vesicles such as the endoplasmic reticulum (ER), endosomes and lysosomes and they sense nucleic acids derived from viruses or dead cells.

TLR2: it is a surface TLR involved in the recognition of a wide range of PAMPs derived from bacteria (peptidoglycan and lipoteichoic acid from Gram positive), fungi (zymosan), parasites (mucines) and viruses (hemagglutinin proteins). TLR2 can dimerize with TLR1 and TLR6, broading the spectrum of recognized PAMPs. TLR2 activation leads primarily to inflammatory cytokine production, especially IL10.

TLR4: located on the plasma membrane, it was identified as the long-sought receptor that responds to bacterial lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria that cause septic shock.[40] TLR4 forms complex with MD2 on the cell surface and together they serve as the main LPS-binding component: 5 of the 6 lipid chains of LPS bind the hydrophobic pocket of MD2, while the remaining chain binds to TLR4.[42] Another key player in the TLR4 signaling is CD14, a GPI-linked protein that is found on the surface of many TLR4 expressing cells[43].

It has been shown that CD14 controls microbe-specific endocytosis of TLR4, promoting signal transduction.[44] Moreover, CD14 is required for TRIF-dependent pathway signaling.[45]

TLR5: it is a surface TLR, recognizes the flagellin protein component of the bacterial flagella. It is highly expressed on gut DC.[46]

TLR3: located intracellularly, it was originally identified as recognizing a synthetic analog of double-stranded RNA (dsRNA), polyinosinic-polycytidylic acid (polyI:C), which mimics viral infection and induces antiviral immune responses by promoting the production of type I and type III interferon[47] and inflammatory cytokines. TLR3 recognizes not only polyI:C but also genomic RNA of reoviruses, dsRNA produced during the replication of single-stranded RNA (ssRNA) viruses, including respiratory syncytial virus, West Nile virus and Hepatitis C virus.

TLR7/8: located on the cytoplasm, they were originally identified as recognizing imidazoquinoline derivatives, i.e. resiquimiod (R848)[48]; it is known they recognizes ssRNA rich in adenosine (TLR8) and guanosine (TLR7), derived form RNA viruses and give rise to the production of large amount of type I interferon. TLR8, which is phylogenetically most similar to TLR7, is functional only in humans[49]; TLR7/8 are expressed in different DC subsets, with pDC expressing TLR7 and TLR9, and TLR8 restricted to myeloid DC.[50]

TLR9: intracellular TLR, recognizes unmethylated 2'-deoxyribo(cytidine-phosphate-guanosine) (CpG) DNA motifs that are frequently present in bacteria and viruses but are rare in mammalian cells. [51]

To be noted that intracellular TLR recognize viral and bacterial nucleic acids in late endosome-lysosome; host's nucleic acid are not normally accessible to these compartments. These TLRs can potentially recognize self RNA and DNA, leading to autoimmune diseases.

Expression and function of TLRs varies significantly in mice and human and in different DC subsets: human plasmacytoid DC express TLR1, 7 and 9, while other DC types do not express TLR9 nor 7, but express TLR8. Notably, human $BDCA1^+$, but not $BDCA3^+$ DC, express TLR4.[52] The latter, on the contrary, expresses high levels of TLR3[53].

Upon recognition of their cognate ligands, TLRs induce the expression of a variety of host defense genes. These include inflammatory cytokines and chemokines, antimicrobial peptides, costimulatory molecules, MHC molecules and other effectors necessary to arm the host cell against invading pathogen. TLRs accomplish this by activating an intracellular signaling pathway conserved from Drosophila to mammals.

Individual TLRs trigger specific biological responses but all culminates in the activation of nuclear factor (NF)-κB and activation protein-1 (AP-1).

The proximal events of TLR-mediated intracellular signaling are mediated by TIR-domain dependent interactions with TIR-domain containing cytosolic adapters such as MyD88, TIRAP, TRIF and TRAM.[54]

MyD88 is used by all TLRs except TLR3 and activates downstream the transcription factor NF-κB and mitogen-activated protein kinases (MAPKs) to induce inflammatory cytokines.[55] In contrast, TRIF is used by TLR3 and TLR4 and induces alternative pathways that lead to the activation of IRF3 and NF-κB and the consequent induction of type I interferon and inflammatory cytokines.[56] Thus, TLR signaling pathways can be largely classified as either MyD88-dependent or TRIF-dependent pathways. TLR4 is the only one that uses both adaptors.

In most cases, a single pathogen can trigger the activation of multiple TLRs together and the host may have evolved to recognize some together as a combinatorial assault and mount immune responses against these combinations in a synergistic manner. This strategy would allow the immune system to rapidly respond to infection. Studies have shown that combinations of TLR ligands can synergistically increase the magnitude and the type[57] of cytokine production by DCs, in particular synergistic surface and intracellular TLR stimulation

The combined activation of different TLRs can result in complementary, synergistic or antagonistic effects that modulate innate and adaptive immunity.

4.1.2 PATTERN RECOGNITION RECEPTORS: C-TYPE LECTIN RECEPTORS

C-type lectin receptors (CLR) were originally defined as a family of soluble and trans-membrane receptors containing carbohydrate recognition domains; however continued research has revealed many structurally homologous domains that are not restricted to carbohydrate binding. These domains are known as C-type lectin-like domains and can be found on C-type lectin receptors.[58] In mammals, 17 CLR subgroups have been identified, classified after their structure and phylogenetic relationship.[59] Subgroups II, V (such as Dectin-1 family) and VI are expressed on myeloid cells, and these CLRs are receptors with the ability to bind, and in same cases respond to PAMPs. Common structures to be recognized are carbohydrates rich in mannose, fucose and glycan, often found in microbial cell walls, but also in endogenous structures.

For several CLRs, the intracellular signaling pathways is not known, but several receptors have been shown to signal via immunoreceptor tyrosine based activation motif (ITAM), expressed either by the receptor itself or via adaptor molecules associated with the receptor.[60] When activated, ITAM is phosphorylated and spleen tyrosine kinase (Syk) is recruited. Upon binding, Syk mediates activation of downstream transcription factors, such as NF-κB and AP-1.[61] Engagement of CLRs often results in Th17 or Th1 responses.[62] Some CLRs express an immunoreceptor tyrosine based inhibition motif (ITIM) with the ability to reduce responses from other PRRs. An example of this is DC immunoreceptor (DCIR), which acts to dampen TLR8-induced IL-12 and TNF-α production.[63]

Even though several CLRs cannot initiate cell activation by themselves, they some acts to enhance the intracellular NF-κB activation and promote transcription of pro-inflammatory cytokines when activated in parallel with TLR8 during binding of a pathogen, even though it does not induce activation when triggered alone.[64] In addition to activation, CLRs can also induce endocytosis when engaged, making them suitable targets for in vivo antigen delivery in vaccine settings.[65] Examples of targeted receptors are DEC-205, Dectin-1, and Clec9A, which all are expressed on several DC subsets[66] and BDCA3$^+$ DCs in particular.[67] When triggered, DEC-205 has not been shown to have immunostimulatory functions per se, while Dectin-1 stimulation can indeed induce DC maturation without additional stimuli[68] and Clec9A has been shown to mediate cross-presentation of endocytosed antigens, although without induction of DC maturation[69]; in addition Clec9A is able to recognize damaged cells through the binding of a filamentous form of actin exposed when the cell membranes are damaged.[70]

4.1.3 PATTERN RECOGNITION RECEPTORS: CYTOPLASMIC DNA SENSOR AND RIG-I RECEPTORS

In contrast to TLRs, which selectively are expressed by defined cell types, most cells express RLRs. This is a group of DExD/H-box RNA helicases responding to viral double-stranded RNA (dsRNA) present in the cytosol, and so far three receptors have been described.

Retinoic acid-inducible gene I (RIG-I) was the first receptor to be characterized in this group,[71] quickly followed by the identification of two additional genes coding for DExD/H-box RNA helicases; melanoma differentiation associated factor 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2).[72,73]

Both RIG-I and MDA5 express a C-terminal domain, a DExD/H-box RNA helicase domain, and at their N-terminus, two caspase activation and recruitment domains (CARDs). The CARD domains are however missing in LGP2. A repressor domain is expressed in the C-terminal domain of RIG-I, which is missing in MDA5. Instead, LGP2 is equipped with one and is hence believed to be a regulator of MDA5. The RLRs recognize a variety of dsRNA virus intermediates present in the cytosol. Flavi viruses, such as dengue virus and West Nile virus, are detected by both MDA5 and RIG-I.[73] Examples of viruses detected by RIG-I are influenza virus and Epstein-Barr virus (EBV)[73], while picorna viruses are detected by MDA5.[74] The receptors respond best to dsRNA that have blunt triphosphorylated 5´ ends, which in the absence of 5´ capping is a sign of non-self RNA.[75] Studies using the synthetic dsRNA analogue poly I:C show that MDA5 preferably recognizes high molecular weight poly I:C, while RIG-I responds to shorter sequences.[76] In addition to RNA, DNA can indirectly also be recognized by RLRs. The enzyme RNA polymerase III senses cytosolic DNA that is rich in A and T nucleotides, and subsequently transcribes it to 5´ triphosphate RNA, which is readily detected by RLRs.

In its inactive form, RIG-I is found with its repressor domain bound to the CARD domain in a closed conformation.[77] Upon binding to a ligand, the repressor domain releases CARD, which then interacts with the adaptor protein interferon-β promoter stimulator 1 (IPS-1), located in the mitochondrial membrane. A signaling complex is formed, involving members of the NF-κB family and IRF3, which upon activation is translocated to the nucleus, where it initiates transcription of pro-inflammatory genes and type I IFNs, respectively.[78]

The RLRs enable most cells and tissue to produce type I IFNs in response to cytosolic RNA, which additionally signals to the surrounding milieu to initiate an antiviral defense. Interferon-β binds to the IFN-α/β receptor in an autocrine or paracrine manner and initiates the transcription of interferon-stimulated genes (ISGs), such as IFN-α, IRF7, and additional PRRs.

Cytosolic DNA is sensed in a similar manner by recently characterized cytosolic DNA sensors. These sensors have previously been described as components in various intracellular type I IFN inducing signaling pathways, but are now shown to bind directly and respond to transfected or viral dsDNA.[79] The two best characterized members in this family are absent in melanoma 2 (AIM2) and DNA-dependent activator of IFN-regulatory factors (DAI).

4.1.4 PATTERN RECOGNITION RECEPTORS: NLRs

A growing family of cytosolic PRRs is the NLRs, with 22 members characterized so far. The NLRs are divided into four subgroups, depending on their structure.[80] The NLRs all express a nucleotide binding domain (NBD) and a LRR in their C-terminus. Additionally, they express various domains at their N-terminus, which divides them into the separate subgroups. The members in the NLRC-group express a CARD domain, which can interact directly with other functional proteins containing CARD domains. The NLRP-group contains a pyrin domain (PYD) that can interact with an adaptor protein consisting of a PYD and a CARD domain, which in turn connects the receptor with additional CARD-expressing effector proteins. The NLRB-group instead has a baculovirus inhibitory domain, and the NLRX group consist of proteins with a variety of N-terminuses that do not fit in the other groups. Among with two members in the dsDNA binding pyrin and HIN200 domain-containing protein (PYHIN) family, several, but not all, NLRs have the ability to form a large, multimeric structure called the inflammasome[81], which has the ability to cleave pro-caspases into their active form. Activation of caspase-1 can mediate inflammatory cell death and cleavage of pro-IL-1β and pro-IL-18 to their active inflammatory forms.[82] So far, no actual interaction between NLR and ligand has been demonstrated, and NLRs are not properly classified as receptors. However, several DAMPs have been shown to activate NLRs and inflammasome formation. It is hypothesized that NLRs are sensitive to changes in the cellular milieu.[83]

Examples of inflammasome forming NLRs are NLRP3 and NLRC4, which are expressed in myeloid and hematopoietic cells, respectively.

Generally, NLRP3 sense self-molecules like adenosine-5′-triphosphate (ATP), cholesterol crystals and monosodium urate (MSU) microcrystals if they are present in an erroneous compartment, such as extracellular ATP.[84]

Exogenous crystals and particles, such as asbestos and silica, can also induce NLRP3 activation, as well as the adjuvant Alum.[84] Microbial components have also been shown to activate the NLRP3 inflammasome, but often in combination with other NLRs, such as NLRC4. Additional structures that activate NLRC4 are the bacterial protein flagellin and the bacterial type III secretion system.[85] A receptor that mediates inflammasome formation upon recognition of dsDNA is the PYHIN family member AIM2, which is activated in cells infected with vaccinia virus and Francisella tularensis, but also in the presence of genomic dsDNA in the cytosol.[86]

4.2 ANTIGEN PRESENTATION

One functional feature that defines DCs is their high capacity to capture, process and present antigens.

Immature DCs endocytose avidly through a variety of mechanisms, including “nonspecific” uptake by constitutive macropinocytosis and “specific” uptake via receptor-mediated endocytosis and phagocytosis[87], such as FcγRII- and mannose receptor-mediated uptake of antigen-antibody complexes[88] or mannosylated, fucosylated antigens[89] respectively.

Internalized antigens are transported normally to late endosomes and lysosomes, loaded onto MHC class II molecules (MHCII), and then presented efficiently to $CD4^+$ T cells.

4.2.1 MHC MOLECULES

Major Histocompatibility Complex (MHC) are special inherited, highly polymorphic protein that play a pivotal role in eliciting an immune response, as they display antigenic peptides for the recognition of T cells.

MHC class I molecules are expressed by all nucleated cells and present protein fragments of cytosolic and nuclear origin at the cell surface. The bulk of fragments that will be loaded on MHC class I molecules are generated by the proteasome; the resulting peptides are translocated into the endoplasmic reticulum (ER) by transporter associated with antigen presentation (TAP) to access MHC I. In the ER, the MHC class I heterodimer is assembled from a polymorphic heavy chain and a light chain called β2-microglobulin. A peptide is the third component required for stability, as it inserts itself deeply into the MHC class I peptide-binding groove, which accommodates peptides of 8-9 amino acids. Without peptides, MHC class I molecules are stabilized by ER chaperone proteins such as calreticulin and tapasin. Tapasin interacts with TAP, thereby coupling peptide translocation into the ER with peptide delivery to MHC I molecules. When peptide binds to MHC I, the chaperones are released and fully assembled peptide-MHC class I complexes leave the ER for presentation at the cell surface.[90]

The MHC class I pathway is designed to present peptides derived from proteins of viruses degraded mainly in the cytosol, that are recognized by $CD8^+$ T cells.

MHC class II molecules are primarily expressed by DCs, macrophages and B cells. They are heterodimeric transmembrane glycoproteins formed by α and β chains that are assembled in the endoplasmic reticulum and associated with an invariant chain. The resulting MHC-invariant chain complex is transported to a late endosomal/lysosomal compartment; the invariant chain is digested, leaving a residual class II associated peptide (CLIP) in the peptide-binding groove of the heterodimer. CLIP is removed by a chaperone protein and peptides are loaded onto MHC II; the complex is transported via vesicles on the plasma membrane to present their peptide cargo to $CD4^+$ T cells. Peptides that are displayed are derived from intracellular proteins. In DCs, this transportation is regulated by the maturation state, which induce higher levels of surface expression of MHC II.[91] Peptides presented on MHC II include exogenous material that is endocytosed from the extracellular environment, and also endogenous components; the cellular turnover implies that intracellular proteins, are segregated within a membrane-bound compartment, and are then fused to a primary nascent lysosome and their contents digested, in a process called autophagy. The signal that provides the routing to the lysosomes is ubiquitination, a post-translational modification which “tag” the proteins to be degraded and its fragments are loaded on MHC II molecules.[92]

In the human system, MHC class I heavy chains are encoded by three highly polymorphic genes, named Human Leukocyte Antigen (HLA) , HLA-A, HLA-B and the rare HLA-C.

Human MHC class II molecules are encoded by HLA-DR, HLA-DP and HLA-DQ genes.

On a first approximation, all DCs efficiently present peptide antigens on their MHC class I and II molecules, as they express high levels of these molecules. Therefore, the question is not whether the DC types differ in their capacity to generate peptide-MHC complexes, but whether they differ in their ability to incorporate peptides that are derived from a given antigen into their presentation pathways.[93] Different DC subsets can differ on the way they capture exogenous antigens[94] and on the expression levels of components of the MHC machinery.[95]

Moreover, another difference relies on the property of some DC subsets to cross-present antigens.

4.2.2 CROSS-PRESENTATION

In the late 1970s, Michael Bevan discovered a new mechanism of antigen presentation, termed cross-presentation[96], which permits some form of extracellular antigens to stimulate $CD8^+$ T cells via the MHC class I pathway, rather than to be loaded on MHC II.

Two main intracellular pathways for cross-presentation have been reported and are referred to as “cytosolic” and “vacuolar”. The first one requires the transfer of exogenous antigens into the cytosol of DCs and the maintenance of an alkaline pH in the phagosomes, thereby limiting the destruction of antigens that are than loaded onto MHC class I molecules. In the vacuolar pathway, exogenous antigens

are degraded directly in endocytic compartments by lysosomal proteases and loaded onto MHC class I molecules by endosomal peptidases. Studies with proteasome[97] or endosomal acidification[98] inhibitors suggests that both the cytosolic and vacuolar pathways for cross-presentation exist in human DCs, but the preferential use of one pathway might depend on the form of antigen. (Figure 4).

Many types of protein antigens have been reported to be cross-presented, including nuclear[99], cytoplasmic and cell surface[100], foreign[101] and self[100], as well as viral[102], bacterial[101] and eukaryotic proteins.[103] Moreover, cross-presentation is important for immunity against tumor antigens[104] and against viruses that don't infect DC.

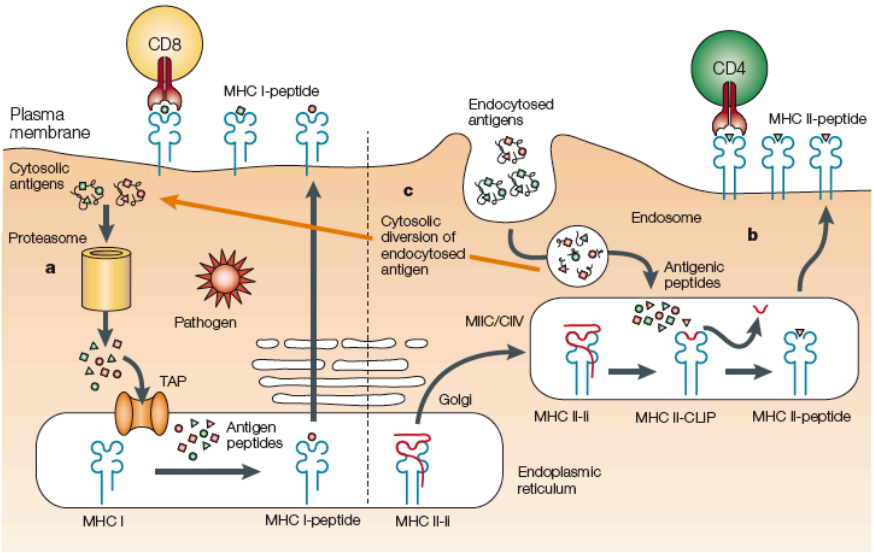

Figure 4. Different antigen-processing pathways for MHC class I and II molecules
a) MHC class I molecules present peptides that are primarily derived from endogenously synthesized proteins of either self or pathogen origin. b) MHC class II molecules present proteins that enter the cell through the endocytic route. c)Dendritic cells can endocytose antigens from other cells and cross-present them to $CD8^+$ cytotoxic T lymphocytes.
From Heath and Carbone, Nat Rev Immunol, 2001

Recent findings have shown different cross-presenting capacity by different subsets, both in mice that in human, as will be discussed further.

5. DC AND T CELL INTERACTIONS

As APCs, dendritic cells exert their primary function by activating both naïve $CD8^+$ and $CD4^+$ T cells in secondary lymphoid organs.

5.1 DC MATURATION

The two well-established maturation states for DCs include the "immature" and "mature" states. Immature, conventional DCs display a phenotype reflecting their specialized function as antigen-capturing cells. They are highly endocytic, able to acquire fluid-phase antigens by macropinocytosis, take up protein or antigen-antibody immune complexes by receptor-mediated endocytosis, and ingest entire cells by phagocytosis. They express relatively low levels of surface MHC-I and MHC-II gene products and costimulatory molecules such as CD80, CD86 and CD40.[105] Although immature DCs can capture antigens, they are unable to process and present them efficiently to T cells; in their immature state, they display self antigens, or protein fragments of dying cells undergoing normal cell turnover, that together with low levels of costimulatory molecules lead to anergy or apoptosis of antigen-specific T cells.[106]

Once DC has been pathogen-primed, DC mature and become immunogenic in that they express cell surface molecules important for T cell activation.

Maturation of DCs is associated with reduced antigen uptake through loss of antigen receptors and down-regulation of macropinocytosis and phagocytosis. However, maturation is also associated with increased efficiency in antigen processing and increased half-life of surface-expressed MHC-peptide complexes. Changes induced also cytoskeleton re-organization, secretion of chemokines, cytokines and proteases, and surface expression of adhesion molecules and chemokine receptors that unable DC to migrate from the peripheral tissues to secondary lymphoid organs.

In particular, mature DCs become particularly sensitive to CCL19.[107] They also lose cell surface expression of CCR1, CCR5 and CCR6, down-regulate CXCR1 and up-regulate expression of CXCR4 and CCR7.[107] The up-regulation of CCR7 promotes responsiveness to CCL19 and CCL21.[108,109] CCL21, a potent chemokine for mature DCs and naïve T cells, leads to co-localization of these two cell types in T cell zones of secondary lymphoid organs leading to cognate T cell activation.[110]

In the secondary lymphoid organs reside naïve T cells, that are activated only following specific antigen recognition and whose fate is determined by three signals that are provided by mature DCs. As shown in Figure 6, the stimulatory signal 1 results from the ligation of the T cell receptor (TCR) to the peptide-loaded MHC class I or class II molecules, determining the antigen specificity of the response. Signal 2 is provided by the costimulatory molecules that are upregulated on the surface of DCs upon maturation (e.g. CD80/CD86/CD40).

The third signal is constituted by the cytokines that are produced upon DC pathogen-induced maturation, which expert a pivotal role in the polarization of T cell responses. (Figure 5).

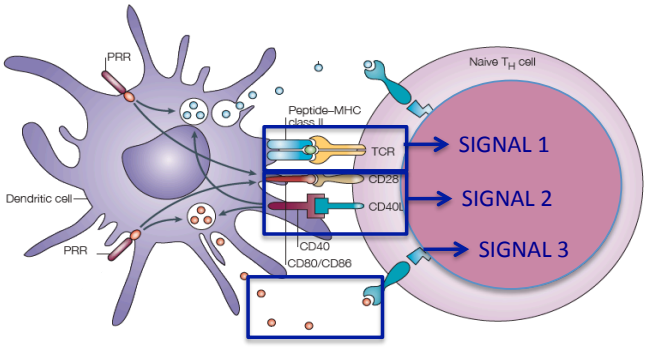

Figure 5: Signals driving T cell activation
Three signals required for a efficient T cell activation
Adapted from Kapsenberg, M., Nat Rev Immunol, 2003

In addition to the nature of the maturation stimulus, the kinetics of activation can influence the capacity of DCs to induce different types of T cell responses. It has to be underlined that cytokines and chemokines are produced with different kinetics and indicate that they might act sequentially in different microenvironments. In addition, secreted cytokines can regulate the production of other cytokines in an autocrine or paracrine fashion. Moreover, another key player in the DC-induced T cell polarization is the duration of T cell-DC interaction: sustained TCR stimulation is required to drive polarization. [111]

5.2 CYTOKINES PRODUCED BY DENDRITIC CELLS

As discussed above, DC maturation induces them to secrete cytokines, that vary among different subsets, both in mice that in humans.

Here below a list of the principal cytokines produced by DCs.

5.2.1. IL-12p70 and IL-23

IL12 family of cytokines comprises IL-12 and IL-23, which share the same subunits.

IL-12 is a cytokine composed of two disulfide-link subunits designated p35 and p40[112]; the genes encoding the subunits are located on different chromosomes (3 and 5 in humans and 6 and 11 in mice), therefore protein expression is independently regulated. While p35 is expressed ubiquitously and constitutively at low levels, p40 expression is limited to microbial infection. In any case, the biological form is the heterodimer p35-p40, known as p70[113], whose generation is limited by p35 levels. Bioactive IL-12p70 is produced by monocytes, macrophages, neutrophils and dendritic cells. Its production depends on the synergistic triggering of both TLR4 and TLR 7-8 by LPS and R848[114]. It is positively regulated by IFNγ[115] and by CD40L.[116] After stimulation, IL-12 is produced only transiently. Its availability in the lymph nodes will be dependent on a continuous flux of recently activated DCs from the inflamed tissues. [111]

IL-12p70 exerts biological effects on both innate and adaptive immune compartments: on the one hand, IL-12 initially induces IFNγ production by NK cells, which increase the cytotoxic activity of phagocytic cells, thereby boosting the innate immune response

On the other hand, it induces proliferation and polarization of naïve T cells towards a IFNγ producing $CD4^+$ T cells, Th1 cells, and $CD8^+$ cytotoxic T cells.

IL-23 cytokine is a heterodimeric protein composed of the p40 subunits of IL-12 covalently linked to p19 subunit. IL-23, alike IL-12, drives Th1 polarization but it acts at a late phase. Moreover, IL-23, differently from IL-12, has a role in the induction of Th17 cells. When IL-12 is produced, it seems to antagonize the Th17 cell formation. The key point of the differential production of these cytokines seemed to be the different response to TLRs.[117]

5.2.2. IL-10

IL-10 is a homodimer protein also known as human cytokine synthesis inhibitor factor (CSIF) and is produced to a great extent by monocytes and dendritic cells, and to a lesser extent by T cells. IL-10 is a cytokine with pleiotropic effects in immunoregulation and inflammation. It downregulates the expression of pro-inflammatory cytokines and IL-12, MHC class II antigens, and co-stimulatory molecules on macrophages. It also enhances B cell survival, proliferation, and antibody production. IL-10 can block NF-kB activity, and is involved in the regulation of the JAK-STAT signaling pathway.

5.2.3. INTERFERONS

Interferons (IFNs) are pleiotropic cytokines that are expressed by a variety of nucleated cells primarily in response to stimulation of a variety of PRRs. IFN enhances cellular killing of viruses by stimulating expression of anti-viral IFN-stimulated genes (ISG),

and indirectly through autocrine loops. IFNs are classified into three classes: type I IFNs, IFNα and IFNβ, type II IFN, IFNγ, and type III IFN or IFNλ.

Interferons are produced by very different type of cells. Among DCs, plasmacytoid DC are the producers of IFNα following TLR9 stimulation by CpG motifs, while conventional murine $CD8\alpha^+$ and human $BDCA3^+$ DC are the major producers of IFNλ by TLR3 triggering.

IFNλ consists of three different isoforms, IFNλ1 (IL-29), the highly identical (>95% sequence identity[118]) IFNλ2 (IL-28A) and IFNλ3 (IL-28B), and the newly discovered IFNλ4.[119]

IFNα act in an autocrine fashion, up-regulating molecules involved in antigen processing, and presentation (MHC I,II) as well as costimulatory molecules and chemokine receptors (e.g. CCR7), thereby increasing antigen-specific $CD4^+$ and $CD8^+$ T cell differentiation. Moreover, IFNα promote IFNγ production by CD8 T cells in a STAT4-dependent manner[120] and promote effector functions of CD8 T cells[121], with antiviral properties.

IFNλ has attracted the attention of numerous scientists, due to its potent antiviral activity, especially in the clearance of Hepatitis C virus (HCV). In patients chronically infected with hepatitis C virus (HCV) genotype 1, GWAS studies identified polymorphisms nearby *interleukin-28B (IL28B)* as important predictor of successful responses to frontline therapy, pegylated interferon (PEG-IFN) and ribavirin (RBV) therapy, as well as of spontaneous clearance of HCV.[122]

Moreover, very recently, it was shown that the polymorphism of IFNλ4 is in high linkage disequilibrium with that of near IL28B, and more strongly associated with spontaneous or treatment-induced HCV clearance than IL28B genotypes, especially in individuals of African ancestry. [123] Current studies are done in order to identify the polymorphism more strongly associated with the outcome of the disease and therefore more likely to be the functional variant.

5.2.4. IL-6

Interleukin-6 is a cytokine produced by DC, macrophages and T cells which exerts pro-inflammatory roles, e.g. during infection and after trauma, especially burns or other tissue damage leading to inflammation[124]. IL-6 also plays a role in fighting infection, as IL-6 has been shown in mice to be required for resistance against bacterium *Streptococcus pneumoniae*.[125]

5.2.5. IL-1β

The Interleukin 1 family (IL-1 family) is a group of 11 cytokines, which induces a complex network of proinflammatory cytokines and via an expression of integrins on leukocytes and endothelial cells, regulates and initiates inflammatory responses.

IL-1β s synthesized as a precursor form protein only after stimulation of innate immune cells; through a complex cleavage[126], the active protein is released into the local environment; IL-1β enhances expansion and effector functions of antigen-specific CD4 and CD8 T cells, both in mouse and in human system.[127]

5.3 T CELL POLARIZATION

Conventional T cells bear a T cell receptor composed of an α- and β-glycoprotein chain (TCR$\alpha\beta$) and recognize antigen in association with MHC molecules displayed on the surface of APCs. DCs are the only cell able to induce the differentiation of naïve T cells, in other words they are able to "prime" naïve T cells, which have higher activation threshold compared to effector T cells.[128] Mature TCR$\alpha\beta$ T cells can be subdivided into functionally distinct populations expressing the membrane proteins CD4 or CD8.

The differentiation to various effector and memory T cell subsets, such as Th17 or Treg cells, is finely tuned and strongly depends upon the strength of stimulation the TCR receives through its TCR interacting with the MHC-peptide complexes and upon the cytokine milieu. The strength of the T cell stimulation is determined in turn by factors such as the concentration of the antigen[129], the presence or absence of co-stimulation, which regulates the extent of signal amplification, and the duration of the interactions between T cells and DCs, which determines the duration of the signaling process. Thus, T cells accumulate signals that drive T cell differentiation by converging in the regulation of transcriptional programs, leading to the different T cell fates.

5.3.1 CYTOTOXIC $CD8^+$ T CELLS

Cytotoxic lymphocytes are characterized by the expression of the CD8 glycoprotein on their surface, as well as by the expression of a T cell receptor (TCR) that specifically recognize peptides loaded on MHC class I molecules.

This feature enables them to become activated by DC displaying fragments of intracellular-invading pathogens, as well as tumor peptides; DC that can cross-present are required to prime cytotoxic T cells that recognize proteins derived from necrotic cells.

A DC bearing such characteristics will promote the differentiation of a naïve $CD8^+$ T cell into cytotoxic T lymphocyte (CTLs) that kill the antigen-infected cells through the secretion of granzymes and perforin, that act together forming pores within the membrane of the target cell, leading to the entry of the granzymes and the apoptosis of the target cell.[130]

$CD8^+$ T cells also elaborate cytokines, including IFNγ and TNFα, as well as chemokines that function to recruit and/or activate the microbicidal activities of effector cells such as macrophages and neutrophils[131]

Dendritic cells subsets that produce IL-12, play a pivotal role in the differentiation of $CD8^+$ into cytotoxic T cells, able to produce high levels of IFNγ, as well as granzymes and perforin.[132]

5.3.2. HELPER $CD4^+$ T CELLS

Naïve $CD4^+$ T helper (Th) cells can undergo different fates depending on the context in which they encounter their specific antigen. Twenty-five years ago, Mosmann and Coffman proposed a paradigm[133], postulating that CD4+ T cells can polarize towards Th1 or Th2 cells which produce different sets of cytokines and mediate protection from intracellular or extracellular pathogens respectively, or may be involved in B cell help.[134] Th1 express T-bet as master transcription factor; they are specialized for the secretion of IFNγ and

lymphotoxin, and are responsible for directing cell-mediated immune responses leading to the eradication of intracellular pathogens, whereas Th2 cells, driven by GATA3 transcription factor, produce IL-4, IL-4, IL-10 and IL-13, mediating protection against extracellular parasites and helminthes.

It is widely accepted that IL-12 and IL-4, produced by the innate arm of the immune system, are key determinants in promoting Th1 and Th2, respectively. It is also known that cytokines produced by different Th subsets can cross-regulate each other function, for example IFNγ by Th1 inhibits Th2 development , whereas IL-10 and IL-4 dampen Th1.[135]

The Th1/Th2 paradigm has been challenged by the discovery of a new subsets of CD4+ T helper cells, so called Th17. They produce IL-17 and exhibit effector functions distinct from Th1 and Th2 cells. The primary function of Th17 cells appears to be the clearance of pathogens that are not adequately handled by Th1 or Th2 cells. However, Th17 cells are potent inducers of tissue inflammation and have been associated with the pathogenesis of many experimental autoimmune diseases.

Production of IL-17 is driven by exposure of naïve cells to different cytokines combinations such as TGFβ, IL-21, IL-1β; IL-6 and the newly discovered cytokine IL-23. [136,137] These cytokines induce the upregulation of RORγT transcription factor and the subsequent production of IL-17.

Mature dendritic cell subsets produce most of the cytokines over mentioned, thereby suggesting a role in skewing towards IL-17 phenotype.

5.3.3. T REGULATORY CELLS

T regulatory cells (Treg) are a specific subset of $CD4^+$ T cells able to suppress T cell responses, maintaining tolerance to self antigens and preventing autoimmune diseases. They express Foxp3 transcription factor and their development is dependent on the presence of TGFβ; notably, addition of IL-6 to TGF-β inhibits the generation of Tregs and induces Th17 cells. On the basis of these data, Kuchroo and colleagues put forward the idea that there is a reciprocal relationship between Tregs and Th17 cells and that IL-6 plays a pivotal role in dictating whether the immune response is dominated by proinflammatory Th17 cells or protective Treg.

As it has been described so far, the immune system shows a remarkable functional plasticity, both in the innate, as well as in the adaptive compartment. Each DC subset has unique biological functions, determining the outcome of DC-T cell contact towards immunity or tolerance.

5.4 CD4 HELP

An efficient $CD8^+$ T cell priming is the outcome of an excellent priming but relies also on the cooperation of $CD4^+$ T cells, the so called “CD4 help”.

The help provided by $CD4^+$ T lymphocytes during the priming of $CD8^+$ T lymphocytes confers a key feature of immune memory: although short-term antigen stimulation by DC might be sufficient to trigger a program of CTL differentiation that includes multiple rounds of division, effector function and contraction, a signal from $CD4^+$ T cells is required to program the final step of differentiation onto

functional memory cells that are capable of rapidly recall response.[138,139] A lot of studies have been done in order to understand which are the mechanisms for CD4 help and the most prominent one is the CD40-CD40L signaling pathway. Findings have established a DC licensing model: DC capture extracellular antigen, process the antigen for MHC class II-restricted presentation to CD4+ T cells, which in turn upregulate CD40L expression and, through interaction with CD40, activate or license the DC to stimulate the response of naïve CD8+ T cells that recognize processed, MHC class-I restricted antigen on the DC.

Other mechanisms of CD4 help, such as the role of cytokines produced by these cells (IL-2 or IFNγ) are currently investigated, due to contrasting results obtained. Schoenberger and colleagues have proposed that IL-2 produced by CD4 T cells is able to "help" effector $CD8^+$ T cells by downregulating the expression of the TRAIL, thereby inhibiting apoptosis.[140]

6. FUNCTIONAL DIFFERENCIES BETWEEN HUMAN AND MOUSE DCs

Intensive studies on mouse DC system clearly showed different subset-specific functions, principally due to the different pattern of receptors expressed on different DC subsets, as discussed above.

While human and mouse pDC are very similar in functions, a very important finding was the discovery of mouse conventional $CD8\alpha^+$ DCs as the subset able to produce high levels of IL-12 and IFNλ and excellent in the cross-presentation of extracellular antigens;

a subset with these characteristics induces a potent $CD8^+$ T cell response and can be a target for anti-HCV therapies; genome-wide association studies (GWAS) recently revealed that certain interleukin-28B (IFNλ3) polymorphisms are strongly associated with responses to therapy in patients chronically infected with hepatitis C virus (HCV) genotype 1, as well as with spontaneous clearance of HCV.[123]

In 2010, different groups published they have found the human analogue of mouse $CD8\alpha^+$DCs: $BDCA3^+$ DC (mDC2).

They showed the two subsets shared the expression of the c-type lectin receptor Clec9A, important for the uptake of necrotic cell components, and the chemokine receptor XCR1. Analysis on murine and human DC systems showed that XCR1 was expressed only in splenic $CD8\alpha^+$DCs and in human $BDCA3^+$ DCs, suggesting these two subsets correspond in murine and human DC system. The ligand for XCR1 is XCL1 and is secreted by NK and $CD8^+$ T cells; $BDCA3^+$ DCs respond to XCL1 by mobilization of intracellular $Ca2^+$ and strong chemotaxis, thus ensuring the contact between DC and NK or $CD8^+$ T cells. XCR1- mediated contact with $CD8^+$ T cells, together with the cross-presentation ability, lead mDC2 to mount an efficient immune response against certain pathogens. [141]

Not only surface markers were shared, but also they retained the same functions, such as IL-12 production and excellent cross-presentation capacity.[53,67,141,142]

In the last year it was emerging the idea that also human $BDCA1^+$ DCs can retain the ability to cross-present exogenous antigens.[143,144] Segura and colleagues showed that human $BDCA1^+$ DC, and also pDC subsets display similar phagosomal pH and efficiently export

internalized protein to the cytosol, all requirements for efficient cross-presentation. To be noted that in the mouse system, these features are restricted to $CD8\alpha^+$ DCs.

7. SCOPE OF THE THESIS

Dendritic cells (DCs) are a heterogeneous population of professional antigen presenting cells that have the unique capacity to initiate adaptive immune response and are thus the physiological inducers of T cell differentiation. The aim of this work is to investigate the functional properties of *ex-vivo* isolated human dendritic cell subsets, in terms of cytokine production and ability to prime naïve T cells, to better understand their role in T cell responses. BDCA3$^+$ DC are thought to be the human orthologue of mouse CD8α$^+$ DC, which efficiently cross-present antigens to naïve CD8$^+$ T cells and produce high levels of IL-12, and are thus potent inducers of cytotoxic T cell responses. We therefore wanted to address if BDCA3$^+$ DC in humans were also specialized to induce cytotoxic T cells, and if BDCA1$^+$ DC might be more important for CD4$^+$ T cell priming.

More in detail we wanted to investigate if:

i. human DC subsets produce different types of cytokines in response to different TLR ligands;
ii. human DC subsets prime preferentially CD4$^+$ or CD8$^+$ T cells;
iii. the role of DC-derived cytokines IL-12 and IL-10 in T cell differentiation.

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CHAPTER 2

Human CD1c$^+$ dendritic cells secrete high levels of IL-12 and potently prime cytotoxic T-cell responses

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A. ABSTRACT

Dendritic cells (DC) have the unique capacities to induce primary T cell responses. In mice, CD8α^+DC are specialised to cross-prime $CD8^+$ T-cells and produce IL-12 that promotes cytotoxicity. Human BDCA-3^+DC share several relevant characteristics with CD8α^+DC, but the capacities of human DC subsets to induce $CD8^+$ T cell responses are incompletely understood.

Here we compared $CD1c^+$mDC1, BDCA-3^+mDC2 and plasmacytoid DC (pDC) in peripheral blood and lymphoid tissues for phenotype, cytokine production and their capacities to prime cytotoxic T cells.

mDC1 were surprisingly the only human DC that secreted high amounts of IL-12p70, but they required combinational Toll-like receptor (TLR) stimulation. mDC2 and pDC produced IFN-λ and IFN-α, respectively. Importantly, mDC1 and mDC2 required different combinations of TLR-ligands to cross-present protein antigens to $CD8^+$ T cells. pDC were inefficient, and also expressed lower levels of MHC- and co-stimulatory molecules. Nevertheless, all DC induced $CD8^+$ memory T-cell expansions upon licensing by $CD4^+$ T cells, and primed naive $CD8^+$ T-cells following appropriate TLR stimulation. However, since mDC1 produced IL-12 they induced the highest levels of cytotoxic molecules.

In conclusion, $CD1c^+$mDC1 are the relevant source of IL-12 for naïve T cells, and are fully equipped to cross-prime cytotoxic T cell responses.

B. INTRODUCTION

Dendritic cells (DC) are professional antigen-presenting cells (APC) that possess the unique capacity to trigger primary adaptive immune responses through the antigen-specific activation of naïve $CD4^+$ and $CD8^+$ T cells[1]. DC are derived from proliferating precursors in the bone marrow that migrate via the blood to lymphoid and non-lymphoid tissues[2]. Immature DC efficiently sample antigenic material, but upon encounter of a pathogen they undergo a complex maturation process that leads to migration to secondary lymphoid organs, cytokine production and enhanced antigen presentation and T cell stimulatory capacities[1]. In particular, the ability to "cross"-present extracellular antigens on MHC class I to $CD8^+$ T cells is important for the priming of cytotoxic T cell responses[3], and this capacity is acquired by DC upon maturation, cytokine signaling and CD40 stimulation by $CD4^+$ helper T cells[4,5].

Two broad subsets of plasmacytoid DC (pDC) and myeloid DC (mDC) with different phenotypes and functions have been identified both in mice and men. mDC respond to bacteria and other pathogens, can secrete IL-12 and induce Th1 responses[6]. Plasmacytoid DC respond to viruses with high IFN-a production[7], can induce Th1 and Th2 responses[8,9] and also cross-present antigens to CTL[10-12]. In the human system, pDC and mDC show complementarities in pathogen recognition[13] and have different migratory behaviors[14]. In particular, pDC respond selectively to TLR9 agonist with IFN-a production, might enter inflamed secondary lymphoid organs via CXCR3[7,15] and induce IL-10 production by T cells via ICOSL[16].

In mice mDC that express CD8a have superior capacities to cross-present antigens to $CD8^+$ T cells in vivo as compared to $CD8a^-$ DC[17,18] and they secrete very high levels of IL-12[19,20]. In humans, BDCA-3^+mDC[21,22] share relevant characteristics with $CD8a^+$DC. Thus, these DC subsets selectively express CLEC9A and XCR1[23-27]. In addition, both mouse $CD8a^+$DC and human BDCA-3^+DC share the dependency on the BATF3 transcription factor for their generation[28], and produce IFN-I[29]. BDCA-3^+DC express also higher levels of TLR3 than $CD1c^+$DC, but lack TLR4[26]. Since BDCA-3^+DC had superior capacities to cross-present antigens and to produce IL-12, it was proposed that BDCA-3^+ DCs are also functionally equivalent of mouse $CD8a^+$DC[30]. However, conflicting results have been published on IL-12 production and cross-presentation of soluble antigens by mDC2 as compared to other DC subsets[23,26,31-34], possibly due to different experimental conditions[3]. Thus, the relative capacities of different human DC subsets to produce IL-12 and to induce $CD8^+$ T cell responses remain a highly relevant open question.

Here we phenotypically and functionally characterized human $CD1c^+$mDC, BDCA-3^+mDC and pDC in peripheral blood as well as in the bone marrow and tonsils, lymphoid tissues where DC are respectively generated and present antigens to T cells. We show that $CD1c^+$mDC1, but surprisingly not BDCA-3^+mDC2, can secrete high levels of IL-12. In addition, we demonstrate that both myeloid DC subsets can efficiently cross-present soluble antigens and prime cytotoxic T cells, indicating that cross-priming is not an exclusive feature of BDCA-3^+mDC2 in humans.

C. METHODS

1. Mononuclear cells from human tissues

Human bone marrow samples (kindly provided by M. Lösch, Department of Anaesthesiology, Intensive Care and Analgesia, and H. Kienapfel, Department of Orthopaedics, Auguste-Viktoria-Klinikum, Charité, Berlin, Germany) were obtained from patients undergoing hip arthroplasty (THA), tonsils specimens surgically removed from pediatric patients and buffy-coat blood of healthy donors provided by IRCCS Policlinico Ospedale Maggiore, Milan, Italy and by Charité Hospital, Berlin, Germany. BMMC (Bone Marrow Mononuclear Cells,), PBMCs (Peripheral Blood Mononuclear Cells) and TMCs (Tonsillar Mononuclear Cells) were isolated by Ficoll-Hypaque gradient (Sigma-Aldrich), according to standard methods.

The ethical committee approved the use of PBMCs, BMMCs and TMCs for research purposes (permissions for BM: EK-No 208-13 and for TMC EA1/107/10) and informed consent was obtained from the subjects involved in this study. Cells were cultured in complete RPMI1640 (EuroClone) containing 10% FCS (EuroClone) or 5% Human Serum (EuroClone), 0.1 % Penicillin/Streptomycin (EuroClone), 0.1 % non-essential amino acids (Lonza) and 0,1% Sodium Pyruvate (Lonza) at 37°C and 5 % CO_2.

2. DC isolation

DC subsets were isolated from BMMC, PBMC and TMC by magnetic enrichment followed by cell sorting. Briefly, cells were incubated with anti-CD1c-FITC (AD5 8E7), anti-BDCA-3-APC (AD5 14H12) and anti-BDCA-4-PE (AD5 17F6, Miltenyi Biotech) antibodies,

non-specific binding to Fc receptors was prevented using FcR blocking reagent (Miltenyi Biotech), and DC were magnetically labeled with anti-FITC, -PE and-APC beads and enriched on columns. The positive fraction was incubated with the lineage markers anti-CD3 (SK7, BioLegend), -CD14 (61D3), -CD16 (CD16), -CD19 (HIB19), -CD56 (B159, e-Bioscience) and -CD11c (3.9, BioLegend). mDC1 were sorted as lineage$^-$CD11c$^+$ CD1c$^+$, mDC2 as lineage$^-$CD11c$^+$BDCA-3^{hi} , pDC as lineage$^-$CD11c$^-$BDCA-4$^+$; in some experiments DC were sorted in addition as HLA-DR$^+$ cells. Purity of all DC subset was >95%. Monocyte subsets were sorted directly from peripheral blood as lymphocyte lineage$^-$ cells (CD19-, CD56- and CD3$^-$) according to CD14 and CD16 or SLAN (DD-1, Miltenyi Biotech) expression.

3. Flow cytometry

Phenotypical analysis was performed by gating DC as lineage$^-$ HLA-DR$^+$ cells and DC subsets according to CD1c, BDCA-3 or BDCA-4 expression. DC were then analyzed for HLA- ABC (B9.12.1, IOTest), HLA-DR (G46-6, BD), CCR5 (3A9, BD), CXCR3 (1C6, BD), ICOS-L (MIH12, eBioscience), CD40 (5C3, BD) and CD86 (2331, BD) expression. Production of IFNγ or cytotoxic molecules in CD8$^+$ T cells was assessed by intracellular staining with antibodies specific for IFNγ (F4S.B3, eBioscience), Granzyme-B (GB11, BioLegend) and Granzyme-K (GM6C3, SantaCruz) according to a standard protocol. Samples were analyzed on a FACSCanto flow cytometer (BD) using FlowJo software (Tristar).

4. DC cytokine production

Sorted DC or monocyte subsets were seeded at 10^4 cells/well in a 96–well plate for 24 hrs with either 100ng/ml LPS (Sigma), 100ng/ml polyI:C (Alexis), 10 μg/ml CpGA (Alexis), 2,5 μg/ml R848, (Alexis), 10ng/ml IFN-g (R&D) or 1ng/ml IL-4 (R&D) or a CD40L–transfected murine cell line (ratio 1:1, JSSB, kindly provided by A. Lanzavecchia, IRB, Bellinzona, Switzerland). Cell-free supernatants were analyzed by ELISA, according to the manufactures' guidelines. ELISAs for IL-12p70 and IFN-λ were from R&D, and IFN-α2a from eBioscience.

5. $CD8^+$ T cell stimulation

$5x10^4$ CFSE-labeled naïve $CD8^+$ T cells with or without $5x10^4$ autologous naïve $CD4^+$ T cells were co-cultured with 10^4 allogeneic purified DC or monocyte subsets for 7 days. DC were stimulated with 100ng/ml LPS (mDC1), 100ng/ml polyI:C (mDC1 and mDC2), 10mg/ml CpGB (pDC, Alexis) with or without 2,5mg/ml R848. T cell priming was assessed by CFSE dilution and calculated as the percentage of divided $CD8^+$ T cells. IFN-γ production was detected by intracellular staining after 4 hours of PMA/ionomycin stimulation; 10 μg/ml Brefeldin A (Sigma) was added for the last 3 hours of stimulation. Cells were stained with labeled antibodies for CD8 and the percentage of $CFSE^{lo}$ $CD8^+$ T cells producing IFN-g or expressing cytotoxic molecules was analyzed. In some experiments, either 1ng/ml recombinant IL-12 or 5 μg/ml neutralizing anti-IL-12 (R&D systems) were added.

6. Cross-presentation

CMV-pp65$_{495-503}$ specific CD8$^+$ T cell lines were generated from HLA-A2$^+$ CMV$^+$ donors by sorting HLA-A2 CMV-pp65$_{495-503}$ dextramer$^+$ (Immudex) CD8$^+$ T cells and expansion with allogenic irradiated PBMC, an EBV cell line, soluble anti-CD3 antibodies (OKT3 30 ng/ml) and 100 U/ml of IL-2. CMV-specific T cell lines were obtained from two different HLA-A2$^+$ donors and maintained in 100U/ml IL-2. Purified DC subsets from HLA-A2$^+$ donors were co-cultured with CMV-pp65$_{495-503}$ specific CD8$^+$ T cells (ratio DC:T = 1:2) in 96-well round-bottom plate in RPMI medium supplemented with 5% HS, with or without CMVpp65 protein (40mg/ml, Miltenyi Biotech) for 20 hrs. IFN-γ production by pp65-specific CD8$^+$ T cells was measured as the read-out of antigen cross-presentation. Unspecific IFN-g production induced by DC in the absence of pp65 was subtracted, and IFN-g production induced with 1µg/ml CMVpp65$_{495-503}$ peptide was set to 100% for each condition. For the stimulation of CMV-specific memory T cells, we adapted a previously published protocol [35]. CFSE-labeled CD8$^+$ T cells from HCMV$^+$ donors were stimulated with autologous DC subsets and a lysate of CMV-infected cells or in some experiments with recombinant pp65 with or without autologous CD4$^+$ T cells. Proliferation of CD8+ T cells was assessed by CFSE dilution.

7. Statistics

Statistical significance was calculated using two-tailed Student's t test in case of Gaussian distribution, otherwise Mann Whitney for unmatched or Wilcoxon for paired groups. $p < 0.05$ (*), $p < 0.005$ (**) $p < 0.0005$ (***) were regarded as statistically significant. One-way ANOVA test was used to compare statistical significance among 3 different groups.

D. RESULTS

1. Frequency and proliferation of human DC subsets in different tissues

Proliferating murine DC precursors migrate from the bone marrow via the blood to seed secondary lymphoid organs where they continue to divide [2]. To analyze human DC subsets in relevant human tissues (bone marrow, blood and tonsils) we identified DC as MHC class II^+ (HLA-DR) cells that lacked lineage markers. DC were further subdivided into subsets of "conventional" $CD11c^+CD1c^+$ myeloid DC ("mDC1"), $CD11c^+$BDCA-3^+ myeloid DC ("mDC2") and $CD11c^-$BDCA-4^+ plasmacytoid DC ("pDC") (Figure 1A).

The three DC subsets had different frequencies in the analyzed tissues, but in all 3 tissues mDC2 was consistently the rarest population (Figure 1B).

In mice, DC subsets have different turn-over rates[2,36]. We analyzed the in vivo proliferation of human DC subsets by staining for the proliferation marker Ki-67. pDC were largely Ki-67^- in peripheral blood of healthy donors and in tonsils, indicating that they proliferated poorly or not at all. In contrast, mDC1 and mDC2 proliferated in peripheral blood, but not in tonsils (Figure 1C)[27]. In hip arthroplasty patients all three DC subsets proliferated in the bone marrow, but only mDC proliferated in the patient's blood (Figure 1D). In summary, mDC2 represent the rarest DC subset, and have a similar in vivo turnover as conventional mDC1 in blood and lymphoid tissues.

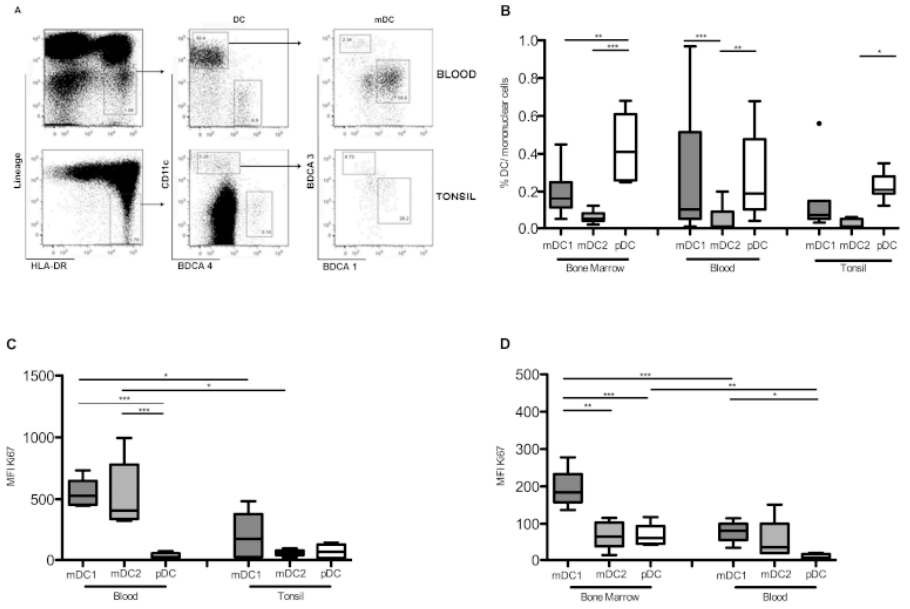

Figure 1. Frequency and proliferation of human DC subsets in bone marrow, peripheral blood and tonsils
(A) Gating strategy for human DC subsets in peripheral blood and tonsils. (B) Frequency of mDC1 (dark grey boxes), mDC2 (light grey boxes) and pDC (white boxes) in total mononuclear cells isolated from bone marrow, blood and tonsils. (C) Mean Fluorescence Intensity (MFI) of the Ki67 proliferation marker in DC subsets from peripheral blood of healthy donors and tonsils. (D) Ki67 expression of DC subsets from bone marrow and peripheral blood of patients that were undergoing hip arthroplasty. Shown are results of six donors in at least two experiments.

2. Surface receptor expression of DC subsets in blood and lymphoid tissues

Expression of MHC and co-stimulatory molecules determines the capacities of DC to stimulate T cells, while chemokine receptors regulate their migration and positioning in tissues. We compared the expression of relevant surface receptors of DC subsets in bone marrow, peripheral blood and tonsils (Figure 2).

While MHC class I was expressed at comparable levels on myeloid DC subsets, pDC expressed lower levels (Figure 2A). MHC class II was expressed at the highest levels by mDC1, at intermediate levels on mDC2 and at the lowest levels by pDC in all tissues (Figure 2B). MHC class II expression was unexpectedly low in tonsils, possibly due to the degradation of surface MHC upon DC contact with activated $CD4^+$ T cells[37]. Conversely, the co-stimulatory molecule CD86 that was also highest on mDC1 and lowest on pDC, was higher in tonsils than in blood (Figure 2C). ICOSL was expressed on pDC but not on mDC1 as expected [16], but the highest levels were expressed on mDC2 in blood and bone marrow, while it was undetectable in tonsils (Figure 2D). Notably, mDC2 also expressed the highest levels of CD40 (Figure 2E). Among chemokine receptors, we found that CXCR3 was undetectable in the bone marrow, but was selectively expressed on pDC and mDC2 in blood and tonsils (Figure 2F). CCR5 expression was highest in the bone marrow, intermediate in peripheral blood and low in tonsils. Moreover, mDC2 expressed the lowest levels of CCR5 in all three tissues (Figure 2G). In summary, each human DC subset has a characteristic, but partially tissue-dependent expression of surface receptors. In particular, pDC express consistently lower levels of MHC molecules and CD86 compared to myeloid DC, indicating that they are less potent to stimulate T cells than mDC.

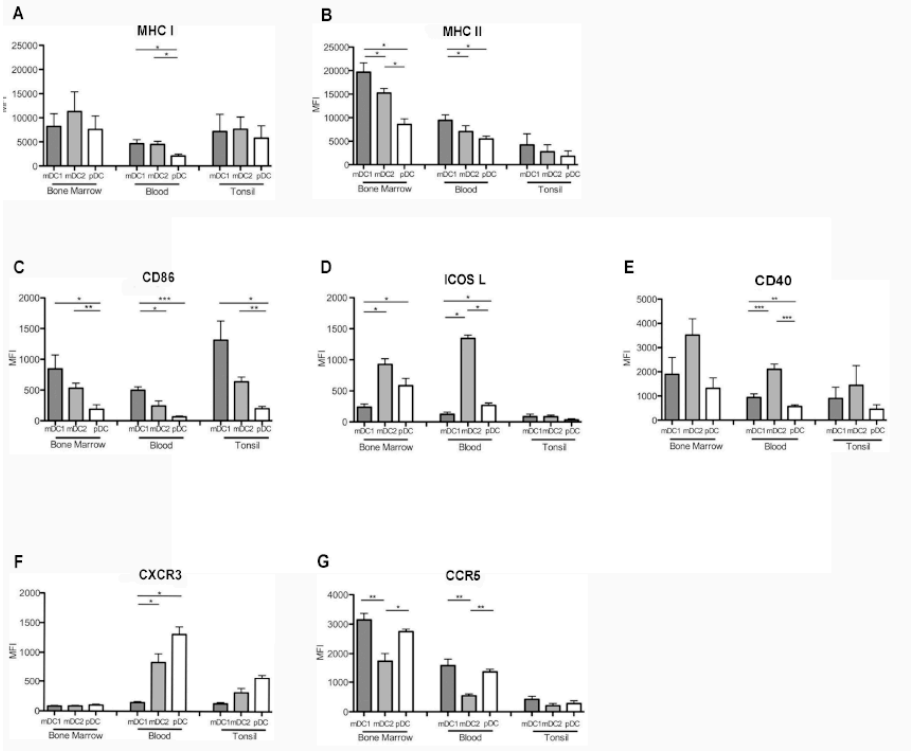

Figure 2. Surface receptor expression on DC subsets in different tissues
Expression of (A) MHC class I, (B) MHC class II, (C) CD86, (D) ICOSL, (E) CD40, (F) CXCR3 and (G) CCR5 on mDC1, mDC2 and pDC in bone marrow, peripheral blood and tonsils. Mean values for at least seven donors in at least three experiments are shown.

3. mDC1 possess high IL-12 producing capacities

Production of bioactive IL-12p70 by DC drives IFN-g production and promotes cytotoxicity in primed naïve T cells and has thus been extensively studied in mouse DCs and in human *in vitro* differentiated monocyte-derived DC. In these DC IL-12 production has a complex regulation and synergistic TLR stimulation, CD40L, IFN-g and IL-4 have been identified as critical factors [38].

However, conflicting results have been published regarding the IL-12 producing capacities of *in vivo* occurring human DC subsets [23,26,31,33,34], possibly due to the different stimulation conditions that were tested.

We found that mDC1 from peripheral blood could produce very high levels of IL-12p70, but required synergistic TLR stimulation by LPS and R848 (Figure 3A). In marked contrast, no IL-12 production was detected by mDC2 stimulated with polyI:C or by pDC stimulated with CpG DNA in the absence or presence of R848 (Figure 3A). IL-12 production by mDC1 was further enhanced by stimulation with IFN-g (Figure 3B) or CD40L (Figure 3C), while IL-4 had surprisingly an inhibitory effect (Figure 3D). Importantly, mDC1 also secreted considerable amounts of IL-12 in response to polyI:C, R848 and IFN-g, while mDC2 produced only very low levels of IL-12 under the same condition (Figure 3E). CD40 stimulation of TLR-activated mDC2 did not increase IL-12 production (data not shown). Notably, IL-12 production by $CD16^+$ and $CD14^+$ monocytes was hardly detectable under the same conditions where mDC1 secreted high levels (supplementary Figure 1). Finally, also mDC1 in tonsils produced detectable IL-12 upon optimal stimulation, while tonsillar mDC2 and pDC did not (Figure 3F). We conclude that mDC1, but not mDC2, are the principal IL-12-producing APC in humans.

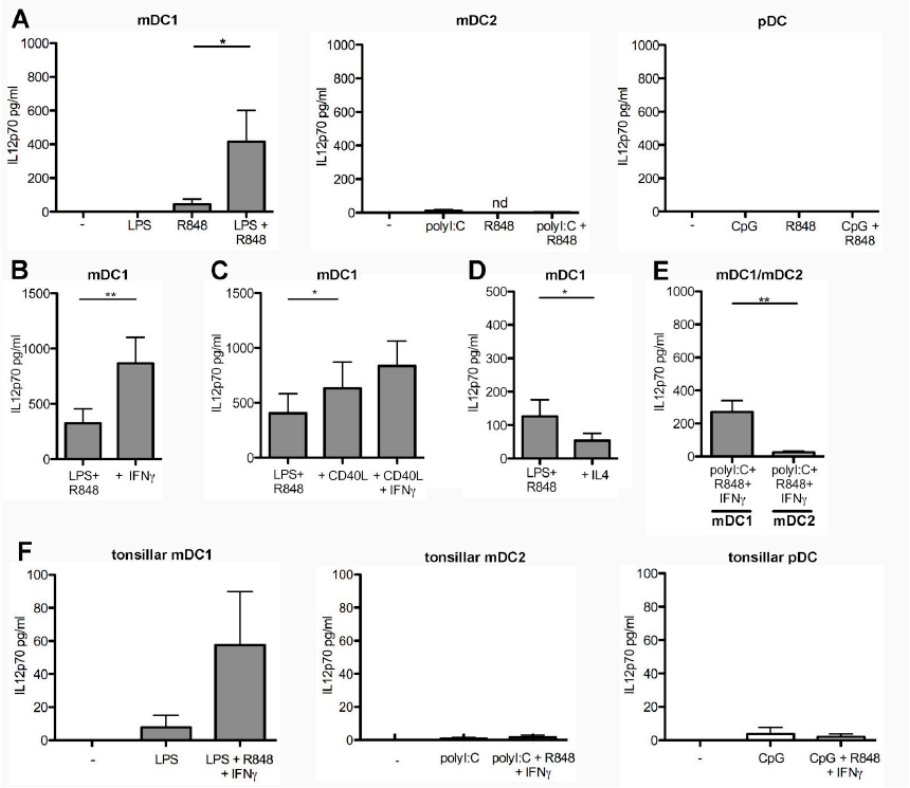

Figure 3. Regulation of IL-12 production by mDC1
(A) IL-12p70 production by peripheral blood DC subsets after 24-h culture in medium alone (-) or in the presence of the indicated TLR agonists. (B) Effects of IFNγ, (C) CD40L and (D) IL-4 on IL12p70 produced by mDC1. (E) IL-12 production by mDC1 (left column) and mDC2 (right column) in response to polyI:C, R848 and IFNγ. Values show the mean of sixteen donors in eight different experiments. (F) IL-12p70 production by DC subsets in tonsils in response to the indicated stimuli. Results from seven donors in five experiments are shown.

4. mDC2 secrete high levels of IFN-λ

Although mDC2 and pDC produced little or no IL-12p70, they could secrete high amounts of IFN-l (Figure 4) and -a (supplementary Figure 2), respectively.

Thus, IFN-λwas secreted at the highest levels by mDC2 (Figure 4A-B) in response to polyI:C. Additional stimulation with R848 and IFN-γ further enhanced IFN-l production by mDC2, while CD40L (data not shown) had no effect. In contrast, pDC secreted only low levels of IFN-l in response to TLR-9 stimulation alone. mDC1 secreted some IFN-l upon stimulation with polyI:C, R848 and IFN-g, but mDC2 secreted much higher levels under the same condition (Figure 4B). Also in tonsils the highest levels of IFN-l were produced by mDC2 (Figure 4C). Finally, IFN-a was as expected exclusively and abundantly produced by pDC both in peripheral blood and tonsils upon TLR-9 stimulation, and it was boosted by CD40 co-engagement (supplementary Figure 2). We conclude that human DC subsets have a specific cytokine profile, and that IFN-l and IFN-a are predominantly or exclusively produced by mDC2 and pDC, respectively.

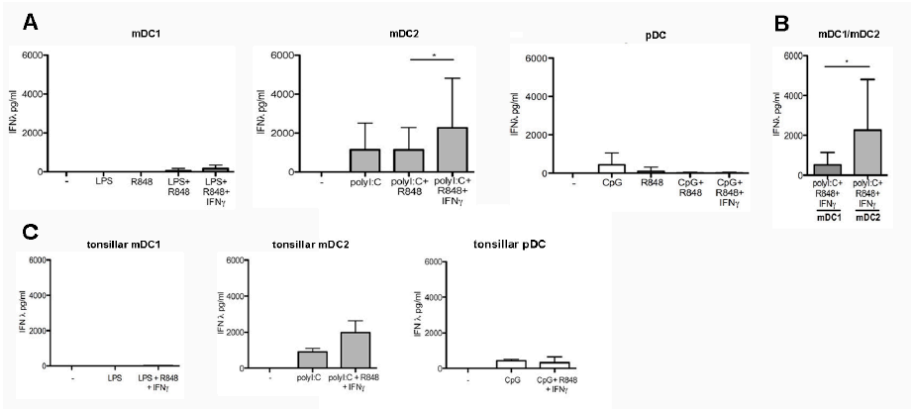

Figure 4. mDC2 secrete high amounts of IFN-λ
(A) IFNλ production by peripheral blood DC subsets after 24-h culture in medium alone (-) or in the presence of the indicated stimuli. (B) IFN-λ production by mDC1 (left column) and mDC2 (right column) in response to polyI:C, R848 and IFN-γ. (C) IFN-λ production by DC subsets in tonsils. Values represent the mean of sixteen PBMC donors in eight different experiments and seven TMC donors in five experiments.

5. mDC subsets efficiently cross-present soluble antigens

mDC2 have been shown to possess superior cross-presenting capacities, but recent reports have questioned this notion. We wondered if these inconsistent results might be due to the different stimulation conditions used[3,39]. To test the cross-presentation capacities of human DC subsets we used the CMV pp65 protein as a model antigen. We stimulated HLA-A2-pp65$_{495-503}$-specific CD8$^+$ T cells with DC subsets from HLA-A2$^+$ donors with soluble pp65 protein. IFN-g produced by the antigen-specific CD8$^+$ T cells was used as a read-out of cross-presentation, and DC loaded with the relevant pp65-derived peptide represented the positive control. As shown in Figure 5A and supplementary Figure 3, cross-presentation by immature DC was hardly detectable, but DC acquired cross-presenting capacities upon TLR stimulation. Thus, low cross-presentation could be detected by mDC1, mDC2 and pDC upon stimulation with respectively LPS, polyI:C or CpG alone. Importantly, mDC2 stimulated with polyI:C and R848 acquired high cross-presentation capacities, while mDC1 were less efficient under the same condition. However, upon stimulation with LPS and R848 also mDC1 cross-presented efficiently. Conversely, cross-presentation by pDC stimulated with R848 in the absence or presence of CpG was undetectable (Fig 5A and data not shown). Moreover, CD40 stimulation of immature DC induced some cross-presentation by mDC1 and mDC2, but not by pDC (Figure 5B). CD40 co-stimulation also increased the cross-presentation capacities of TLR-stimulated DC, but the effect was weak and did not reach statistical significance (data not shown).

In order to address the role of CD4 help in cross-presentation and $CD8^+$ T cell activation in a more physiological system, we measured proliferation of CFSE-labeled $CD8^+$ T cells from CMV^+ Donors with autologous DC subsets with CMV-derived proteins in the absence or presence of autologous $CD4^+$ T cells. All DC induced efficient proliferation of CMV-specific $CD8^+$ T cells when $CD4^+$ T cells were present. In the absence of $CD4^+$ T cells only low $CD8^+$ T cell proliferation was induced in some donors by mDC2 (Figure 5C).

We conclude that both mDC1 and mDC2 can efficiently cross-present soluble protein antigens, but have different requirements for TLR stimulation. In contrast, pDC are less efficient but could nevertheless contribute to secondary expansions of $CD8^+$ memory T cells.

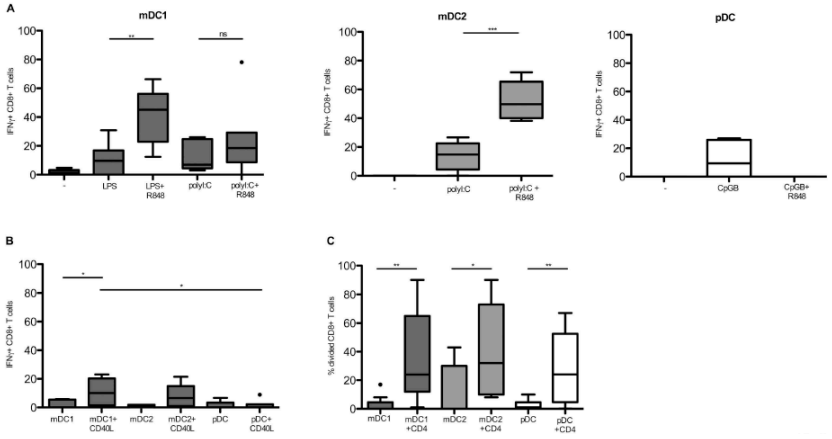

Figure 5. Efficient antigen cross-presentation by TLR-stimulated mDC
DC subsets obtained from HLA-A2$^+$ donors were co-cultured for 20 hours with HLA-A2 pp65-specific CD8$^+$ T cells with recombinant soluble CMV pp65 protein, in the absence (-) or presence of TLR agonists (A) or CD40L-transfectants (B). Shown is the percentage of IFNγ production by CD8$^+$ T cells normalized on IFN-γ production in response to $pp65_{495-503}$ peptide. Data are from seven donors that were analyzed in four experiments. Primary data of one representative experiment is shown in supplementary Figure 3. (C) 5x10^4 CFSE-labeled CD8$^+$ T cells with or without 5x10^4 autologous CD4$^+$ T cells were incubated with autologous purified DC subsets in the presence of CMV-derived proteins and the fraction of proliferating CD8$^+$ T cells analyzed. Shown are results of ten HCMV$^+$ donors in different experiments.

6. DC require CD4 help and TLR stimulation for optimal CD8$^+$ T cell priming

A unique feature of DC is their ability to prime naïve T cells, but the relative capacities of *in vivo* occurring human DC subsets to prime naïve CD8$^+$ T cells have not been analyzed yet. We therefore also assessed the capacities of DC subsets to prime naïve CD4$^+$ and CD8$^+$ T cells in the absence and presence of TLR stimulation. FACS-purified, naïve CFSE-labeled CD4$^+$ and CD8$^+$ T cells were incubated alone or together with_allogenic DC subsets in the absence or presence of TLR agonists, and proliferation of CD4$^+$ or CD8$^+$ T cells was assessed by CFSE dilution.

mDC1 and mDC2, and to a lesser extend pDC, induced naïve $CD4^+$ T cell proliferation in the absence of TLR stimulation, while $CD14^+$ monocytes or $SLAN^+$ cells failed to do so (Figure 6A). However, DC induced only low or undetectable proliferation of naïve $CD8^+$ T cells under the same conditions (Figure 6B). Importantly, all DC subsets that received either appropriate TLR stimulation or CD4 help induced naive $CD8^+$ T cell proliferation, but optimal proliferation required both CD4 help and TLR stimulation (Figure 6B). Interestingly, while TLR-9 stimulation alone was sufficient for pDC to acquire priming capacities, mDC1 required combinational TLR stimulation. The higher capacity of TLR-stimulated DC to prime $CD8^+$ T cells was associated with up-regulation of MHC class-I and CD86 expression as expected (Figure 6C). Thus, all DC subsets can prime $CD4^+$ and $CD8^+$ T cells, and they require TLR stimulation or CD4 help for $CD8^+$ T cell priming.

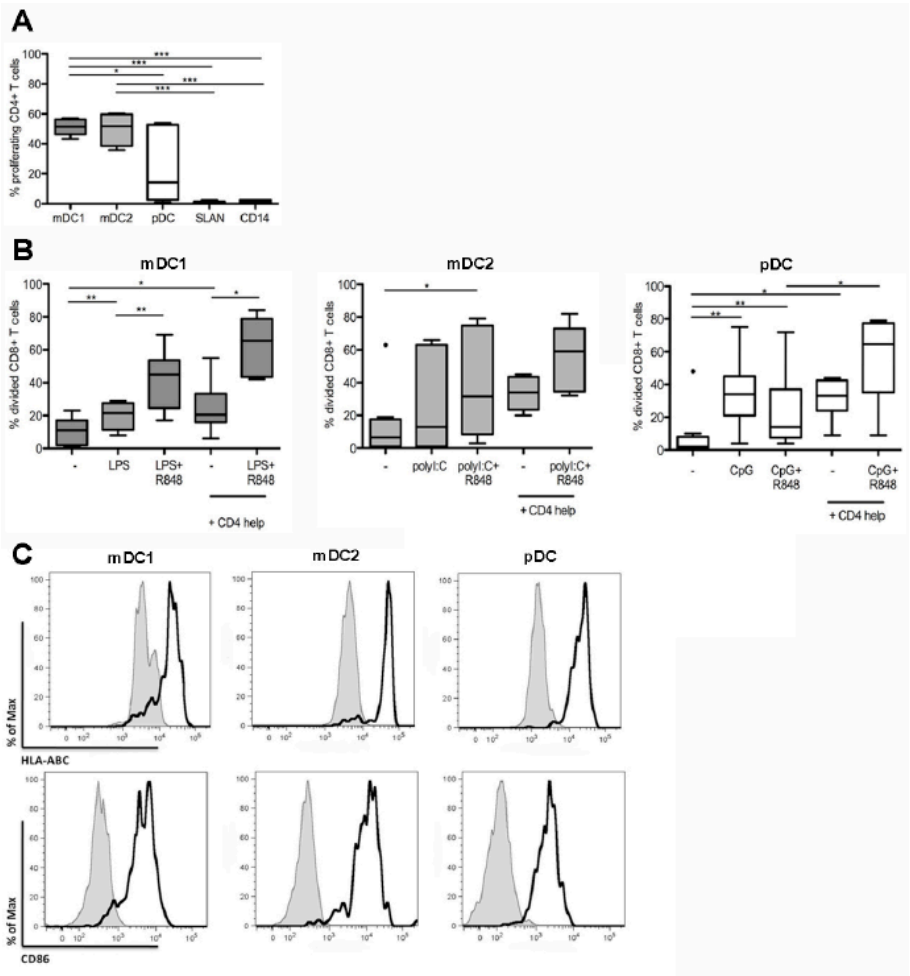

Figure 6. TLR stimulation and CD4 help license DC to prime CD8$^+$ T cells

(A/B) Autologous naïve CD4$^+$ or CD8$^+$ T cells from peripheral blood were CFSE-labeled and cultured alone or together with allogeneic DC or monocytes subsets in the absence (-) or presence of TLR agonists as indicated. Proliferation was assessed after 7 days by CFSE dilution and is shown as the percentage of divided CD4$^+$ T cells (A) or CD8$^+$ T cells (B). (C) MHC class-I (upper panels) and CD86 (lower panels) expression on DC before (filled histograms) or after stimulation (open histograms) with LPS + R848 of mDC1 (left panels), with polyI:C + R848 of mDC2 (central panels) and with CpG of pDC (right panels). One representative donor out of three is shown.

7. mDC1 efficiently induce cytotoxic molecules via IL-12

We next analyzed if proliferating naïve CD8+ T cells primed by different DC subsets differentiated to cytotoxic T cells, which unlike naïve cells secrete IFN-g and express cytotoxic molecules.

Naïve $CD8^+$ T cells that proliferated in response to TLR-stimulated DC subsets differentiated, since they acquired IFN-g producing capacities (Figure 7A), and expressed cytotoxic molecules (Figure 7B/C). Following combinational TLR stimulation mDC1 induced very high levels of IFN-g production as well as Granzyme-B (Figure 7B) and –K (Figure 7C). Also pDC induced IFN-g producing capacities and Granzyme-B, but not Granzyme-K. Interestingly, upon optimal stimulation with polyI:C and R848 mDC2 induced IFN-g production as efficiently as mDC1 (Figure 7D), but they induced significant lower levels of Granzyme-B and –K (Figure 7E/F). Importantly, neutralizing anti-IL-12 antibodies significantly reduced Granzyme-B induction by mDC1, while they had no effect on Granzyme-B induced by mDC2 (Figure 7E). Moreover, anti-IL-12 antibodies strongly reduced Granzyme-K expression in mDC1-primed CTL, while the addition of physiological amounts of IL-12 induced high levels of Granzyme-K in mDC2-primed CTL (Figure 7F).

In summary, while all DC can induce IFN-g production upon $CD8^+$ T cell priming, mDC1 induce the highest levels of cytotoxic molecules because they produce high amounts of IL-12.

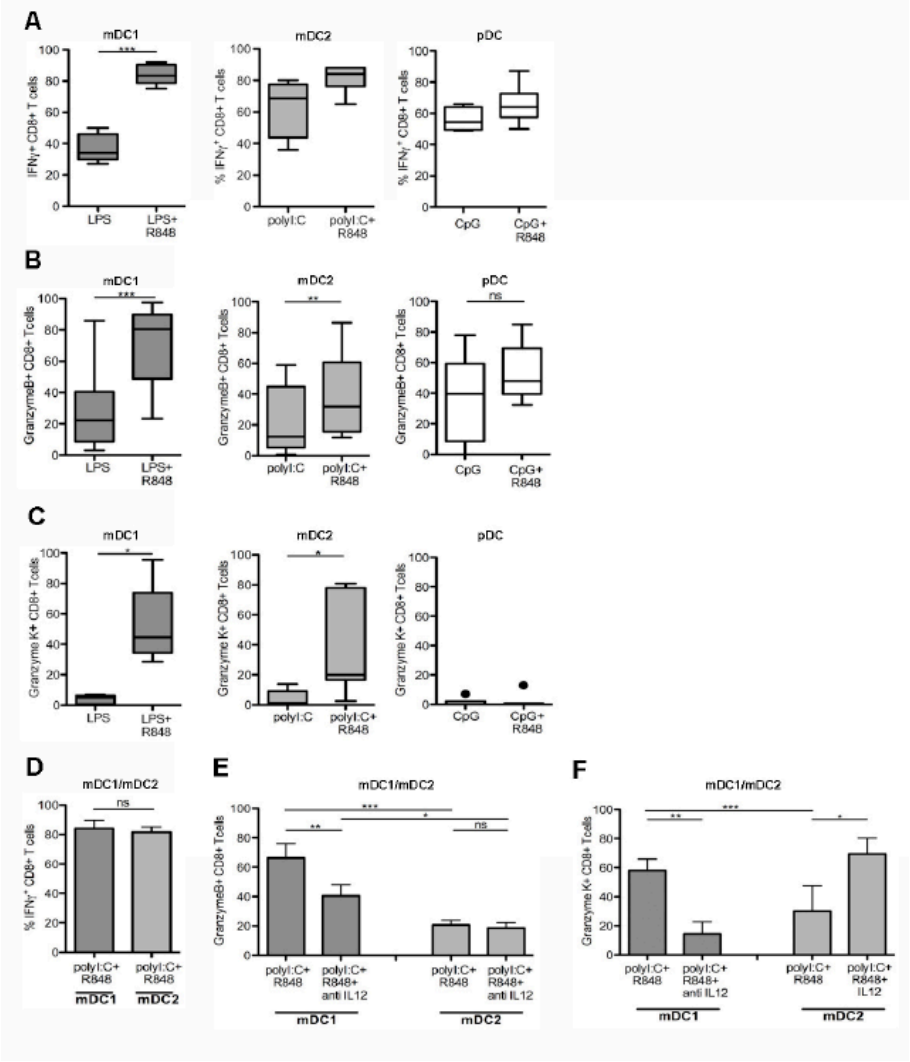

Figure 7. Efficient and IL-12-dependent CTL generation by mDC1
Naïve CFSE-labeled $CD8^+$ T cells were co-cultured for 7 days with allogeneic DC subsets in the presence of TLR agonists. (A) IFN-γ production of proliferating $CD8^+$ T cells was assessed following brief re-stimulation with PMA and Ionomycin. Shown is the percentage of IFN-g production among divided $CD8^+$ T cells primed by mDC1 (left panel) mDC2 (central panel) or pDC (right panel), which had been stimulated with the indicated TLR agonists in nine donors in different experiments. Expression of intracellular Granzyme B (B) or Granzyme K (C) in proliferating $CD8^+$ T cells primed by the indicated DC subsets stimulated with the indicated TLR agonists. (D) Mean percentage of IFN-g producing cells among $CD8^+$ T cells that had divided with mDC1 or mDC2 matured with polyI:C and R848. Granzyme-B

(E) and Granzyme-K (F) expression in divided naïve $CD8^+$ T cells primed by mDC1 or mDC2 matured with Polyl:C and R848 in the absence or presence of neutralizing anti-IL-12 antibodies or 1 ng/ml IL-12 as indicated. Shown is the mean of at least four donors in at least three experiments.

E. DISCUSSION

BDCA-3^+ DC (mDC2) are thought to be the human equivalent of murine CD8a $^+$ DC, that are characterized by the production of high levels of IL-12 and their abilities to cross-prime $CD8^+$ T cell responses. We report here that human conventional mDC1, but not BDCA-3^+mDC2, are potent producers of bioactive IL-12, and that they can cross-present antigens and promote cytotoxic $CD8^+$ T cell responses more efficiently than mDC2.

Human BDCA-3^+mDC2 were shown to share several relevant characteristics with $CD8a^+$DCs in the mouse, such as XCR1 and CLEC9A expression and the dependence on the transcription factor BATF3, but inconsistent results were published on the capacities of human DC subsets to cross-present soluble antigens and to secrete IL-12[23,26,31,33,34]. Notably however, there are also some relevant differences between the DC networks in men and mice. For example, murine myeloid DC express TLR9 and secrete IFN-a[19,23], while in humans these are exclusive features of pDC[13].

We identified here unequivocally conventional $CD1c^+$mDC1 as the most potent human IL-12 producing APCs. Notably, mDC1 both from peripheral blood and tonsils produced IL-12. The high IL-12 producing capacity of mDC1 was missed in several previous studies, because it is tightly controlled and absolutely requires combinational stimulation of Toll-like receptors[33].

The low amounts of IL-12 (<50pg/ml) produced by mDC2 that we detected here are consistent with the low levels of mDC2-derived IL-12 reported previously[23,26,31]. However, we detected IL-12 production by mDC2 only in response to polyI:C in combination with R848 and IFN-g. Under this condition mDC2 secreted very high amounts of IFN-l, while mDC1 produced substantial amounts of IL-12. Thus, the characteristic cytokine of mDC2 in response to polyI:C is IFN-l[29], and not IL-12. Since IFN-l is induced upon TLR3 stimulation by double-stranded RNA and mediates anti-viral protection, mDC2 might be particular relevant to fight viruses that are not efficiently recognized by pDC, like the Hepatitis C Virus[40,41]. IL-12 production by mDC1 was boosted by IFN-g and CD40L, showing that mDC1 have a similar regulation of IL-12 as human monocyte-derived DC and murine $CD8a^+$DC[38]. A notable exception to this rule is the negative effect of IL-4 on IL-12 produced by mDC1, because IL-4 paradoxically enhances IL-12 production in other DC[42]. Moreover, *in vitro* differentiated monocyte-derived DC can secrete even higher levels of IL-12[33]. Nevertheless, since mDC1 efficiently prime naïve T cells upon TLR stimulation, they are probably the relevant source of IL-12 in primary human T cell responses *in vivo.*

$CD8a^+$ DC in mice are considered to be the principal DC subset to cross-prime cytotoxic T cell responses[17,18,43], although contributions from other DC subsets have also been reported under certain conditions[11,39,44]. Notably, cross-priming and secondary expansions of $CD8^+$ T cells in mice critically depends on CD4 help that is mediated largely by CD40/CD40L interactions[4,45-47]. The role of CD4 help on human cytotoxic T cell responses has been much less investigated,

but given the strong evidence in mice it is considered to be relevant[48].

We defined here for the first time the different stimulation requirements of *in vivo* occurring human DC subsets to prime cytotoxic T cell responses. We showed that DC maturation by toll-like receptor agonists and licensing by $CD4^+$ T cells determined the capacity of all DC subsets to induce proliferation and differentiation of naïve $CD8^+$ T cells. As expected, this enhanced capacity of TLR-stimulated DC to stimulate $CD8^+$ T cells was associated with an enhanced expression of MHC class I and of co-stimulatory molecules. Interestingly, priming of $CD4^+$ T cells was quite efficiently induced by immature mDC subsets, consistent with the view that $CD8^+$ T cell priming has more stringent requirements.

Previous reports suggested that mDC2 have an higher intrinsic capacity to cross-present antigens in particular from necrotic cells[26], which might be particularly relevant for tolerance induction under steady-state conditions[49-51]. Our findings that immature mDC2 could activate CMV-specific $CD8^+$ memory T cells in the presence of soluble antigens in some donors is consistent with this notion. Nevertheless, efficient cross-presentation by both mDC1 and mDC2 required combinational TLR stimulation, possibly explaining inconsistent results of previous studies. Combinational TLR stimulation was shown to be important for IL-12 production[33], and we found here that it is also required for cross-presentation and CTL priming by mDC. This requirement for synergistic stimulation of surface and intracellular pathogen-sensing receptors might license DC that have taken up microbes, and consequently contain the highest levels of relevant antigens[38], to cross-prime cytotoxic T cells.

Consistent with our findings, two very recent studies showed that all three DC subsets can cross-present antigens if they are derived from tonsils or if antigen is delivered via appropriate surface receptors[52,53]. Importantly, we identified here the different signals that induce cross-presentation *in vitro* and that probably mimick the stimuli that were received by tonsillar DC *in vivo*. Interestingly, mDC1 performed best upon TLR-4 plus TLR-8 stimulation, while mDC2 required agonists of TLR-3 and TLR-8. Given these subset-specific requirements different adjuvants of protein vaccines could selectively license mDC1 or mDC2 to cross-prime cytotoxic T cell responses[48]. However, since mDC1 are much more abundant and efficiently induce CTL expressing high levels of cytotoxic molecules, they are more promising candidates than mDC2. Moreover, since we found that pDC express lower levels of MHC class-I and CD86, are inefficient to cross-present antigens and fail to induce Granzyme-K, pDC are probably also less suited for anti-tumor vaccines than mDC1[12].

In summary, although BDCA-3$^+$mDC2 share many relevant characteristics including IFN-I secretion with murine CD8a$^+$DC, our findings also evidence some species-specific aspects of DC biology that have to be considered when results from mouse models are translated into the clinics. In particular, the tightly regulated capacities of conventional CD1c$^+$DC to secrete high amounts of IL-12, cross-present antigens and prime cytotoxic T cells have important implications for the design of human vaccines.

F. ACKNOWLEDGMENTS

We thank P. Jähn (Miltenyi Biotech, Bergisch Gladbach, Germany) and A. Lanzavecchia (IRB, Bellinzona, Switzerland) for reagents and helpful discussion. This work was supported by the DFG, the Romeo and Enrica Invernizzi Foundation and the Cariplo Foundation.

G. AUTHORSHIP CONTRIBUTION

G.N., J.K., A.W., S.S., P.G., and A.B. performed research and analysed data, B. S., M.M. and MC. C assisted research, K. S., S. T., L. P. and C. S provided clinical samples, F. F., C. R., P. N., R. DF and S. A. provided advice and assisted in the writing of the manuscript, J.G. designed the study, analysed data and wrote the paper.

H. CONFLICT-OF-INTEREST DISCLOSURE

The authors declare no competing financial interests.

I. SUPPLEMENTAL INFORMATION

Fig S1

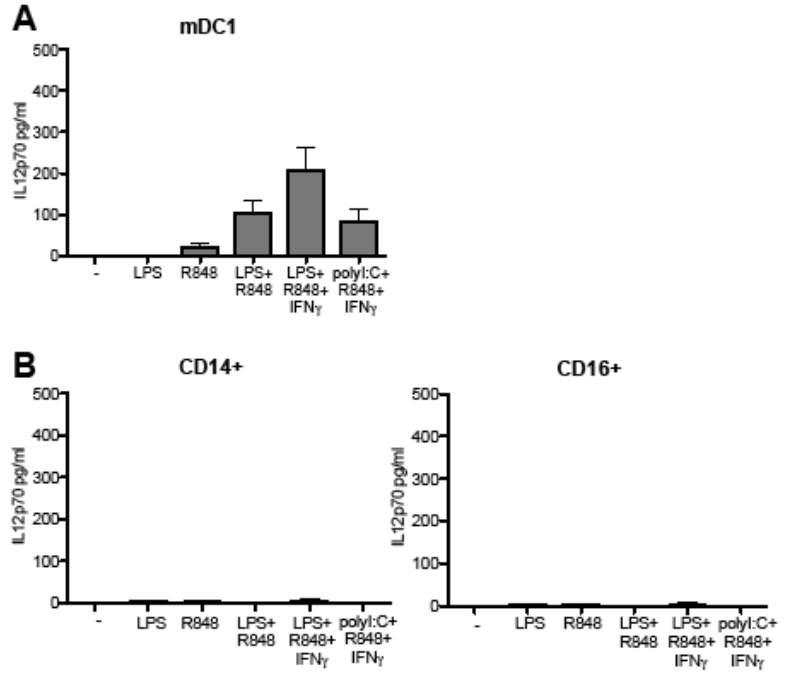

Figure S1. IL12p70 production by mDC1 and monocytes

Bioactive IL-12p70 production by peripheral blood mDC1 (A) and CD14+/CD16+ monocytes after 24-h culture in medium alone (-) or in the presence of indicated stimuli. Error bars represent mean ± SEM of 3 donors in 3 different experiments

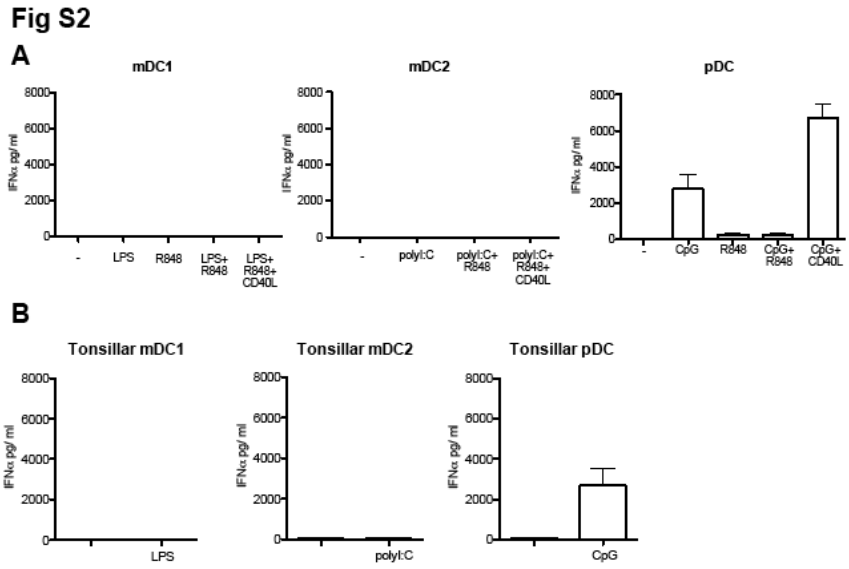

Figure S2. Potent IFNα production by human pDC
IFNα production by peripheral blood (A) and tonsillar (B) DC subsets following TLR stimulation,as indicated.
Error bars represent mean ± SEM for 16 PBMC donors in 8 different experiments and 7 TMC donors in 5 experiments.

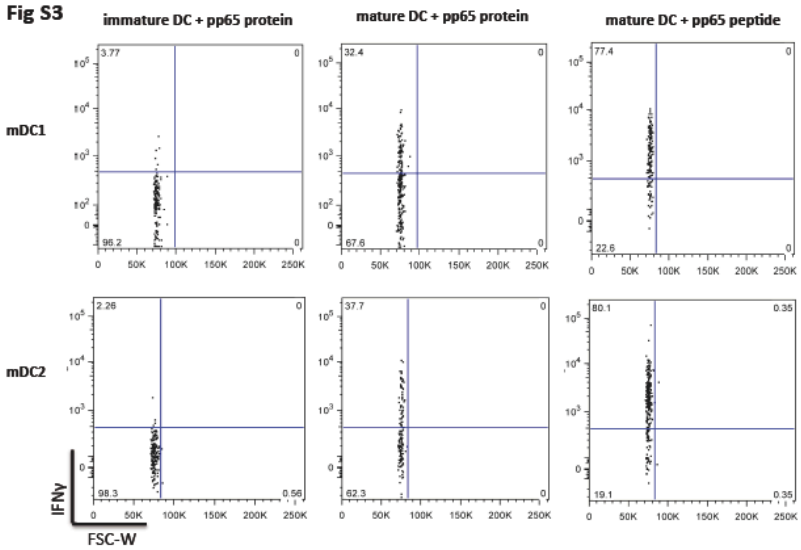

Figure S3. Cross-presentation by mature myeloid DC
DC subsets obtained from HLA-A2+ donors were co-cultured with HLA-A2 pp65-specific CD8+ T cells (ratio 1:2), in the presence of recombinant pp65 protein or $pp65_{495\text{-}503}$ peptide, togheter with TLR agonists. Here is shown IFNγ production by pp65-specific CD8+ T cells [immature DC left panel; mature DC (LSP+R848 mDC1, polyI:C+R848 mDC2)+ pp65protein, middle panel; mature DC + pp65 peptide, right panel). One representative donor is shown.

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CHAPTER 3

Role of IL-10 produced by human DCs in the priming of T cells

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The data presented in this chapter are part of an ongoing project.

A. ABSTRACT

IL-10 is a cytokine with anti-inflammatory properties and has a pivotal role in preventing inflammatory and autoimmune diseases. It is produced by a large number of cells, including T cells, dendritic cells (DC), macrophages and natural killer (NK) cells.

Here we investigated the human DC and monocyte compartments and found that IL-10 production is restricted to type 1 myeloid dendritic cells (mDC1) and to $CD14^+$ monocytes. Notably, the regulation of IL-10 production is different in the two cell populations: LPS induced quite high IL-10 levels in $CD14^+$ monocytes, whereas R848 alone on in combination with LPS induced high IL-10 production in mDC1, but was actually inhibitory for monocytes.

mDC1 but not $CD14^+$ monocytes are professional antigen presenting cells (APCs) and are able to prime adaptive immune responses and it is known that IL-10 targets directly APC, decreasing their capacity to activate Ag-specific T cell responses *in vitro*. We here confirmed that IL-10 inhibited the production of IL-12p70, thus dampening Th1 responses, and it also inhibited T cell priming.

The role of IL-10 on T cell activation is well known, but the role of DC-derived IL-10 in the priming of cytotoxic T cell responses is unclear. Here we show that IL-10 produced by mDC1 prevents the priming of undifferentiated T cells, but has no direct positive effect on CTL priming. It is possible that IL-10 positively impacts on $CD8^+$ memory T cells, rather than on the priming of CTL.

B. INTRODUCTION

Interleukin-10 (IL-10) is a cytokine discovered in 1989 by Mossmann and colleagues as a novel immune mediator secreted by mouse type 2 T-helper (Th2) cell clones, able to inhibit the synthesis of IL-2 and IFNγ in Th1 clones.[1] IL-10 is produced by a large number of cells, including T and B lymphocytes, natural killer (NK) cells, macrophages and dendritic cells.[2,3] The main effect of IL-10 is to dampen immune responses, that is the reason why IL-10 is referred to as an anti-inflammatory cytokine but IL-10 as is emerging a multifaceted cytokine.

The anti-inflammatory role of IL-10 is mainly due to its effect on antigen presenting cells (APCs): they are producers and targets of IL-10. This cytokine can act in an autocrine or in a paracrine way, leading to the downregulation of surface expression of antigen presenting molecules (MHC II), as well as of co-stimulatory (i.e. CD86) and adhesion (i.e. integrins) molecules.[4] IL-10 can also negatively modulate the secretion of inflammatory cytokines[5]; to be underlined that this function has a critical role for the immune system in limiting the host damage due to aberrant cytokine production. Thus, IL-10 affects adaptive immune responses by directly acting on APCs, that become bad T-cell stimulators. The final effects of IL-10 on T cells are antithetical; it is documented that IL-10 inhibits Ag-specific $CD4^+$ T cell proliferation[6] and can act directly on Th17 cells by controlling their expansion, thereby avoiding inflammatory diseases, such as inflammatory bowel disease (IBD).[7] Moreover, IL-10 produced by a specific subsets of dendritic cells, called “DC-10”, was shown to induce the expansion of tolerogenic $CD4^+$ type 1 regulatory cells (Tr1).[8]

The effects on $CD8^+$ T cells are controversial[9]. In fact, IL-10 was reported to induce cytotoxicity in CD8+ T cells[10], thereby being important for tumor cell clearance: Neven and colleagues very recently found that a deficit in IL-10 receptor leads to development of non-Hodgkin's lymphoma, due to the absence of cytotoxic tumor infiltrating lymphocytes.[11] On the contrary, further evidence indicated a pathogenic role for IL-10 in the development of diabetis in diabetic NOD mice.[12]

It is clear that IL-10 is a very complex cytokine and the studies regarding its role on T cells were carried out on antigen-experienced T cells; in this study we investigate the role of IL-10 produced by human dendritic cells, the most potent APCs, in the priming of naïve T cells.

We here demonstrate that IL-10 is produced only by type 1 myeloid DC (mDC1) and $CD14^+$ monocytes and prevents the priming of undifferentiated $CD4^+$ and $CD8^+$ T cells.

C. METHODS

1. Mononuclear cells from human peripheral blood

Buffy-coat blood of healthy donors was provided by IRCCS Policlinico Ospedale Maggiore, Milan, Italy; PBMCs (Peripheral Blood Mononuclear Cells) were isolated by Ficoll-Hypaque gradient (Sigma-Aldrich), according to standard methods.

The ethical committee approved the use of PBMCs for research purposes and informed consent was obtained from the subjects involved in this study in accordance with the Declaration of Helsinki. Cells were cultured in complete RPMI1640 (EuroClone) containing 10% FCS (EuroClone), 0.1% Penicillin/Streptomycin (EuroClone),

0.1 % non-essential amino acids (Lonza) and 0,1% Sodium Pyruvate (Lonza) at 37°C and 5 % CO_2.

2. DC isolation

DC subsets were isolated from PBMC by magnetic enrichment followed by cell sorting. Briefly, cells were incubated with anti-CD1c-FITC (AD5 8E7), anti-BDCA-3-APC (AD5 14H12) and anti-BDCA-4-PE (AD5 17F6, Miltenyi Biotech) antibodies, non-specific binding to Fc receptors was prevented using FcR blocking reagent (Miltenyi Biotech), and DC were magnetically labeled with anti-FITC, -PE and-APC beads and enriched on columns. The positive fraction was incubated with the lineage markers anti-CD3 (SK7, BioLegend), -CD14 (61D3), -CD16 (CD16), -CD19 (HIB19), -CD56 (B159, e-Bioscience) and -CD11c (3.9, BioLegend). mDC1 were sorted as lineage$^-$CD11c$^+$ CD1c$^+$, mDC2 as lineage$^-$CD11c$^+$BDCA-3^{hi} , pDC as lineage$^-$CD11c$^-$BDCA-4$^+$. Purity of all DC subset was >95%. Monocyte subsets were sorted directly from peripheral blood as lymphocyte lineage$^-$ cells (CD19-, CD56- and CD3$^-$) according to CD14 and CD16 expression.

3. Flow cytometry

Cytokine production by DC was assessed by intracellular staining with antibodies specific for IL-10 (9D7, BioLegend), IL-12p70 (20C2, BD) and TNFα (MAb11, BD).

Production of IFN-γ or cytotoxic molecule in CD8$^+$ T cells, as well as IFN-γ and Foxp3 in CD4$^+$ T cells was assessed by intracellular staining with antibodies specific for IFN-γ (F4S.B3, eBioscience), Granzyme-B (GB11, BD) and Foxp3 (PCH101, eBioscience) according to a standard protocol.

Samples were analyzed on a FACSCanto flow cytometer (BD) using FlowJo software (Tristar).

4. DC cytokine production

Sorted DC or monocyte subsets were seeded at 10^4 cells/well in a 96–well plate for 24 hrs with either 100ng/ml LPS (Sigma), 100ng/ml polyI:C (Alexis), 10 μg/ml CpGC (IDT), 10 μg/ml CpGA (Alexis), 2,5 μg/ml R848, (Alexis), 10μg/ml zymosan (Sigma-Aldrich), 10 ng/m IFN-γ (R&D) and CD40L–transfected murine cell line (ratio 1:1, JSSB, kindly provided by A. Lanzavecchia, IRB, Bellinzona, Switzerland). In some cases 10μg/ml αIL-10 (JES3-9D7, Miltenyi Biotec), 4μg/ml αIFN-λ1 (247801, R&D) and 10μg/ml αIFN-alpha (eBioscience) neutralizing antibodies were used. Cytokine production was assessed by intracellular staining (mDC1 and pDC) and by ELISA. ELISAs were performed on cell-free supernatants , according to the manufactures' guidelines. ELISAs for IL-1β (BioLegend), IL-6 (eBioscience), IL-10 (BD), IL-23 (eBioscience), IFN-λ2/3 (PBL InterferonSource), TNFα (eBioscience).

5. T cell stimulation

5x10^4 CellTrace™-labeled naïve $CD4^+$ or $CD8^+$ T cells were co-cultured with 10^4 allogeneic purified DC or monocyte subsets for 7 days. DC were stimulated with 100ng/ml LPS (mDC1), 100ng/ml polyI:C (mDC2), 10μg/ml CpGC (pDC), with or without αIL-10, αIFN-λ1 and 10μg/ml αIFN-alpha, respectively.

T cell proliferation was assessed by CellTrace™ dilutions and calculated as the percentage of divided $CD4^+$ or $CD8^+$ T cells. IFN-γ production was detected by intracellular staining after 4 hours of

PMA/ionomycin stimulation; 10 µg/ml Brefeldin A (Sigma) was added for the last 3 hours of stimulation. GranzymeB and Foxp3 induction was detected by intracellular staining, according to standard protocols.

IFN-γ, GranzymeB and Foxp3 production was calculated in two different ways, as percentage of cytokine produced by CellTrace ^{low} proliferating T cells, or as percentage of cytokine produced on total T cells.

In other experiments, $5x10^4$ CellTrace™-labeled naïve $CD4^+$ T cells were co-cultured with 10^4 autologous purified DC subsets for 7 days. DC were stimulated with 10 pg/ml TSST (Sigma Aldrich).

6. Statistics

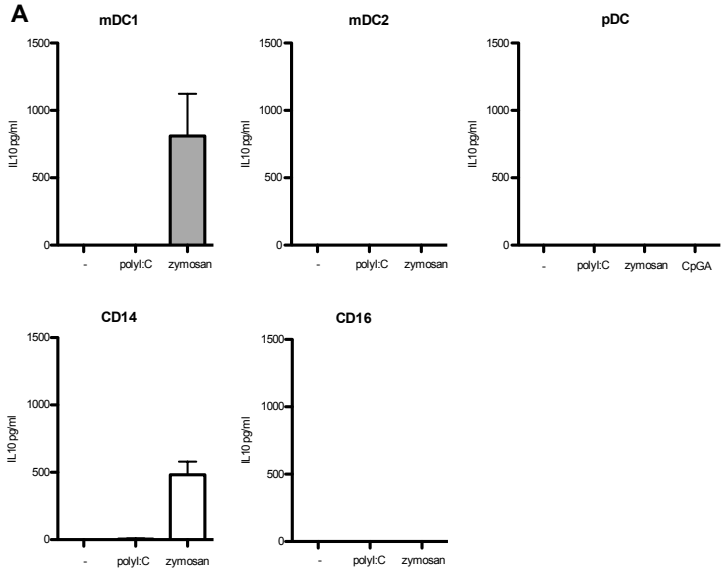
Statistical significance was calculated using two-tailed Student's t test in case of Gaussian distribution, otherwise Mann Whitney for unmatched or Wilcoxon for paired groups. $p < 0.05$ (*), $p < 0.005$ (**) $p < 0.0005$ (***) were regarded as statistically significant.

D. RESULTS

1. IL-10 production by DC and monocyte subsets

Studies in mice indicated that macrophages and all DC subsets can secrete IL-10 in vivo following TLRs stimulation, in particular TLR2, TLR4 and TLR9.[14,15] We compared human sorted dendritic cell subsets i. e. $CD1c^+$mDC1, BDCA-3^+mDC2 and BDCA-4^+pDC, and monocyte populations i. e. $CD14^+$, $CD16^+$ as well as $CD14^+16^+$ for IL-10 production following TLR triggering by selected agonists. Interestingly, only mDC1 and $CD14^+$ monocytes were able to produce IL-10 upon triggering of TLR2 by zymosan, while polyI:C had no

effect as expected (Figure 1A). mDC1 and monocytes subsets, that express TLR4 and 8, were also stimulated with LPS (TLR4L) and R848 (TLR7/8L) alone or in combination and IL-10 secretion assessed. Surprisingly, monocytes and mDC1 showed a different regulation of IL-10 production: thus, LPS induced quite high IL-10 levels in $CD14^+$ monocytes, whereas R848 alone on in combination with LPS induced high IL-10 production in mDC1, but was actually inhibitory for monocytes (Figure 1B). Notably, also upon TLR4/8 stimulation IL-10 was not induced in $CD16^+$ or $CD14^+16^+$cells, although the latter were reported to produce high levels of IL-10 under these conditions and to contain so-called "DC10" cells[8] (Figure 1B). Finally, LPS-induced IL-10 production was enhanced by stimulation with CD40L in both mDC1 and $CD14^+$ monocytes (Figure 1C).



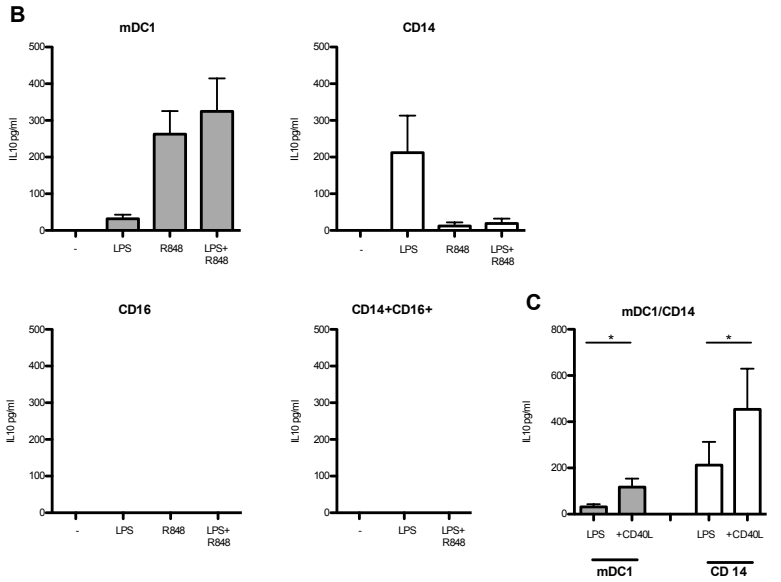


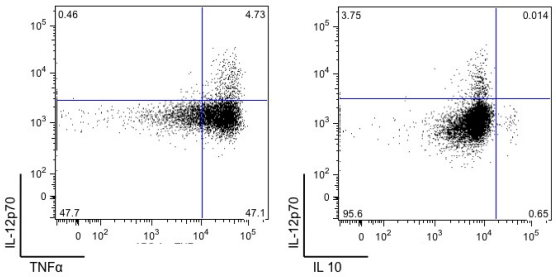
Figure 1. Regulation of IL-10 production in mDC1 and CD14$^+$ monocytes. (A-B) IL-10 production by purified peripheral blood DC and monocyte subsets after a 24-hour culture in medium alone (-) or in the presence of the indicated TLR agonists. (C) Effect of CD40 stimulation on IL-10 production. 6 donors in 4 independent experiments are shown. *p<0.05

We conclude that in humans, IL-10 production is restricted to selected DC and monocyte subsets, i. e. mDC1 and CD14$^+$ monocytes.

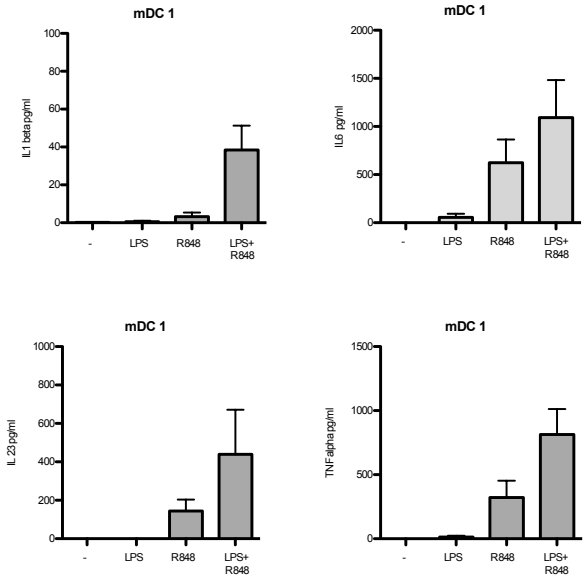
2. Human mDC1 produce high levels of pro-inflammatory cytokines

Cytokines secreted by DC play a pivotal role in the skewing of naïve T cells into different T cell subsets. mDC1, the most frequent DC subset in human peripheral blood[16], was highly efficient to secrete several pro-inflammatory cytokines upon stimulation with TLR4 and 8 agonists, including IL-6, IL-1β, TNFα and IL-23 (Figure 2A/B).

A


0.46
4.73
47.7
47.1
IL-12p70
TNFα
3.75
0.014
95.6
0.65
IL-12p70
IL 10

B


mDC 1
IL1 beta pg/ml
mDC 1
IL6 pg/ml
mDC 1
IL 23 pg/ml
mDC 1
TNF alpha pg/ml
-
LPS
R848
LPS+ R848

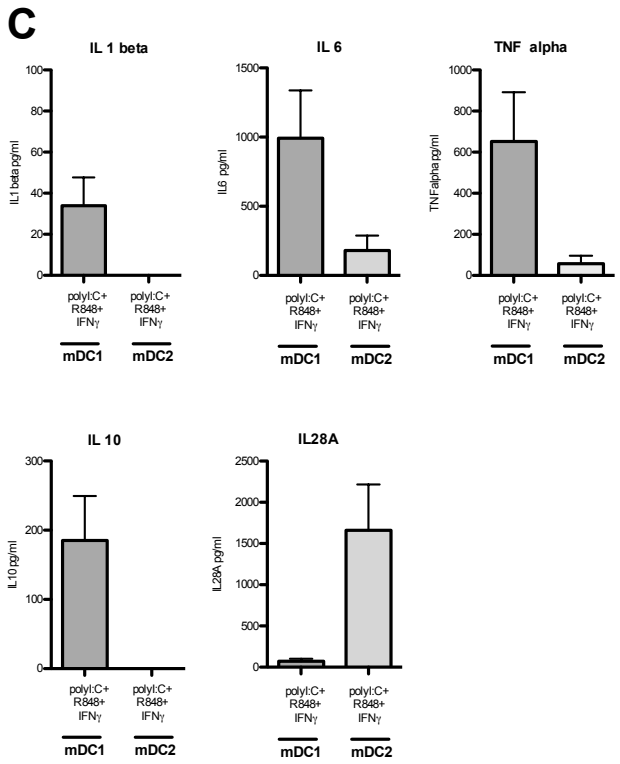


Figure 2. Pro- and anti-inflammatory cytokine production by mDC1 and mDC2. Peripheral blood mDC1 and mDC2 cytokine production after a 24-hour culture in medium alone (-) or in presence of the indicated TLR agonists. (A) Intracellular staining of IL-12p70, TNFα and IL-10 (one representative staining out of three) and (B) ELISAs performed on mDC1 show the production of IL-1β, IL-6, IL-23 and TNFα. (Mean of 6 donors/ 4 experiments) (C) Cytokine production by mDC1 and mDC2 following stimulation with polyI:C, R848 and IFNγ.

Cytokine production by mDC1 following TLR stimulation revealed that co-stimulation with LPS and R848 was able to induce the production of high levels of pro-inflammatory cytokines such as IL-1β, IL-6, and TNFα, IL-23p19/p40, as well as IL-12p70, as reported in chapter 2 (Nizzoli et. al. Blood 2013). Interestingly, intracellular staining revealed that while IL-12 was co-expressed with TNFα,

IL-12 and IL-10 were secreted by different cells. Moreover, we wondered whether the other myeloid DC subset, mDC2, had the same capacities to produce pro-inflammatory cytokines. To address this point we used the stimulus that most strongly activated cytokine production by mDC2 (i.e.polyI:C+R848+IFNγ, see Chapter 2 and data not shown), for both DC subsets. As shown in Figure 2C, mDC1 produced significantly higher levels of the pro-inflammatory cytokines IL-6, and TNFα than mDC2, and only mDC1 but not mDC2 produced IL-1b and IL-10. On the contrary, although mDC1 were able to produce some IL-29/IFN-l1 (see Chapter 2) only mDC2 produced IL-28A/IFN-l2 under these conditions. This data further supports the notion that mDC1 and mDC2 have largely different cytokine profiles.

3. IL-10 selectively decreases IL-12p70 production of mDC1

The main focus of this work is to define the role of IL-10 produced by DC in the shaping of T cell responses. It is well known that anti-inflammatory functions of IL-10 can be exerted by limiting or inhibiting the production of inflammatory cytokines.[17]

The role of DC and monocyte-derived IL-10 was assessed by neutralizing IL-10 with specific antibodies. Anti-IL-10 antibodies were added to stimulated mDC1 and $CD14^+$ monocytes, and supernatants were analyzed for cytokine production: neutralizing IL-10 increased IL-12p70 levels in mDC1 stimulated with LPS and R848 as expected (Figure 3A)[16], but notably there was no induction of IL-12 in monocytes or in mDC1 stimulated with LPS alone. Surprisingly, IL-10 neutralization had also no effect on IL-6 production (Figure 3B).

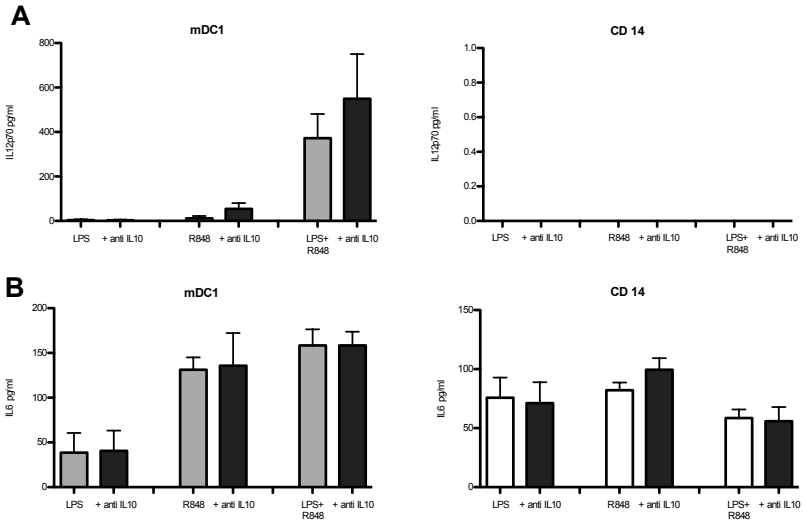


Figure 3. Effect of IL-10 neutralization on cytokine production. Cytokines produced by peripheral blood mDC1 and $CD14^+$ monocytes after a 24-hour culture in medium alone (-) or in presence of the indicated TLR agonists. (A) Anti-IL-10 enhances IL-12p70 production in mDC1 stimulated with LPS and R848, while (B) it has no effect on IL-6 produced by DC or monocytes. Mean of 3 donors in 2 experiments.

4. IL-10 inhibits T cell priming

It is well known that IL-10 inhibits either directly or indirectly by its effect on APC the proliferation of $CD4^+$ and $CD8^+$ T cells, and it was proposed that IL-10 produced by tolerogenic, IL-10 producing DC ("DC-10") promotes IL-10 production by activated T cells[5,18]. However the role of IL-10 produced by conventional DC in T cell priming is unclear. We therefore investigated the role of IL-10 produced by ex vivo isolated mDC1 and $CD14^+$ monocytes on allogeneic T cell priming. The other two principal DC subsets, i.e. mDC2 and pDC, do not produce IL-10 (Figure 1), but produce high levels of IFNλ and IFNα, respectively (Nizzoli et al.). Since also IFNs are anti-

proliferative cytokines, we also investigated the effects of mDC2 and pDC-derived IFNs on T cells priming. To be noted that IFNλ and IL-10 share the same receptor chain, IL10 receptor beta (IL10RB), and that the role of type III IFN in T cell priming is largely unknown.

To analyze whether IL-10, IFNλ and IFNα impact on T cell proliferation, DC subsets and $CD14^+$ monocytes were matured with TLR ligands that induced these cytokines by DC. Stimulated DC subsets were co-cultured with CellTracer-labeled allogeneic naïve $CD4^+$ and $CD8^+$ T cells in the presence of antibodies neutralizing IL-10, IFNλ or IFNα,.and proliferation of T cells was assessed after 7 days by CellTracer dilutions.

We observed that mDC1 primed $CD4^+$ and $CD8^+$ T cells, and that blockade of mDC1-produced IL-10 significantly increased both $CD4^+$ (Figure 4C) and $CD8^+$ (Figure 4B) naïve T cell proliferation. On the contrary mDC2 and pDC were less potent, and no significant effect was found for IFNλ and IFNα produced by mDC2 and pDC, respectively. $CD14^+$ monocytes failed to prime naive T cells (Figure 4) even in the presence of neutralizing anti-IL-10 antibodies and were therefore not considered further.

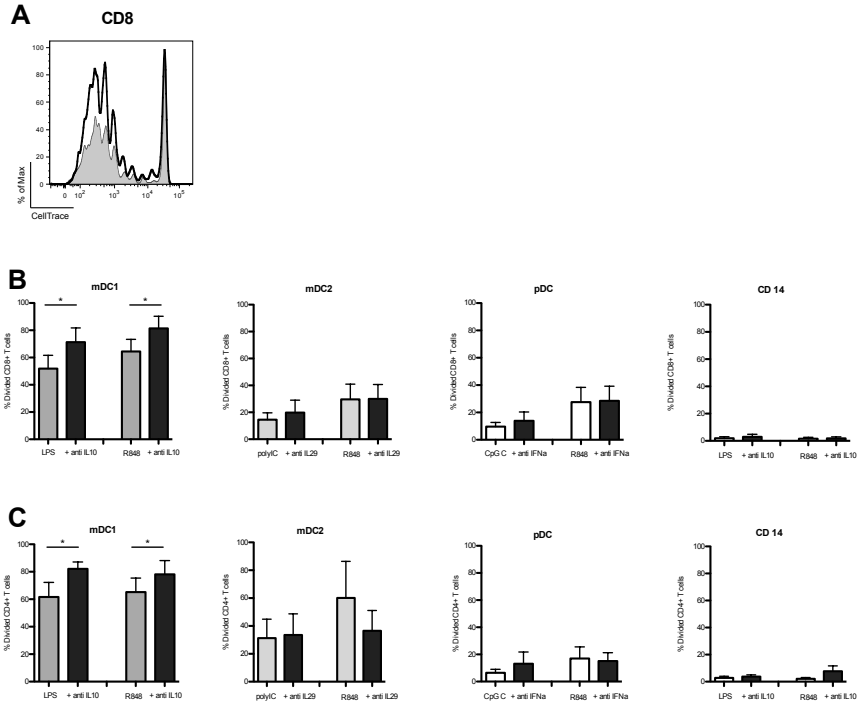


Figure 4. IL-10 neutralization increases T cell proliferation. Peripheral blood DC subsets and $CD14^+$ cells where matured in the presence of the indicated stimuli and co-cultured for 7 days with allogeneic $CD4^+$ and $CD8^+$ T cells. (A) T cell proliferation induced by mDC1 stimulated with LPS alone (tinted line) or LPS+ anti-IL-10 (bold black line). One representative experiment is shown. Mean percentage of proliferating $CD8^+$ (B) and $CD4^+$ (C) naïve T cells induced by DC subsets and monocytes in six donors. *p<0.05

5. IL-10 prevents the priming of undifferentiated $CD8^+$ T cells

The role of IL-10 on CTL responses is poorly understood, because it can have positive and negative effects on cytotoxic T cells.[9,19] A very recent work suggested a pivotal role for IL10 in the development of human CTLs, since patients lacking IL10R developed non Hodgkin's lymphomas that was associated with a lack of tumor-infiltrating cytotoxic T cells (CTL)[11].

A positive effect on CTL responses was previously reported for diabetic NOD mice, in which IL10 accelerate the onset of disease.[12]

To address the role of mDC1-derived IL-10 in CTL priming, naïve CellTracer-labeled $CD8^+$ T cells were stimulated with matured DC subsets and monocytes in the presence or absence of neutralizing antibodies against IL-10, IFNλ or IFNα. Seven days later, $CD8^+$ T cells were analyzed for Granzyme B (GrzB) expression and IFNγ producing capacities. IL-10 neutralization diminished the fraction of Granzyme B^+ and IFNγ^+ cells among proliferating T cells (Figure 5A/B and D/E), suggesting that IL-10 might promote $CD8^+$ T cell differentiation. However, a more accurate analysis revealed that the decreased percentage of differentiated $CD8^+$ T cells was caused by an expansion of undifferentiated $CD8^+$ T cells that lacked Granzyme B and IFN-g expression upon IL-10 neutralization ((Figure 5A/C and D/F), but not by a decrease of differentiated CTL.

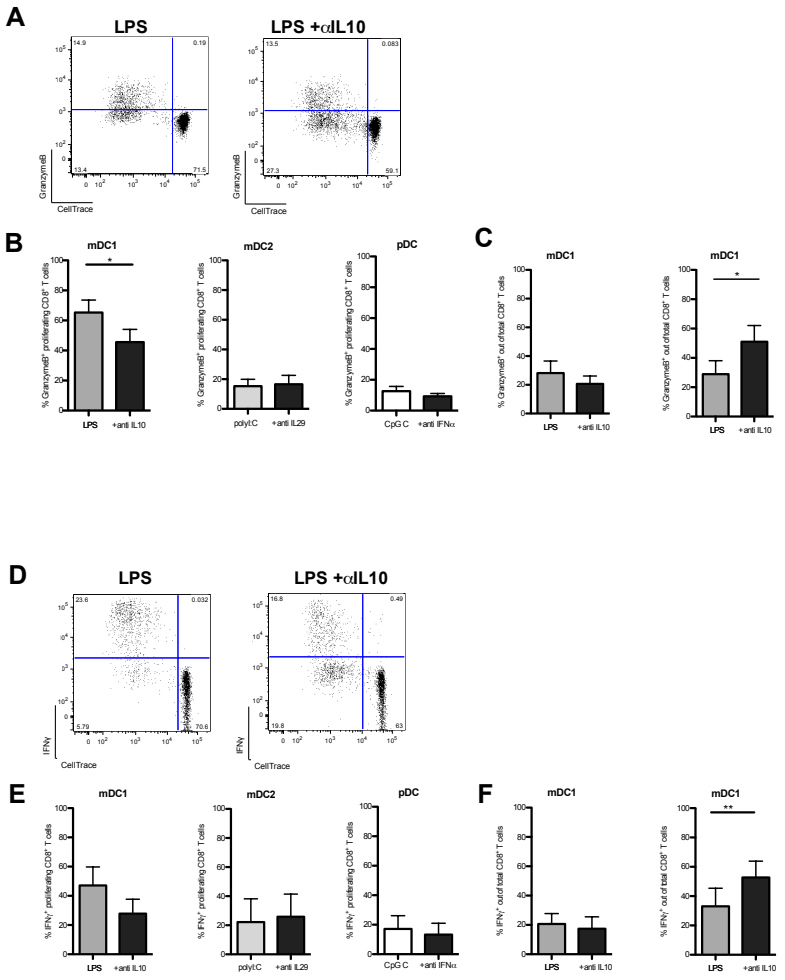


Figure 5. IL-10 neutralization induces the outgrowth of GrzB$^-$ and IFNγ$^-$ T cells. Peripheral blood DC subsets where matured in the presence of indicated stimuli and co-cultured for 7 days with naïve allogeneic CD8$^+$ T cells. GrzB (A) and IFNγ (D) induced by mDC1 stimulated with LPS alone (left panel) or LPS+ anti-IL-10 (right panel). One representative experiment is shown. (B) Percentage of GrzB$^+$ proliferating (cellTrace$^-$) CD8 T cells and (C) percentage of GrzB$^+$out of total CD8 T cells are shown. Mean of six donors. *p<.05

As observed previously, mDC2 and pDC induced CTL differentiation to a lower extent as compared to mDC1 (Nizzoli et al.). Moreover, no significant effect was observed for anti-IFNλ or anti-IFNα neutralizing Abs on CTL differentiation. These findings suggest that IL-10 produced by mDC1 inhibits the priming of undifferentiated $CD8^+$ T cells, while neutralization of IFNa produced by pDC or of IFNl produced by mDC2 have no clear effect on CD8+ T cell priming.

6. IL-10 prevents the priming of undifferentiated $CD4^+$ T cells

It is possible that IL-10 has different effects on $CD4^+$ and $CD8^+$ T cells. We therefore investigated the effects of IL-10 blockade also on $CD4^+$ T cell priming by allogeneic mDC1. As observed for $CD8^+$ T cells, addition of anti-IL-10 to LPS-matured mDC1 led to a decreased percentage of $IFN\gamma^+$ cells among proliferating $CD4^+$ T cells, but again this effect was caused by the expansion of cells that did not produce IFN-g (Figure 6A/B). mDC2 and pDC induced lower levels of Th1 cells, and there was again no effect of neutralizing antibodies to IFN-l and a, respectively .

We also analyzed the induction of Foxp3 in $CD4^+$ T cells by the three DC subsets to understand if they were able to induce regulatory T cells that express Foxp3. As shown in Figure 6D, mDC1 and mDC2 induced Foxp3 in a fraction of $CD4^+$ T cells, while pDC failed to do so. The percentage of $Foxp3^+$ cells diminished upon IL-10 neutralization, but again this could be largely explained by the expansion of $Foxp3^-$ $CD4^+$ T cells. (Figure 6C/E). Conversely, anti-IFNl antibodies had no effect on Foxp3 induction by mDC2, and anti-IFNa antibodies failed to induce Foxp3 expression on $CD4^+$ T cells primed by pDC.

In summary, mDC1-derived IL-10 also inhibits the accumulation of undifferentiated $CD4^+$ T cells.

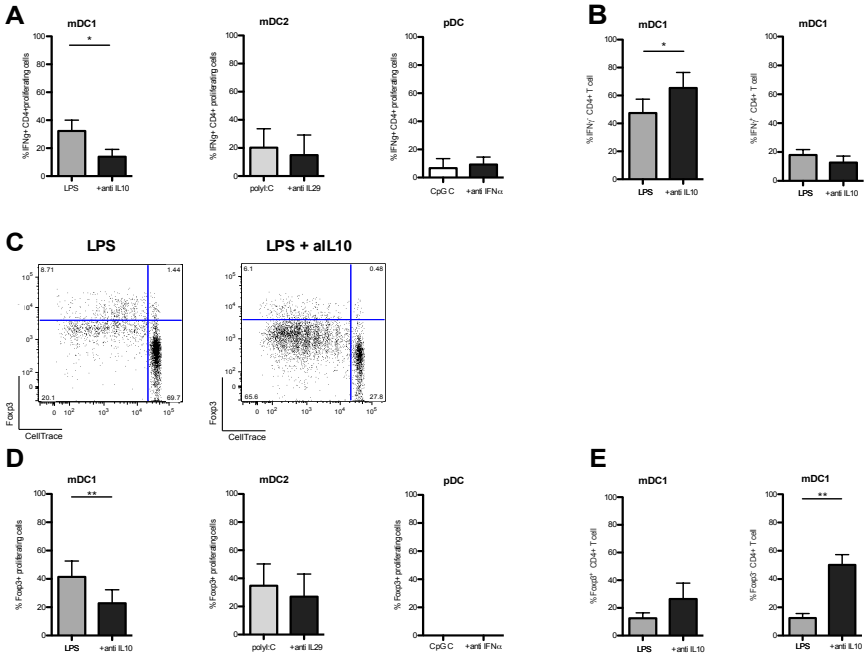


Figure 6. IL-10 inhibits the outgrowth of $IFN\gamma^-$ and $Foxp3^-$ T cells. Peripheral blood DC subsets where matured in the presence of the indicated stimuli and co-cultured for 7 days with naïve allogeneic $CD4^+$ T cells. Percentage of $IFN\gamma^+$ (A) or $Foxp3^+$ (D) proliferating ($CellTracer^-$) CD4 T cells and percentage of $IFN\gamma^+$(B) or $Foxp3^+$ (E) out of total CD4+ T cells are shown. Mean of six donors. Foxp3 (C) induced by mDC1 stimulated with LPS alone (left panel) or LPS+ anti-IL-10 (right panel). One representative experiment is shown. *p<0.05

7. pDC induce IL10 production in $CD4^+$ T cells.

It was proposed that IL-10 producing DC induce IL-10 producing type 1 T regulatory cells (Tr1)[8]. We therefore compared the ability of different DC subsets to induce IL-10 production in primed $CD4^+$ T cells.

Since we observed little IL-10 production in $CD4^+$ T cells primed by allogenic DC, we stimulated them with autologous DC subsets that were loaded with the superantigen TSST-1 in the presence or absence of neutralizing antibodies to the relevant cytokines. Intracellular staining performed on T cells revealed that pDC, that lack IL-10 producing capacities, induced the highest levels of IL-10 in $CD4^+$ T cells (Figure 7). Conversely, mDC1 induced only low levels of IL-10, and IL-10 neutralization had no effect. Interestingly, mDC2 induced intermediate levels of IL-10. Previous reports suggested a role for IFNα in IL-10 induction [20,21]. Consistently, we found a tendency (p=0.09) for less IL-10 production in pDC-primed $CD4^+$ T cells when IFNα was blocked.

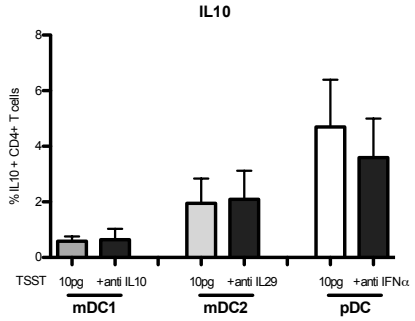


Figure 7. pDC induce IL-10 production in naïve $CD4^+$ T cells via IFNα. Peripheral blood DC subsets loaded with 10 pg/ml of TSST where matured with the indicated stimuli and co-cultured for 7 days with naïve autologous $CD4^+$ T cells, with (black bars) or without neutralizing antibodies to the indicated cytokines. Shown is the mean percentage of Il-10 producing T cells in three donors as assessed by intracellular staining.

E. DISCUSSION

It is known that the ability to synthesize IL-10 is not limited to certain T cell subsets (Th), but is a characteristic of almost all leukocytes.[22] A very important source in vivo are monocytes, macrophages and dendritic cells[3]. In this study we aimed to shed light into the role of IL-10 produced by human DC and monocyte subsets. We showed that IL-10 production is restricted to mDC1 and $CD14^+$ cells and is dependent on the triggering of TLRs as expected, However, LPS induced quite high IL-10 levels in $CD14^+$ monocytes while R848 alone on in combination with LPS induced high IL-10 production in mDC1, but was actually inhibitory for monocytes. Notably, the triggering of TL2, that in mouse induces very high levels of IL-10 in all DC subsets, in human cells led to IL-10 production only by mDC1 and CD14+ monocytes. Further, IL-10 production was enhanced by CD40L.

mDC1, the most frequent human DC population in the blood[16], not only produced IL-10, but also a very large number of pro-inflammatory cyotokines: as previously shown, mDC1 are the major producers of IL-12p70 (see chapter 2). Moreover, we here observed that synergistic TLR activation induced production of IL-1β, IL-6, IL-23 and TNFα. The other subsets of human DCs, mDC2 and pDC produce IFNλ and IFNα, respectively (see chapter 2).

The primary biological effect of IL10 is to dampen immune responses, principally inhibiting APCs functions. Studies showed that IL-10 can act in an autocrine or a paracrine way, reducing APC-cell surface expression of major histocompatibility complex class II (MHCII) molecules[4] and of co-stimulating (e.g. CD86) and adhesion

(e.g. CD54) molecules.

Not only, IL-10 can impair immune responses[18] by limiting or inhibiting the production of inflammatory cytokines.[17] We here observed that the addition of anti-IL10 neutralizing Ab to stimulated mDC1 and $CD14^+$ led to a reduction of IL-12p70 production by mDC1, as expected, but it has no effect on IL-6. It seems likely that IL-10 also inhibits other pro-inflammatory cytokines produced by mDC1.

It is well known that IL-10 inhibits either directly or indirectly the proliferation of $CD4^+$ and $CD8^+$ T cells, acting through its effect on APC, but less is known about the role of DC-produced IL10 on the priming of naïve T cells. We here show that mDC1 efficiently stimulate the proliferation of naïve T cells and IL-10 inhibits the proliferation of both $CD4^+$ and $CD8^+$ T cells. $CD14^+$ cells poorly stimulate T cells and were not considered further. The inhibitory effect of IL-10 on T cell priming is probably mediated by reduced expression of MHC and co-stimulatory molecules, but also direct inhibitory effects on T cells might contribute. We also investigated the effect of IFNs produced by mDC2 e pDC, as they have anti-proliferative activity[23]. As shown, mDC2 and pDC were less potent than mDC1 in T cell stimulation, and no significant effect was found for IFNλ and IFNα.

The effect of IL-10 on $CD4^+$ and $CD8^+$ T cells is controversial: IL-10 was shown to act directly on $CD4^+$ Th17 cells, limiting their expansion thereby avoiding inflammatory diseases[7]; IL10 produced by so called “DC-10” can lead to the expansion of type 1 T regulatory cells (Tr1)[8].

The effect of IL-10 on $CD8^+$ T cells is dual: it was shown to induce cytotoxicity and be important for the prevention of lymphomas[11], on the other hand IL-10 was shown to promote diabetes in diabetic NOD mice.[12]

Importantly, we observed that IL-10 produced by mDC1 inhibits the proliferation of undifferentiated $CD4^+$ and $CD8^+$ T cells; on the contrary, when IL-10 is blocked, $CD8^+$ naïve T cells that proliferate are $GrzB^-$ and $IFN\gamma^-$, while $CD4^+$ are $IFN\gamma^-$ and $Foxp3^-$. Neutralization of IFNa produced by pDC or of IFNl produced by mDC2 have no clear effect on T cell priming.

Since It was proposed that IL-10 producing DC induce IL-10 producing type 1 T regulatory cells (Tr1)[8], we therefore compared the ability of different DC subsets to induce IL-10 production in primed $CD4^+$ T cells. Interestingly, data revealed that pDC, that lack IL-10 producing capacities, induced the highest levels of IL-10 in $CD4^+$ T cells via $IFN\alpha$. Conversely, mDC1 induced only low levels a of IL-10, and IL-10 neutralization had no effect.

In summary, human mDC1 are the relevant source of IL-10 in primary adaptive immune responses and shapes T cell responses to obtain properly differentiated cells.

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CHAPTER 4

SUMMARY, CONCLUSION AND TRANSLATIONAL IMPACT OF THE PROJECTS

A. mDC1 as potential targets for anti-tumor vaccines

My entire PhD work was focused on dissecting the functional properties of *ex vivo* isolated human dendritic cell subsets: myeloid DCs, $CD1c^+$ (mDC1) and $BDCA3^+$ (mDC2), and plasmacytoid DCs (pDC), as they are the relevant cells that prime human helper and cytotoxic T cell responses *in vivo*.

Previous studies suggested that human mDC2 could be the human counterpart of mouse $CD8\alpha^+$ DCs; this subset was shown to produce high levels of IL-12p70 as well as IFNλ, and to be excellent in cross-presentation.[1-4] These features are the requirements to elicit a strong antigen-specific CD8 T cell response and thus $CD8\alpha^+$ DC play a fundamental role to prime cytotoxic T cell responses in mice.

We asked if the capacity to prime cytotoxic T cell responses in humans was also restricted to mDC2 or if appropriate stimulation of other DC subtypes could induce similar functional features.

As shown in chapter 2, in the human DC system, mDC1 and not mDC2, are the principal producers of bioactive IL-12p70, but only when TLR4 and TLR8 were synergistically triggered. Notably, mDC2 shared with mouse $CD8\alpha^+$ DCs the ability to produce high levels of IFNλ. We also tested the ability of myeloid DC subsets to cross-present soluble protein antigens and, interestingly, both mDC1 and mDC2 acquired this capacity upon appropriate synergistic TLR stimulation. TLR-induced maturation, of DC together with CD4 help was also needed to efficiently prime naïve $CD8^+$ T cells. However, due to the high levels of IL-12 produced by mDC1 they were more efficient than other DC subsets to induce cytotoxic T cell differentiation.

In the second part of the project (chapter 3) we focused our attention on IL-10, a cytokine that was also selectively produced by mDC1 as well as by $CD14^+$ monocytes. IL-10 is a cytokine with pleiotropic functions, and in particular it inhibits $CD4^+$ effector T cells and promotes IL-10 producing regulatory T cells[5,6], but might be required for the generation of $CD8^+$ cytotoxic T cell responses.[7,8]

These paradoxical effects on $CD4^+$ and $CD8^+$ T cell responses prompted us to investigate the role of IL-10 produced by *in vivo* occurring DC, i. e. mDC1, in the priming of naïve $CD4^+$ and $CD8^+$ T cells. We found that DC-derived IL-10 inhibited the proliferation of both $CD4^+$ and $CD8^+$ T cells, and that it prevented the accumulation of poorly differentiated T cells rather than to selectively induce cytotoxicity. It is possible that a pro-cytotoxic effect of IL-10 is not exerted upon CTL priming, but rather on the expansion and differentiation of $CD8^+$ memory T cells. Upon priming DC-derived IL-10 might be rather important to prevent the expansion of poorly functional low affinity cells. Finally, pDC, but not mDC, efficiently induced IL-10 producing capacities of $CD4^+$ T cells, and pDC-derived IFNα seemed to play a relevant role. Conversely, no clear effect of mDC2-derived IFNλ on T cell priming and differentiation could be observed.

In conclusion we discovered that in the human system mDC1, and not mDC2, are the main producers of IL-12p70, a cytokine that potently promotes the differentiation of cytotoxic $CD8^+$ T cells as well as of $CD4^+$ Th1 cells. Importantly we showed that, if properly stimulated, all DC subsets could prime naïve $CD4^+$ and $CD8^+$ T cells in vitro.

Our results have relevant implications for vaccines that are designed to induce cytotoxic T cell responses. Conventional vaccines induce mainly $CD4^+$ helper T cells and antibodies, but are inefficient to induce cytotoxic responses that are particular important to control tumor growth. Our study indicate mDC1 are a very promising target for vaccination strategies that aim to induce CTL responses: IL-12p70 production, together with their cross-presentation abilities could lead to a strong induction of antigen-specific $CD8^+$ T cells. Moreover, our study clearly showed that mDC1 are a potent source of a broad panel of pro-inflammatory cytokines, when stimulated with synergistic TLR ligands. If TLR8 agonists with low cytotoxicity could be identified, the synergistic stimulation of mDC1 by adjuvants might lead to efficient CTL priming in the absence of too severe side effects.

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