Regulation of the multifaceted functions of human plasmacytoid dendritic cells: a polyphonic policy

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GENERAL INTRODUCTION

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‘The plasmacytoid dendritic cell as the Swiss army knife of the immune system: molecular regulation of its multifaceted functions.’

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1. THE IMMUNE SYSTEM

The immune system consists of a wide range of specialized white blood cell subsets that circulate in the blood and lymphatic system to detect and destroy intruding pathogens (like viruses, bacteria, fungi and parasites) that have passed physical barriers. Cells of the innate immune system (e.g. macrophages, dendritic cells, granulocytes, mast cells, basophils, eosinophils, NK cells and neutrophils) are able to quickly respond and provide a non-specific immune response. The innate immune system senses pathogens via pattern recognition receptors (PRRs) which include Toll-like receptors (TLRs) and C-type lectin receptors (CLRs) that are able to discriminate between defined pathogen associated molecular patterns (PAMPs) that may contain conserved proteins, carbohydrates, lipids and nucleic acid structures that derive from the invading pathogen. Different innate immune subsets express a different arsenal of PRRs. Still, this response is aspecific and pathogens might overcome this part of our defense. Engagement of PRRs by PAMPs leads to the induction of a signaling cascade that activates the immune cells to clear the invading pathogen and the infected cells.

Some innate immune cell subsets have the ability to take up, process and present antigens to the adaptive immune cells. These subsets are referred to as antigen presenting cells (APCs). Cells of the adaptive immune system are T and B cells, which respond when specific cell surface receptors present on these cells recognize the unique antigen that is presented by the APC. Cells of the adaptive immune system induce a specific immune response to pathogens and have the capacity to form immunological memory. This ensures that upon second invasion of the same pathogen the immune response is induced much faster and with greater magnitude.

Dendritic cells (DCs) are APCs that play a major role in antigen presentation. They recirculate through the body and in the tissues they act as sentinels for invading pathogens. In case of an infection they migrate to the draining lymph nodes (LNs) where they activate T cells. After 40 years of intensive research our knowledge about this diverse family of DCs has significantly increased. DCs are the main and most efficient APC subtype that sense pathogens and initiate an adaptive response through production of immune-modulating cytokines and the presentation of antigens particularly to naïve T cells. Two major types of DCs are known; conventional DCs (cDCs, also called myeloid DC) and plasmacytoid dendritic cells (pDCs). These distinct subsets are characterized by the different expression of PRRs and cell surface molecules. Human blood derived cDCs are characterized by surface expression of HLA-DR and CD11c, and either blood dendritic cell antigen (BDCA)-1 or BDCA-3. cDCs are the professional APCs as they are most efficient in antigen uptake, processing and presentation to T cells. The second major human DC subtype are the pDCs, which express BDCA-2 and BDCA-4. Non-activated pDCs have a round shaped appearance with an abundant endoplasmic reticulum. pDCs are found in T cell areas of LNs and spleen, mucosa-associated lymphoid tissues, thymus, tonsil, liver and peripheral blood. Human pDCs express CD123 (IL-3Rα), CD45RA, HLA-DR and CD4, and lack expression of CD11c and CD14. The equivalent pDCs identified in the mouse with similar functional characteristics expresses CD11c, HLA-DR, bone marrow and stromal cell antigen 2 (BST-2), B220 and Siglec-H.
2. PLASMACYTOID DENDRITIC CELLS

As mentioned, pDCs belong to the innate immune system, as they can directly respond towards a broad range of viruses and bacteria and produce massive amounts of type I Interferons (IFN) affecting other immune cells. This, together with the notion that pDCs mature into APCs with the capacity to present antigens to T cells have put pDCs forward as a link between innate and adaptive immunity. Conversely, pDCs can also induce tolerance instead of immunity, which is a balance that is still incompletely understood. The work presented in this thesis contributes to a better understanding of the molecular mechanisms that regulate the multifaceted pDC functions.

2.1 Development of plasmacytoid dendritic cells

pDCs derive from CD34+ hematopoietic progenitor cells (HPCs) in the bone marrow. First, CD34+ HPCs develop into common myeloid and common lymphoid progenitors (CMPs and CLPs). As shown in the mouse both CMPs and CLPs can give rise to a common dendritic cell progenitor which can generate cDCs and pDCs. pDCs can also derive from CLPs through an intermediate precursor that generates B cells (reviewed in7). pDC precursors have been found in human cord blood, fetal liver, and fetal bone marrow, which suggests that pDCs develop from within these primary lymphoid tissues. It is not yet exactly clear how the commitment to different DC subsets during DC ontogeny is induced. There are different transcriptional regulators required during DC ontogeny. Recently, it was shown in mice that a Flt3+ common DC precursor for both cDCs and pDCs exists, which cannot give rise to any other cell lineage. Irrespective whether the progenitor is of myeloid or lymphoid origin, pDC development in human and in mice is strictly Fms-like kinase 3 ligand (Flt3L)-dependent. Isolation of CD34+ HPCs from fetal liver, cord blood or thymus, gave rise to pDCs in vitro by addition of recombinant Flt3L. In line with this, it has recently been shown in the mouse that BCL11a is required for pDC development in vivo by controlling the expression of Flt3 in early HPCs.

Spi-B, an E-Twenty-Six (ETS) transcription factor, which is expressed in pDCs and B cells is an important transcription factor in pDC development. In human B cells Spi-B was shown to regulate plasma cell differentiation. Spi-B is crucially involved in human pDC development since decreased levels of Spi-B by RNA interference in HPCs resulted in strong inhibition of development of pDCs in vitro and in vivo. Furthermore, Spi-B overexpression in HPCs stimulated pDC development, while this impaired the development of other lineages like B, T and NK cells. Spi-B is highly homologous to transcription factor PU.1, which is also required for the development of multiple other hematopoietic lineages. Mice lacking Spi-B do not show abnormalities of myeloid or lymphoid development which may be due to the redundancy between Spi-B and PU.1. PU.1 is exclusively expressed in human cDCs, but not in pDCs, whereas Spi-B is exclusively expressed in pDC but not cDC. However, downregulation of PU.1 in human HPCs blocked the development of both pDCs and cDCs in vitro, which may suggest that PU.1 already acts at an early progenitor stage prior to pDC commitment. Based on the notion that Inhibitor of DNA binding (Id)2 and Id3 when overexpressed in CD34+ HPCs blocked pDC development, suggests that E-proteins are involved. E proteins, including E12, E47, HEB and
E2-2, are transcription factors that contain a basic helix-loop-helix (bHLH) structure. The E protein E2-2 is primarily expressed in pDCs and crucial for pDC development in humans and in mice. Furthermore, it was shown that E2-2 can directly bind the promoter regions of pDC-specific genes like Spi-B, IFN regulatory factor (IRF)-7 and IRF-8. In normal conditions, pDCs are constantly generated in the bone marrow and migrate towards lymphoid organs. This may rely on Runx2, which in mice was shown to be expressed in pDCs in an E2-2 dependent manner, and required for pDCs to migrate from the bone marrow into the periphery.

3. PDCS IN IMMUNITY

pDCs are present in blood, bone marrow and lymphoid organs and can be recruited into T cell rich areas of lymphoid organs which is associated with the expression of CD62L and CCR7. Under steady state conditions pDCs are hardly detected in other peripheral tissues like skin and lung. Human pDCs selectively express PRRs TLR7 and TLR9, which are expressed in the endosomes and can respond to PAMPs via recognition of single-stranded (ss) RNA and double stranded (ds) DNA respectively. pDCs have multifaceted functions in immunity, as they not only have an antiviral function through type I IFN production, but are also able to induce antiviral immune responses by activating antigen specific T cells and play a role in tolerance. Although pDCs are only present at low frequency (0.2-0.8%) among PBMCs, they are able to dedicate 60% of the induced transcriptome to expression of type I IFN genes (IFN-α, -β, -ω, -τ) and type III genes (IFN-λ1-3) in the first six hours and can produce 100-1000 times more type I IFN compared to any other blood cell types after viral infection. Type I IFN induces a strong antiviral state by induction of antiviral molecules, such as MxA, that increase resistance of cells to viral infections and promotes apoptosis of the infected cells. Thereby, pDCs are very important players in antiviral immunity. The main functions of pDCs will be discussed in the following paragraphs.

3.1 Sensing nucleic acids from pathogens

APCs are able to sense a broad spectrum of pathogens by recognizing diverse PAMPs like nucleic acids, lipoproteins, proteins and lipids. TLRs are a family of conserved membrane-spanning molecules that can recognize different PAMPs. To date, 13 different TLRs have been detected, each shaping a specific immune response upon infection. Human pDCs selectively express TLR7 and TLR9 in their intracellular endosomal-lysosomal compartments, where these TLRs are involved in detection of microbial nucleic acids. TLR7 senses viral ssRNA and synthetic oligoribonucleotides. TLR7 is required to respond to ssRNA viruses such as influenza virus and vesicular stomatitis virus (VSV). TLR9 senses dsDNA rich in unmethylated CpG sequences and synthetic CpG oligonucleotides. TLR9 was found to be required for pDCs to respond to DNA viruses like herpes simplex virus 1 and 2 (HSV-1, -2) and murine cytomegalovirus (CMV). Engagement of TLR7/9 in the early endosomes leads to the production of type I IFN but poorly induces differentiation of pDCs, whereas TLR engagement in the late endosomes leads to maturation of pDCs and fails to induce a type I IFN response.
TLR9 or signaling molecules downstream of TLRs, completely lost the ability to produce IFN-α when challenged with the TLR9 ligand CpG ODNs. Given that human pDCs do not express TLR2, TLR4, TLR5 and TLR3, which are expressed on cDCs, pDCs are not able to respond to lipopolysaccharide (LPS), flagellin, peptidoglycans and dsRNA.

The signaling cascade downstream of TLR7/9 depends on the adapter protein myeloid differentiation primary response protein 88 (MyD88). MyD88 is critical for TLR signaling since mice lacking functional MyD88 showed increased susceptibility to virus infections. MyD88 complexes with IL-1 receptor-associated kinase (IRAK-1) and IRAK-4, tumor-necrosis factor receptor-associated (TRAF)-6 and TRAF-3 and IRF-7. Both TLR7 and TLR9 signaling pathways culminate in activation of nuclear factor -κB (NF-κB), which depends on phosphorylation of inhibitory (I) -κB proteins by the kinases IKKα and IKKβ and subsequent degradation. Known subunits of the NF-κB family are RelA/p65, RelB, cRel, p52, p50 that form homo- or heterodimers. The RelA/p50 heterodimer is most frequently activated after TLR signaling. RelA/p50 dimers are directly responsible for expression of co-stimulatory molecules (i.e. CD40, CD80, CD86), while IRF-5 together with NF-κB and mitogen-activated protein kinase (MAPK) activation is crucial for the production of IL-6 and TNF-α. Phosphorylation of IRF-7, likely mediated by PI3K activation, leads to IRF-7 nuclear translocation where it initiates IFN-α/β gene transcription. A schematic overview of the TLR pathways in pDCs can be found in Figure 1.

3.2 Professional IFN type 1 producing cells
pDCs constitutively express high levels of IRF-7 which upon phosphorylation after TLR activation can enhance the IFN gene expression very rapidly. It was shown in mice that IRF-7 is essential for type I IFN production, since pDCs isolated from IRF-7-/- mice lost the ability to produce IFN-α and IFN-β after stimulation. After IFN-α or IFN-β binding to the IFN receptor, multiple interferon stimulated genes (ISG) are transcribed, such as dsRNA dependent protein kinase (PKR), the GTPase MxA, and 2’-5’oligoadenylate synthetase (OAS-1), that all have antiviral effects. Type I IFNs produced by pDCs are pleiotropic cytokines that also initiate antiviral functions of cells of both the innate and adaptive immune system. These cytokines increase NK cell mediated cytotoxicity and IFN-γ production, and differentiation and maturation of cDCs leading to secretion of cytokines like IL-12, IL-15, IL-18 and IL-23. pDCs also respond to the IFNs in an autocrine or paracrine manner by up-regulation of IRF-7 thereby creating a positive feedback loop. In addition, IFN-α/β promote pDC survival via induction of anti-apoptotic genes. Activated pDCs also produce IL-6 and TNF-α which are regulated via NF-κB activation after TLR stimulation and enhance the adaptive immune response by supporting pDC maturation. pDC mediated adaptive immunity is affecting T cell functions by inducing survival and activation, Th1 differentiation and IFN-γ production and antiviral CTL responses. pDC activation and cytokine secretion increase the ability of cDCs to cross-present exogenous antigens to CD8+ T cells and promote their clonal expansion. Moreover, by producing type I IFN and IL-6 pDCs are involved in differentiation of B cells into plasma cells and production of immunoglobulines. Mouse and human pDCs also produce chemokines (CXCL19, CXCL10, CCL3, CCL4 and CCL5) by which they can attract NK and activated CD4+ and CD8+ T cells to sites of infection.
3.3 Antigen presentation

In contrast to cDCs, pDCs are hardly found in the peripheral tissues under steady state conditions. While pDCs circulate in the bloodstream and reach secondary LNs through high endothelial venules, other DC subsets enter secondary lymphoid organs via lymph vessels. After inflammation, pDCs leave the bloodstream and migrate to the infectious site to take up antigens, migrate to the LNs and present the antigens. Activated pDCs produce different chemokines and cytokines, acquire a DC morphology, up-regulate expression of the T cell co-stimulatory molecules CD40, CD80 and CD86. A complex of TRAF-3, BTK, IRAK-1, inhibitor of NF-κB kinase α (IKKα) and phosphoinositide 3-kinase (PI3K) activates and phosphorylates Interferon Response Factor (IRF)-7, which then translocates to the nucleus and initiates expression of type I IFN (IFN-α, IFN-β, IFN-γ, IFN-ω) genes.

The view that pDCs are inferior to cDCs in antigen presentation has reduced the focus to do research in this direction. The limited antigen presentation capacity of pDCs may be due to their limited ability to capture antigens, the lower expression levels of MHC class I and II and more rapid turnover of these molecules on the pDC surface compared to cDCs. Although it is clear that pDCs are well capable of presenting endogenous antigens, they appear to be
less efficient in presenting antigens captured from the extracellular environment. This may be due to their reduced ability to internalize antigens by phagocytosis or macropinocytosis. Internalization of exogenous antigens may act through receptor-mediated endocytosis. C-type lectins DEC-205, DC immunoreceptor (DCIR), FcγRIIa and BDCA-2 are expressed on pDCs and able to act as antigen uptake receptors resulting in antigen-specific CD4+ T cell activation. In mice CpG-matured pDCs efficiently induced CD8+ T cell responses against endogenous antigens, although this was not related to the ability of cDC priming. Currently, the efficiency of pDCs to cross-present exogenous antigens to CD8+ T cells is being re-evaluated. Accumulating evidence suggests that pDCs are endowed with an efficient cross-presenting machinery and able to effectively cross-present antigens. Taking together, although pDCs are less efficient APCs compared to cDCs, they can be considered as professional APCs that efficiently present exogenous antigens to T cells. Their antigen presentation capacity in combination with their ability to produce type I IFN, makes pDCs very interesting targets for anti-tumor therapy research.

3.4 Tolerance induction

Although pDCs are able to induce immune responses, they are also known to induce tolerance. The tolerogenic functions of pDCs are of great interest and currently under investigation. It has been shown that pDCs contribute to peripheral T cell tolerance in transplantation, tumor escape, and oral and mucosal tolerance. TLR9 ligation in pDCs induces the expression of the immunosuppressive enzyme indoleamine-2,3-dioxygenase (IDO), which is able to degrade the essential amino acid tryptophan, thereby suppressing T cell responses. In many solid tumors, pDCs are present and contribute to the immunosuppressive environment. Another tolerogenic mediator on activated pDCs is the expression of inducible co-stimulatory ligand (ICOS-L) which binds inducible T cell co-stimulator (ICOS) on naive CD4+ T cells and leads to the differentiation of IL-10-producing Tregs. In ovarian cancer patients, pDCs and ICOS Foxp3+ Tregs were found to be strong predictors for disease progression, suggesting an essential role for pDCs and ICOS-L immunosuppression in ovarian cancer. Melanoma progression in humans is associated with tumor-infiltrating pDCs promoting pro-inflammatory Th2 and Tregs through OX40L and ICOS-L, respectively. In addition, a subset of pDCs expressing lymphocyte activation gene 3 (LAG3) negatively regulated T cell activation while positively regulating Treg functions by production of IL-6. Furthermore, BST-2 is endogenously expressed in tumors and engages immunoglobulin-like transcript (ILT)-7 expressed on pDCs which suppresses the production of type I IFN in response to TLR ligation and thereby reduces the anti-tumor response. A subset of ‘tolerogenic pDCs’ has been identified in mouse gut and thymus and express the gut and thymus homing chemokine receptor CCR9, which is lost upon TLR triggering correlating with reduced ability to prime tolerance. In humans, an equivalent tolerance inducing pDC subset has not yet been identified. The tolerogenic character of pDCs in the human thymus was illustrated as they induced differentiation of CD4+CD8+ double positive T cells into regulatory T cells (Tregs) that produced IL-10.
Another way to induce tolerance may act via the serine protease granzyme B (GrB) in pDCs, that is up-regulated in response to IL-3, either alone or in combination with IL-10 and has been found to suppress T cell proliferation in a perforin-independent manner.\[123\] We show in chapter 4 that IL-21 was also capable to induce GrB in pDCs, which impaired CD4\(^+\) T cell proliferation and that inhibition of GrB activity restored pDC-induced T cell activation. Neither IL-21 nor IL-3/IL-10 alone altered pDC cytokine production or co-stimulatory molecule expression. Ample evidence suggests that IL-21 has potent anti-tumor activity mainly by activating NK cells and CD8\(^+\) T cells.\[124\] Conversely, however, it can be envisioned that IL-21 may add to create an immunosuppressive environment by stimulating intra-tumoral pDCs to produce GrB and hence dampening anti-tumor T cell responses. In Figure 2 an overview of the multifaceted functions of pDCs is shown.

### 4. PDC PATHOGENESIS

pDCs efficiently respond to viral or bacterial nucleic acids, but normally do not respond to self-nucleic acids that are released in the extracellular environment. Free self-nucleic acids are rapidly degraded and do not enter the endosomal compartment of pDCs. However, in certain autoimmune disorders it became clear that pDCs play an important role in the initiation or maintenance of these

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**Figure 2:** The multifaceted functions of pDCs in the immune system. pDCs have multiple functions in the immune system. After TLR activation, pDCs produce cytokines, such as type I IFNs, IL-6 and TNF-\(\alpha\), that have pleiotropic functions in the immune system (see main text). In the antiviral response, type I IFNs produced by pDCs directly repress viral replication. Furthermore, pDCs secrete IL-6 and TNF-\(\alpha\), and express co-stimulatory molecules, including CD40, CD80 and CD86, which enable pDCs to present antigens to T cells. pDCs can also express Granzyme B, which is involved in induction of both T cell tolerance and cytotoxicity. Expression of TNF-Related Apoptosis Inducing Ligand (TRAIL) is also involved in cytotoxicity.
diseases as they did sense self-nucleic acids via TLRs expressed in the endosomal compartments.\textsuperscript{125,126} Uncontrolled and unwanted production of IFN-α plays a role in autoimmune diseases like psoriasis,\textsuperscript{127} systemic lupus erythematosus (SLE)\textsuperscript{128,129} and Sjögren’s syndrome (SS).\textsuperscript{130}

In the skin autoimmune disorder psoriasis free self-DNA forms complexes with the cationic antimicrobial peptide LL-37, which is highly expressed in skin lesions.\textsuperscript{131} These complexes are able to enter the endosomal compartments of pDCs where they trigger TLR9, which induces chronic type I IFN production that triggers T-cell mediated immunity and disease development.\textsuperscript{126,127}

In SLE, patients can be diagnosed to have high concentrations of IFN-α in their blood. In this disease, self-nucleic acids are complexed with auto-antibodies directed against nucleic acids or nucleoproteins.\textsuperscript{125,131,132} Binding to FcγRII on the surface of pDCs induced transport of the self-nucleic acids into the endosomes were they ligate TLR7 or TLR9. This results in continuous activation of pDCs to produce type I IFNs leading to activation and maturation of cDCs that stimulate auto-reactive T cells,\textsuperscript{133} and promote the differentiation of auto-reactive B cells into auto-antibody secreting plasma cells.\textsuperscript{134} It was shown that the IFN-signature correlates with disease activity and severity and could be used as a biomarker.\textsuperscript{128,134,135} While pDC numbers in blood of SLE patients are reduced, pDCs do infiltrate in skin and renal lesions.\textsuperscript{136} In Figure 3 an overview of pDC functions in pathogenesis are shown.

![Figure 3: Role of pDCs in pathogenesis](image)

**Figure 3: Role of pDCs in pathogenesis.** In auto-immune diseases, such as SLE and psoriasis, it was shown that pDCs are able to respond to self-nucleic acid complexes that results in unwanted production of copious amounts of type I IFNs. Viruses may escape the antiviral type I IFN response by binding to inhibitory receptors, which downregulate TLR- induced type I IFN production. Last, pDCs may be involved in tumor escape as they induce Tregs, which reduce tumor specific T cell activation.
5. REGULATION OF THE TLR RESPONSE

5.1 Regulating cell surface receptors

Based on the notion that pDCs are potent inducers of the immune system and are important for linking the innate and adaptive immune system, pDC activation needs to be tightly controlled. pDCs express several cell surface receptors that enable regulation of the TLR-induced type I IFN response. The receptors that can modulate the type I IFN response are BDCA-2, ILT-7, natural killer protein 44 (NKp44), DCIR, high-affinity immunoglobulin Ig(E) receptor (FcεRI), adenosine diphosphate P2Y receptors, a nitric oxide-induced cGMP-dependent receptor, prostaglandin E2 receptors, and CD300a/c.

While natural ligands for most of these receptors are still unknown, ILT-7 binds to the IFN induced protein BST-2 which can be expressed on surrounding cells and act as negative feedback necessary to prevent overactivation.

In this thesis we have focused on BDCA-2, which is a type II CLR and is selectively expressed on pDCs and associates with FcγR. BDCA-2 signaling interfered with TLR9-induced activation of pDCs, thereby inhibiting IFN type I production.

**Figure 4: C-type lectin BDCA-2 signaling can interfere with TLR9-induced activation of pDCs.** Several known ligands for the C-type lectin BDCA-2 expressed on human pDCs are HIV-glycoprotein 120 (gp120), asialo-galactosyl oligosaccharides and Hepatitis C virus E2 protein. Binding of these ligands to BDCA-2 leads to inhibition of the TLR-induced cytokine production of human pDCs. BDCA-2 signals via association with the γ-chain of FcεRI, which contains an immunoreceptor tyrosine-based activation motif (ITAM). This pathway inhibits TLR-induced type I IFN, and also IL-6 and TNF-α production by pDCs.
I secretion (Figure 4). Furthermore, crosslinking of BDCA-2 with monoclonal antibodies led to receptor internalization, antigen uptake, Ca\textsuperscript{2+}-influx and signaling via FcɛRIγ-dependent pathway.\textsuperscript{67,144} BDCA-2 antibody-derived peptides were presented via MHC class II to T cells, which shows efficient antigen delivery and processing in pDCs. Although we start to obtain some knowledge about the function of BDCA-2, the regulation of BDCA-2 expression remains elusive. In chapter 5 we describe Spi-B as a new regulator of BDCA-2 expression in human pDCs.

Interestingly, some viruses express glycoproteins that bind to BDCA-2 and thereby down-regulate the orchestrated antiviral response by inhibition of type I IFN production. Known viral ligands able to bind BDCA-2 are glycoprotein gp120 on human immunodeficiency virus (HIV)-1\textsuperscript{146}, E2 protein on hepatitis C virus (HCV)\textsuperscript{147} and hepatitis B surface antigen (HBsAg) of hepatitis B virus (HBV).\textsuperscript{148} While the natural ligand for BDCA-2 remains elusive, BDCA-2 can bind to asialo-galactosyl-oligosaccharides.\textsuperscript{149} In chapter 6 we describe a cell population in the thymus that expresses a natural ligand for BDCA-2.

5.3. Micro-RNAs; a new level of regulation

MicroRNAs (miRNAs) were first discovered in 1993.\textsuperscript{150} MiRNAs are small noncoding RNA sequences that are endogenously produced and exist in animals, humans, plants and viruses.\textsuperscript{151-153} They play a role in many processes like organ development, cell differentiation and diseases and have been identified as a new layer of regulation.\textsuperscript{154,155} MiRNA genes can be found under their own promoter, as well as located in introns or exons of coding and noncoding genes. After transcription by RNA polymerase II, pri-miRNAs are cleaved by Drosha and exported into the cytoplasm. Enzyme Dicer will cleave the stem-loop motif and the pre-miRNA will become a mature double stranded miRNA of which one strand will associate with the RNA-induced silencing complex (RISC) (Figure 5). The ~19-24 nucleotides of the miRNA act by down-regulating gene expression through targeting the 3’-untranslated region of messenger RNAs (mRNAs) resulting in mRNA degradation and/or inhibition of mRNA translation. Because miRNAs are able to bind target mRNA by imperfect complementarity, one miRNA can regulate multiple mRNAs.\textsuperscript{156} MiRNAs have emerged as regulators of TLR signaling pathways in a broad range of human immune cells, including macrophages, monocytes and T cells.\textsuperscript{155} It has been shown that miR-155 and miR155* are able to regulate the type I IFN response after stimulation of TLR7 in pDCs.\textsuperscript{157} Furthermore, miR-29b and miR-29c are involved in TLR control of glucocorticoid-induced apoptosis by directly targeting Bcl-2 and Mcl-1 in human pDCs.\textsuperscript{158} It is interesting that in various cell types, miRNAs are induced upon TLR activation thereby creating a negative feedback loop.\textsuperscript{155,159} In chapter 3 we show that miR-146a expression is induced upon TLR7 and TLR9 triggering in human pDC and interferes with TLR-induced cytokine production, maturation and survival. Together with mouse data\textsuperscript{160,161}, miR-146a is recognized as a brake of the immune response by downregulating IRAK-1 and TRAF-6 expression and hence dampening of TLR-induced responses. Reduced miR-146a expression has been found in PBMC of SLE patients and this may add to the observed elevated concentrations of IFN-α and IL-6 in the serum.\textsuperscript{162} Accordingly, miR-146a polymorphisms are associated with SLE.\textsuperscript{163-165}
6. NOTCH RECEPTORS

Notch receptors are type I transmembrane glycoproteins that consist of an extracellular and intracellular domain. Notch encodes an evolutionary conserved transmembrane receptor that is involved in many differentiation processes. There are four Notch receptors identified in mammals (Notch1-4), which can be engaged by five different transmembrane ligands: Jagged1 (Jag1), Jag2, Delta-like (DLL)1, DLL3 and DLL4. In the hematopoietic system, Notch receptors are best-studied for their effects in lymphocyte development in the thymus, cell differentiation, proliferation and survival of T cells. Activation of Notch receptors typically occurs via cell-cell contact as Notch receptors are engaged by their ligands that are expressed on the surface of a neighboring cell. This allows translocation of the intracellular Notch domain (NICD) by a two-step proteolytic cleavage involving a metalloprotease followed by γ-secretase. NICD releases co-repressors from the transcription factor CSL/CBF-1 (called RBP-Jκ in the mouse), which allows the recruitment of co-activators to form a complex leading to transcription of Notch target genes. Many target genes are regulated by Notch receptors, but the best-characterized targets are members of the basic helix-loop-helix (bHLH) transcriptional repressor hairy enhancer of split (HES) family, including HES1 and HEY which are transcriptional repressors that function as feedback inhibitors of Notch signaling. Other Notch targets that have been described are C-MYC, CD25, NF-κB, p21 and cyclin D. A schematic overview of Notch ligation and NICD translocation is shown in Figure 6.

It was shown that human pDCs express Notch1, Notch2, Jag1, Jag2, DLL1 and DLL4 and TLR7 signaling increased expression of Notch1. A role for Notch in pDC functions was suggested.
Figure 6: Notch4 pathway in human plasmacytoid dendritic cells. The transmembrane receptor Notch4 is activated via binding of one of the Notch-ligands, including DLL1, DLL2, DLL3, Jag1 or Jag2. Thereafter, the intracellular domain of Notch4 is cleaved by an ADAM-metalloprotease and γ-secretase. This cleavage releases the intracellular domain of Notch4 (NICD4), which then translocates to the nucleus. NICD4 regulates gene expression by binding to CBF1 suppressor of hairless Lag-1 (CSL), which is converted from a repressor of Notch target gene transcription into a transcriptional activator.

since the expression of IFN-α, CD83 and CD86 were reduced after HSV-2 infection in the presence of γ-secretase inhibitor DAPT.\textsuperscript{180} Notch functions in pDCs remain poorly understood and there are only limited studies on the role of Notch4. In chapter 2 we show that Notch4 is expressed on human pDCs and was induced after TLR ligation. Notch4 ligation has an effect on development and function of pDCs, which is an addition of our understanding how pDCs are functioning in conditions were Notch ligands are expressed and TLR ligands are lacking.

6. THYMIC PDCS

As mentioned earlier, pDCs are present in the thymus both in human and mouse.\textsuperscript{181,182} The thymus is a lymphoid organ in the upper anterior thorax, just above the heart. Bone marrow progenitors enter the thymus via the bloodstream and are instructed to become non-self-peptide recognizing T cells before they are allowed to migrate into the periphery. In fetal stages and in young children, the thymus is the source of large numbers of newly generated T cells. After puberty, development of new T cells slows down and the thymus involutes.\textsuperscript{183} Thymic tissue consist of several lobes, that each contain an outer cortex and inner medulla. Multipotent progenitors that arrive from the bone marrow enter at the cortico-medullary junction.\textsuperscript{184} The early stages of T cell development take place in the cortex, which is packed with immature proliferating thymocytes that interact with stromal epithelial cells and respond to interleukin-IL7. Thymic precursors express CD34\textsuperscript{185} and upregulate CD1a when transitioned into the T cell lineage (Figure 7). The next process is β-selection in which the TCRβ locus in thymocytes that
now express CD4, is rearranged and in combination with a pre-TCR α chain lead to survival and proliferation. After completion of the TCRαβ rearrangement developing double-positive (CD4+CD8+) thymocytes die unless they become rescued by low-affinity interactions with self-peptide-MHC complexes by a process called positive selection. After positive selection thymocytes differentiate into either CD4+ or CD8+ single positive thymocytes. When the TCR on thymocytes recognize self-peptide-MHC by a high-affinity interaction these thymocytes will undergo apoptosis due to a process called negative selection.\textsuperscript{186} Only 5% of the developing thymocytes survives selection criteria and migrate into the periphery.\textsuperscript{187}

![Diagram of T cell development in the human thymus.](image_url)

**Figure 7: T cell development in the human thymus.** The human thymus consist of multiple lobes that each have a medullary area and a cortex. Different types of cells are present in the human thymus, like cortical epithelial cells (CTECs), medullary epithelial cells (MTECs), macrophages (Mφs), dendritic cells (DCs) and plasmacytoid dendritic cells (pDCs). pDCs are located in the medulla and at the corticomedullary junction (CMJ) of the human thymus. CD34+ hematopoietic progenitor cells (HPCs) enter the thymus at the CMJ and migrate deep into the cortex committ into the T cell lineage. HPCs will upregulate CD1a after T cell commitment and will undergo TCR-β selection. Double positive CD8+CD4+ thymocytes are either positively selected, negatively selected or die by neglect. Developing T cells that are positively selected migrate into the medulla where they differentiate into single positive CD8 or CD4 T cells able to migrate out of the thymus into the blood stream.
In the thymus, pDCs are located at the cortico-medullary junction and in the thymic medulla.\textsuperscript{181,182} Thymic pDCs can phenotypically be distinguished from peripheral pDCs by expression of CD2, CD5, and CD7.\textsuperscript{182} The functions of pDCs in the human thymus are still unknown, but previously it was shown that pDCs that become activated via thymic stromal lymphopoietin (TSLP) or CD40 ligand (CD40L) plus IL-3, play a role in the induction of regulatory T cells which produced IL-10 and TGF-β.\textsuperscript{37,38}

Furthermore, thymic pDCs retain the ability to secrete IFN-α after CpG activation.\textsuperscript{188} Although IFN-α is known for its antiviral effects, it may also be important for normal functioning of the adaptive and innate immune system at low, physiological concentrations. Previously, we showed that recombinant IFN-α suppresses the development of human T cells \textit{in vitro} by inhibiting early steps of T cell development.\textsuperscript{189} Furthermore, we showed that IFN-α and thereby MxA, are constitutively expressed in postnatal and fetal thymus tissues in the medulla where the pDCs are localized.\textsuperscript{190} These findings suggest that pDCs might play a role in normal T cell development or might alter the requirements for negative selection. In \textit{chapter 7} we show that IFN-α is affecting T cell development and that both MxA and S1P-R1 expression are present at different T cell development stages and may regulate T cell selection and migration.


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