Developmental and cholera toxin-induced alterations in the expression of intracellular signalling molecules

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Gothenburg 2013

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Homo volans (from Faustus Verantius: Machinae Novae, plate 38. Venetiis, ca. 1615) (Faust Vrančić (1551-1617), Croatian polyhistor, inventor, lexicographer, writer, philosopher, and bishop)

Developmental and cholera toxin-induced alterations in the expression of intracellular signalling molecules

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Mom tati Miši

(to my father Milivoj)

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ABSTRACT

The innate immune system represents the first line of host defence that reacts promptly to microbial attacks. This system relies on several families of pathogen-recognition receptors (PRRs), which recognize pathogen-associated molecular patterns (PAMP) on a broad range of pathogens. Toll-like receptors (TLRs), the best-characterised PRRs, act through the signal transduction pathways to induce production of the pro-inflammatory cytokines that participate in innate responses against pathogens and also provide signals for the activation of adaptive immunity.

At birth, the immune system is characterised by immaturity and undeveloped functions. This is reflected in the greater susceptibilities of neonates to various pathogens, in particular viruses, such as herpes simplex virus (HSV), respiratory syncytial virus, and cytomegalovirus. The aim of this thesis was to characterize in umbilical cord blood cells the expression profiles of PRRs that sense viral nucleic acids, and to ascertain whether these profiles represent a molecular basis for the inadequate neonatal responses to viral infections. Neonatal natural killer (NK) cells, normally involved in anti-viral and anti-tumour defences, were found to lack TLR3 mRNA and protein expression. Consequently, they could not respond to the TLR3 ligand poly(I:C) by producing IFN-γ, which, in contrast, was abundantly secreted by adult NK cells. The neonatal NK cell cytotoxicity against tumor cells, HSV-infected targets, and in stimulation with HSV was also impaired. In similarity to the cord blood NK cells, TLR3 mRNA expression was low in decidual NK cells obtained from placentas at full-term delivery, but not in mononuclear

blood cells from pregnant women. In adult mononuclear blood cell populations, the highest level of TLR3 expression was associated with the cytotoxic $CD56^{dim}$ NK cell subset.

Dendritic cells (DCs) link the innate and adaptive immunity systems through TLR signalling. DCs also induce tolerance to host antigens, and regulate the magnitude of immune responses by suppressing immune reactions, partly mediated by the tryptophan-degrading enzyme indoleamine-2,3 dioxygenase (IDO). Cholera toxin (CT), which is a strong bacterial immunogen and a DC-maturation-promoting adjuvant, was investigated in the context of IDO induction. CT-pulsing of DCs induced the expression of IDO mRNA but not the production of IDO protein. However, CT primed for CD40L-induced IDO mRNA and protein activity. The CT-pulsed DCs potently stimulated allogeneic and autologous T-cell responses, and these activities were not regulated by IDO. However, CD40L-induced IL-12p40 production was dependent upon IDO.

The mechanism of CT adjuvanticity has been addressed with respect to interference with viral-recognition receptor pathways. Among the different viral nucleic acid-sensing receptors, CT showed selective inhibition of TLR7 mRNA expression. Although they represent the main mechanisms through which CT exerts its effects, the induction of cyclic adenosine monophosphate and PKA activation were not linked to the CT-mediated down-regulation of TLR7 mRNA. Instead, the PKC signalling pathway was implicated, as was IL-6.

Overall, the results presented in this thesis reveal new thinking about the ways in which TLRs sense infections and how CT acts as an adjuvant, with implications for innovative vaccine development and elucidation of the immune pathways that protect us against infections.

Keywords: dendritic cells, IDO, NK cells, TLRs, newborns

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SAMMANFATTNING PÅ SVENSKA

Vi lever i en värld full av mikroorganismer. För att kunna överleva har alla högre organismer därför, under evolutionen, utvecklat olika former av försvarsmekanismer som dels hindrar mikroorganismerna från att komma in i vår kropp och dels dödar de mikroorganismer som ändå lyckats komma in. Dessa försvarsmekanismer kallar vi för vårt immunsystem. Hos människan brukar vi traditionellt prata om en medfödd immunitet som reagerar snabbt och brett på alla former av mikroorganismer, och om förvärvad immunitet som är specifik bara för just den infektion som vi drabbats av, som tar lång tid att utveckla men som har en minnesfunktion och därmed kan reagera ytterst snabbt och effektivt vid ny smitta.

De vita blodkroppar som utgör stommen i det medfödda immunsvaret har olika strategier för att omedelbart veta om en mikroorganism har kommit in i vår kropp. Framförallt uttrycker de speciella receptorer både på cellytan och inne i cellerna, som känner igen strukturer som är unika för mikroorganismer, dvs som inte finns på våra egna celler och organ. I min avhandling har jag studerat regleringen av två sådana receptorer. Jag har dels undersökt om nyfödda barn, som vi vet är ytterst infektionskänsliga, inte har like mycket receptorer i sina celler som vi vuxna, och dels om bakterieprodukter kan modifiera uttrycket av dessa receptorer. Jag har kunnat visa att barn saknar en speciell receptor som heter TLR3. Denna receptor är viktig för att vi ska skydda oss mot herpesvirus, och jag har i mina försök kunnat visa att celler från nyfödda barn saknar förmågan att avdöda herpesvirus-infekterade celler. Dessutom visar mina studier att ett bakterietoxin påverkar uttrycket av en annan receptor, TLR7. Celler som normalt har höga halter av denna receptor slutade helt att syntetisera receptorn när de kom i kontakt med toxinet. Detta visar ett nytt sätt som bakterier manipulerar vårt immunförsvar på.

Vårt immunsvar ska reagera på allt som är farligt och icke kroppseget, men ska inte reagera mot vår egen kropp och inte heller mot det vi andas in eller äter. Om immunsystemet gör det så utvecklas vi autoimmuna sjukdomar och allergier. Immunsystemet har därför en mängd olika kontrollsteg som ska styra när och hur det ska reagera. Jag har studerat ett enzym som påverkar vårt specifika immunsystem, och stänger av det. Enzymet brukar kallas för IDO. Vi vet sedan tidigare att IDO behövs för att en gravid kvinna inte ska stöta bort sitt foster. Dessvärre kan IDO även vara ett hinder när vi vill att immunsystemet ska attackera en tumör. Forskargruppen jag arbetat tillsammans med under min doktorandtid har utvecklat en immunologisk terapi som, i alla fall hos möss som har en tumör, leder till att tumören

försvinner. Terapin bestod av celler, tumörantigen och bakterietoxin. För att säkerställa att behandlingen inte gav upphov till IDO-aktivering har jag undersökt hur bakterietoxinet påverkar IDO-syntesen och den biologiska aktiviteten hos IDO. Mina resultat visar att bakterietoxinet inte bidrog till mer IDO aktivitet vilket var bra.

Sammanfattningsvis har jag studerat hur olika receptorer och molekyler som finns inne i våra vita blodkroppar påverkas dels av vår ålder och dels av vår omgivning. Min forskning har därmed bidragit med några små pusselbitar till den komplicerade värld som vårt immunsystem utgör.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I Slavica L, Nurkkala-Karlsson M, Karlson T, Ingelsten M, Nyström J, Eriksson K. IDO expression and functional activity in dendritic cells exposed to cholera toxin. *Scandinavian Journal of Immunology* 2012; 76(2): 113-22.

II Slavica L, Nordström I, Nurkkala Karlsson M, Valadi H, Kacerovsky M, Jacobsson B, Eriksson K. TLR3 impairment in human newborns. *Journal of Leukocyte Biology* 2013; 94(5): 1003-11.

III Zenić L, Nordström I, Karlsson A, Eriksson K. Cholera toxin selectively blocks TLR7 expression in human lymphocytes.

In manuscript

CONTENT

ABBREVIATIONS

ADP	adenosine diphosphate
ALRs	AIM-2-like receptors
APCs	antigen-presenting cells
BDCA	blood dendritic cell antigen
cAMP	cyclic adenosine monophosphate
CBMCs	cord blood mononuclear cells
CD	cluster of differentiation
CpG ODN	CpG oligonucleotide
CT	cholera toxin
CTA	cholera toxin A subunit
CTB	cholera toxin B subunit
CTLs	cytotoxic T lymphocytes
CTLA-4	cytotoxic T lymphocyte antigen 4
DAMPs	damage-associated molecular patterns
DCs	dendritic cells
GM-CSF	granulocyte/macrophage-colony stimulating factor
HLA	human leukocyte antigen
HPV	human papillomavirus
HSV	herpes simplex virus
IDO	indoleamine 2,3-dioxygenase
IFI-16	interferon gamma-inducible protein 16
IFN	interferon
IL	interleukin
IRF	interferon regulatory factor
LPS	lipopolysaccharide
MDA-5	melanoma differentiation-associated gene 5
MDDCs	monocyte-derived dendritic cells
MHC	major histocompatibility complex
1-MT	1-methyltryptophan
MyD88	myeloid differentiation factor 88
NF-κB	nuclear factor κB
NK cells	natural killer cells
NO	nitric oxide
PAMPs	pathogen-associated molecular patterns
PBMCs	peripheral blood mononuclear cells
pDCs	plasmacytoid dendritic cells
PGE_2	prostaglandin E_2
PKA	protein kinase A

PKR	protein kinase R
poly(I:C)	polyinosinic-polycytidilic acid
PRRs	pattern-recognition receptors
RIG	retinoic acid-inducible gene I
RLRs	RIG-I-like receptors
SLE	systemic lupus erythematosus
TCR	T-cell receptor
TGF	transforming growth factor
Th	T helper
TRAF	TNF receptor-associated factor
TRIF	TIR-domain-containing adapter-inducing interferon β
TLRs	Toll-like receptors
TNF	tumor necrosis factor

1 INTRODUCTION

"Yet it was with those who had recovered from the disease that the sick and the dying found most compassion. These knew what it was from experience, and had now no fear for themselves; for the same man was never attacked twice - never at least fatally." [1] Without knowing the cause and the nature of the disease, the Greek historian Thucydides described the plague of Athens in 430 B.C. and noticed that those who had survived contagion were exempt from further episodes of the disease. The "exempted" ones are what we today call "immune". The term originates from the Latin *immunitas* ("protected from"), which in ancient Rome denoted the exemption from civic duties afforded to senators, as well as individuals who were excused from the military or other service. Although the Roman poet Marcus Annaeus Lucanus (39–65 A.D.) was the first to use the word "immunes" in his epic poem "Pharsalia" to describe the resistance to snakebite of the Psylli tribe of North Africa [2], it was not until the late 19th century (almost 2000 years later!) and the discoveries of the great immunologists Pasteur, Koch, Metchnikoff and others, that "immunity" became a commonly used expression in biology to define protection against certain diseases, especially infectious diseases.

1.1 The immune system – an overview

Central to the defence of an organism against disease is the immune system, which is defined as a complex system of cells, tissues, and organs that work together to combat attacks by different microorganisms and subsequent disease development. The foundation of the immune defence is the ability to discriminate normal from foreign entities, such as the disease-causing microbes (pathogens), as well as the detection of intrinsic tissues that have become transformed. This capability to discern "self" from "non-self" can be traced to a very early stage in evolution: genetically related sponges tend to fuse into large colonies, while unrelated sponges destroy the cells in the contact zone, thereby demonstrating a primitive form of tissue rejection.

In vertebrates, the immune system is comprised of two functional entities; the innate and adaptive. The innate immune system represents the first line of immune defence and is active in a normal individual from birth. Its components are present in the blood, the skin, and the mucous membranes that line the gastrointestinal and respiratory tracts, which constitute both physical and chemical barriers to the invading pathogens. It involves leukocytes, such as neutrophils, monocytes/macrophages, natural killer cells,

and dendritic cells, and their secreted products (cytokines, chemokines, and complement proteins). The main feature of the innate immunity is a rapid but "non-specific" response that provides a basic level of resistance to an infectious disease.

In contrast, the adaptive immune system becomes active only after an encounter with foreign substances, i.e., antigens, and is thus being acquired throughout life. It consists of T and B lymphocytes and their products, i.e., cytokines and antibodies, which circulate in the blood, lymphoid system, and tissues. These cells have a high specificity for a given antigen, ensuring the clearance of an infection, but also retrieving immunological memory, which enables the body to mount a quicker and stronger immune response upon a subsequent microbial/antigen encounter.

1.2 Immune cells relevant for this thesis

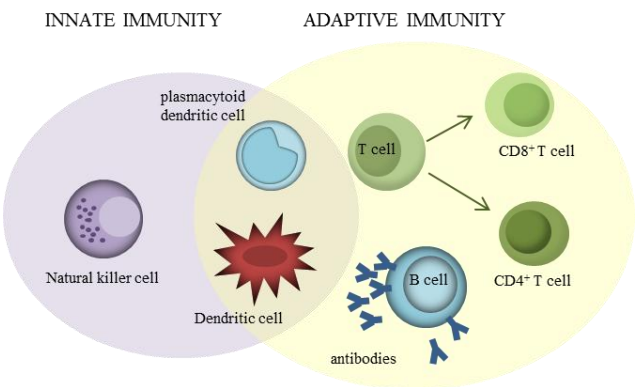

Figure 1. Components of the immune system with relevance to this thesis.

1.2.1 Dendritic cells and the control of immune responses

It is now 40 years since Ralph Steinman and Zanvil Cohn, in trying to understand how T cells are activated, discovered a new cell type that they called the 'dendritic cell' (DC) [3]. Despite initial scepticism on the part of scientific community, the role of DCs in coordinating the immune response to foreign antigens by activating adaptive immunity, as well as in inducing tolerance to self-antigens, is now firmly established, and has given rise to DC-based therapeutic interventions.

1.2.2 DC subsets

DCs make up a heterogeneous cell population that resides in almost all peripheral tissues. In humans, they are generally divided into two major subsets based on origin: myeloid DCs and lymphoid (plasmacytoid) DCs (Table 1). Myeloid DCs are usually referred to as "classical" or conventional DCs. Differentiation of DCs along the myeloid lineage gives rise to several different subsets of myeloid DCs with specific localisations: i) Langerhans cells in the skin epidermis; ii) interstitial DCs in the dermis and most other organs, mainly in connective tissues; and iii) blood myeloid DCs, which can be further divided into BDCA-1^+ and BDCA-3^+ cells [4]. Plasmacytoid DCs (pDCs) are of lymphoid origin, and are found in the blood and in the T-cell zones of lymphoid organs.

Table 1. The main human DC subsets, their origins, and tissue distributions

DC type	origin	location
Langerhans cells	myeloid	epidermis (skin)
interstitial DC	myeloid	most organs, connective tissue
blood DC	myeloid	blood
plasmacytoid DC	lymphoid	blood

The type and function of a DC are dynamically influenced by external stimuli. Monocytes that infiltrate an inflammatory site can differentiate into macrophages or monocyte-derived DCs (MDDCs) [5]. However, these MDDCs are different than the DCs that exist under homeostatic conditions [6], and their existence and the rate of monocyte-to-DC conversion *in vivo* remain subjects of debate [7]. Nonetheless, MDDCs are easily generated *in*

vitro by culturing monocytes with granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 and are widely used for *in vitro* studies of human DC function. This is something that we have taken advantage of in our studies.

In mice, DCs are categorised into myeloid DCs and pDCs, while the resident DCs in lymphoid tissues are subdivided into $CD8\alpha^-$ DCs (myeloid-origin), and $CD8\alpha^+$ DCs (lymphoid-origin); the human counterpart of the letter has only recently been assigned as blood BDCA-3^+ DCs [8]. Besides the existence of many DC subsets in both species, their levels of homology are still not completely understood and their functional equivalences remain to be determined.

1.2.3 T lymphocytes

Highly specialised T cells comprise a pool of numerous clones, each with surface T-cell receptors (TCRs) that are specific for a single antigen. There are two well-defined subpopulations of T cells:

Cytotoxic T lymphocytes (CTL), generally defined as $CD8^+$ T cells, destroy cells infected with intracellular pathogens (viruses or bacteria), tumour cells, and the cells of a grafted foreign tissue. The mechanism underlying this T cell-mediated cytotoxicity involves the release of cytoplasmic granules that contain perforin proteins, which create perforations in cells, and granzymes, which induce programmed cell death (apoptosis) in target cells.

T-helper (Th) lymphocytes ($CD4^+$ T cells) coordinate immune responses by releasing cytokines. Depending on the type of cytokine that it secretes, the Th cell can contribute to the activation of cytotoxic T cells, B cells, macrophages, and many other cell types.

1.2.4 DC maturation and T-cell activation

In peripheral tissues, immature DCs constantly sample antigens from the microenvironment. The antigens that are internalised (by phagocytosis, endocytosis or micropinocytosis) are processed into peptides and loaded onto *major histocompatibility complex* (MHC) class I (MHC I) and class II (MHC II) molecules for antigen presentation. Microbial components as well as the inflammatory cytokines released by other cell types in response to microbes, represent a "danger" signal that induces DC maturation [9, 10]. This process is characterised by the up-regulation of MHC and co-stimulatory molecules,

cytokine production, and altered expression of chemokine receptors. Eventually, the DCs migrate to the secondary lymphoid organs (spleen, lymph nodes, lymphoid tissues lining the mucosal surfaces), where they present antigens to naive $CD4^+$ and $CD8^+$ T cells, which recognise a specific antigen-MHC complex, thereby providing a "Signal 1" for T-cell activation (Figure 2). The TCRs of the $CD8^+$ T cells, together with the CD8 co-receptor, recognise antigens on the MHC I molecules that present peptides of intracellular proteins (intrinsic proteins or those from intracellularly replicating pathogens). The TCRs of $CD4^+$ T cells, in combination with the CD4 co-receptor, recognise the peptide of extracellular antigens on MHC II molecules. DCs interact *via* the co-stimulatory molecules CD80 and CD86 with CD28 molecules on T cells, generating the signal referred to as a "Signal 2". A final DC maturation step is achieved when the up-regulated co-stimulatory CD40 molecules bind to their CD40 ligands (CD40L) on $CD4^+$ T cells, resulting in: a) further up-regulation of co-stimulatory molecule expression and augmentation of the T-cell-stimulatory capacity; b) T cell-derived IL-2 production, which drives T-cell proliferation; and c) DC-derived IL-12 production, which governs a Th1-type $CD4^+$ polarisation and CTL differentiation; the letter supplies a polarising "Signal 3". Thus, during pathogen invasion, the DCs ensure that harmful antigens collected at the periphery, together with the co-stimulatory signals and the secreted cytokines, alert the antigen-specific T cells and harness the powerful adaptive system.

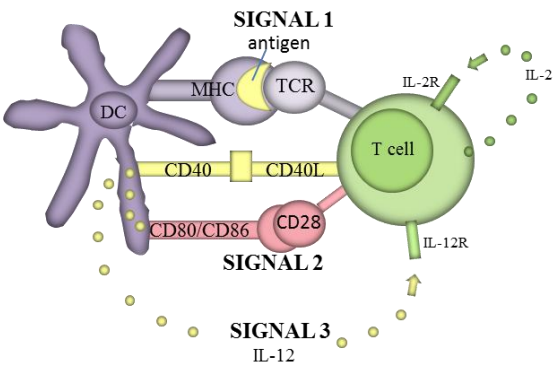

Figure 2. The main receptors involved in DC-T cell interactions.

1.2.5 DC-primed Th cell polarisation

In addition to their effects on DC maturation, the danger signals (i.e., pathogen components, pathogen-sensing receptors, and pro-inflammatory cytokines), influence DC-mediated polarisation of naive Th cells. Upon sensing a pathogen, the DCs (and other cell types) secrete cytokines that are instrumental in initiating an immune response. Depending on cytokine that is present in proximity to the naive Th cell (i.e., a Th0 cell) when it recognises an antigen presented by a DC, the Th0 cell will mature and differentiate into one of the following types of Th cell (Figure 3):

- **Th1 cells**, which secrete "Th1 cytokines", in particular IFN-γ. They trigger cell-mediated immunity (e.g., cytotoxic T cells), crucial for combating intracellular pathogens and tumours, and play roles in both inflammation and autoimmunity;
- **Th2 cells**, which secrete "Th2 cytokines", such as IL-4, IL-5, and IL-13, and activate macrophages and B cells, which function in defences against extracellular pathogens;
- **Regulatory T cells** (Tregs; defined as $CD4^+CD25^+Foxp3^+$), which regulate the magnitude of an immune response by inhibiting the proliferation of innate and adaptive immune cells, so as to counterbalance excessive reactions. As part of normal homeostasis, Tregs participate in immune tolerance (non-reactivity) to self-antigens;
- **Th17 cells**, which secrete IL-17, IL-21, and IL-22 and activate neutrophils, are involved in eradicating extracellular microbes. Th17 cells are also implicated in several autoimmune disorders.

CD40L [11], IFN-γ [12], and lipopolysaccharide (LPS) [12] stimulate potent IL-12 production and Th1 induction by DCs. In contrast, anti-inflammatory molecules, such as IL-10, transforming growth factor β (TGF-β), prostaglandin E2 (PGE_2) [12], and the DC co-stimulatory molecule OX40L [13], are stimulators of Th2 development. Other factors, such as the duration of DC maturation, also contribute to the Th1/Th2 balance; at early stages of activation, DCs secrete higher levels of IL-12 [14], whereas during prolonged activation, DCs cease IL-12 production and preferentially induce Th2 responses [15].

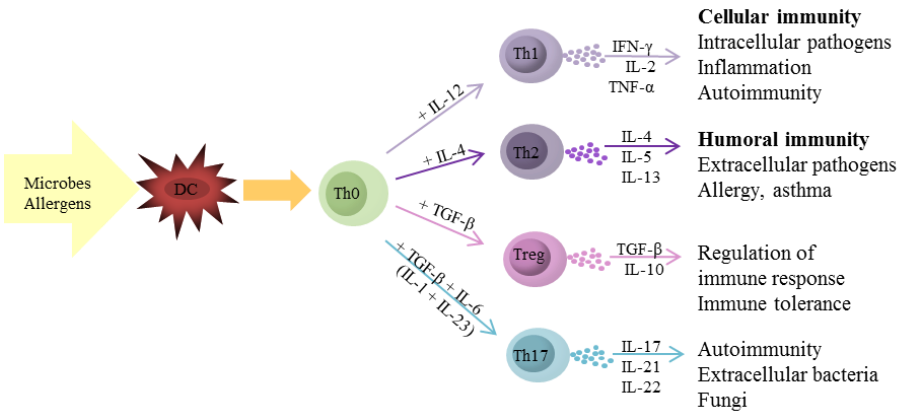

Figure 3. Dendritic cell-mediated polarisation of naive Th cells.

1.2.6 B lymphocytes

B cells are specialised in antibody production in response to infection or vaccination. In addition, they act as antigen-presenting cells (APC), particularly during recall responses, and as cytokine-secreting regulatory cells to suppress inflammatory responses [16]. The immune responses mediated by B cells are part of humoral immunity (from the Latin, *humor* = body liquid, where the components are found) and are mainly aimed at combating extracellular pathogens, although antibodies also inhibit intracellular pathogen entry into cells and neutralise bacterial toxins.

1.2.7 Natural killer (NK) cells and their roles in viral infections

NK cells play important roles in innate immunity to viruses and tumours. Based on the density of the CD56 marker on the cell surface, NK cells are categorised into two subsets: 1) the $CD56^{bright}CD16^{dim/-}$ subset, which accounts for 10% of the NK cells in the blood and upon activation mainly produces high levels of cytokines, primarily IFN-γ [17, 18]; and 2) the $CD56^{dim}CD16^{+}$ subset, which accounts for more than 90% of circulating NK cells and mainly mediates natural cytotoxicity (without the need for prior

sensitisation with an antigen) and antibody-dependent cytotoxicity [19, 20]. The activation of NK cells is primarily determined by the predominant signals received from the activating receptors and inhibitory receptors. These receptors recognise host proteins that are produced during the stress of malignant transformation, as well as the MHC I or MHC I-like molecules that are normally present on all nucleated cells. The recognition of stress ligands by activating receptors, in combination with the absence of MHC I ligands for inhibitory receptors, trigger NK cell-mediated responses. This represents an "alternative" recognition strategy that is based on the absence of typical self-MHC I molecules on the surface of the target cell - the "missing self" hypothesis proposed in 1986 by the Swedish immunologist Klas Kärre [21].

Initially discovered based on their ability to kill rapidly tumour cells in the absence of antigen-specific recognition [22], NK cells were subsequently identified as innate lymphocytes that are involved in controlling certain viral infections. A large body of evidence supports the role of human NK cells in antiviral defence. This is exemplified by immunocompromised individuals who have a primary immunodeficiency of NK cells and who suffer from severe, recurrent, and often life-threatening viral infections [23]. Patients with impaired NK cell function experience severe herpesvirus infections, and when there is a complete absence of NK cells, disseminated herpes simplex virus (HSV) disease [24]. The association between NK cell deficiency and susceptibility to HSV underlines the importance of NK cells in defence against HSV infection. *In vitro* studies have confirmed that NK cells play a crucial role in inhibiting HSV replication [25]. This knowledge has been validated in several murine models of viral infection. Murine and human NK cells have similarities in terms of function, activation, and signalling pathways. However, the murine NK cell receptors are structurally unrelated to their human counterparts (e.g., inhibitory receptor Ly49 versus KIR), and since there is no murine homologue of CD56, the existence of two analogous subsets in mice remains unresolved. Murine NK cells can be distinguished based on their expression of $CD27^{low}$ and $CD27^{high}$ [26], and these subsets show both similarities and dissimilarities to human CD56 subsets [27]. Therefore, results obtained using murine NK cells should be extrapolated with caution to the human situation. In this thesis, the focus is on human NK cells.

1.3 Receptors in the innate immune system

The innate immune system combats a broad spectrum of intruders with such efficiency that activation of adaptive immunity is not always necessary. Innate immune responses to pathogens are based on the recognition of specific molecular structures of the invading micro-organism, so-called pathogen-associated molecular patterns (PAMPs), by specific receptors on the innate immune cells, which are termed 'pattern-recognition receptors' (PRRs). These PRRs are distributed on the surface of and inside the host cell and recognise a large variety of PAMPs. Apart from the different microbes, PRRs recognise transformed host molecules and complexes as "danger" signals, which are produced during cellular stress, infection or inflammation [28]. Host molecules that are released from damaged cells and found in "ectopic" (not natural) locations can be recognised as 'damage-associated molecular patterns' (DAMPs) and they also act as endogenous PRR ligands. Some PRRs are phagocyte receptors that trigger the ingestion of the recognised pathogen, while others are chemotaxis receptors that govern the migration of macrophages and neutrophils from the blood to the site of infection. The third group of PRRs includes signalling PRRs, which are specialised in boosting both the innate responses and subsequent adaptive responses.

1.3.1 Toll-like receptors (TLRs)

The significance of Toll-like receptors is best illustrated by the fact that Bruce Beutler and Jules Hoffmann were awarded the 2011 Nobel Prize in Medicine or Physiology for their discovery of these receptors. Charles Janeway postulated the existence of such PRRs in mammals already in 1989 [29], and more than two decades of research conducted by his group and other groups since then have led to the identification of TLRs and their respective ligands, as well as the elucidation of their roles in linking innate and adaptive immunity [30-32]. TLRs represent an evolutionary conserved family of signalling receptors (being present in plants, insects, and vertebrates) that sense a large repertoire of PAMPs. To date, ten TLRs have been identified in humans, each specialised for different structures (Figure 4). TLR1, TLR2, TLR4, and TLR6 are expressed on the cell surface and recognise lipid complexes, while TLR5 detects the flagellin protein of the flagellum organelle. TLR3, TLR7, TLR8, and TLR9 detect viral and bacterial nucleic acids and are located on intracellular vesicles, mainly endosomes. While the ligand for TLR10 is unknown, there is homology between this receptor and TLR1. The different cellular locations of the TLRs reflect their roles as sentinels for pathogens that enter the cell *via* various routes.

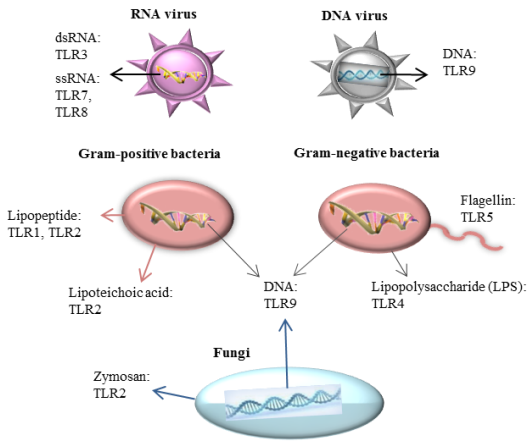

Figure 4. Recognition of PAMPs by their respective TLRs (adapted from: Christmas, P. (2010) Toll-like receptors: Sensors that detect infection. Nature Education 3(9):85)

After ligand binding, TLR dimerization occurs (thereby forming homodimers or heterodimers, such as TLR1/TLR2, TLR2/TLR6, and TLR7/TLR8), and this initiates a signalling cascade that involves various cytoplasmic adaptor molecules, kinases, and transcription factors. For the TLRs, signalling engages the MyD88 adaptor molecule, with the exceptions of TLR3, which uses TRIF, and nuclear factor-κB (NF-κB), which initiates transcription of the genes for pro-inflammatory cytokines and chemokines (e.g., IL-1, IL-6, IL-8, IL-12, TNF-α), leading to inflammatory responses. Signalling from the endosomal TLRs can also recruit the interferon-regulatory factors (IRF-3 and, IRF-7), resulting in the secretion of type I interleukins (IFN I; IFN-α and IFN-β), which are the main cytokines implicated in anti-viral responses (Figure 5). Other consequences of TLR signalling are the induction of adhesion molecules that enable leukocyte migration to the site of infection, DC maturation, and ultimately, T-cell activation.

While TLRs are present in most tissues, their expression levels are higher in tissues that are exposed to the external environment (e.g., lung and gastrointestinal tract) and lymphoid organs, such as the spleen [33]. TLRs are prominently expressed on antigen-presenting cells, such as DCs, macrophages, and B cells. Although immune cells can express the mRNA species for all TLRs, leukocytes have a cell type-specific pattern of TLR expression, which tailors their activities against microbes.

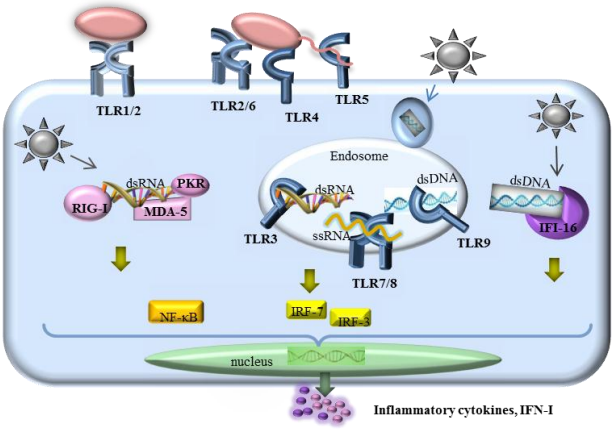

Figure 5. Schematic of the TLRs and other PRR signalling molecules in cells.

1.3.2 Why do TLRs not react to self-compounds?

Surface-localised TLRs mainly recognise PAMPs that are typical of microbes, as these structures, such as the LPS of Gram-negative bacteria or the lipoteichoic acid of Gram-positive bacteria, are not found in the host. Endosomal TLRs bind nucleic acids, some of which are also typical of pathogens (e.g., double-stranded RNA [dsRNA] or unmethylated CpG oligodeoxynucleotide [CpG ODN] motifs in DNA). However, studies have revealed that appropriate compartmentalisation of the ligands rather than a species-specific structure forms the basis for self-versus non-self discrimination; thus, both host and foreign nucleic acids can activate cells *via* TLRs, provided that the nucleic acid is located in the endosomes [34, 35]. Nonetheless, some intrinsic structures are recognised as endogenous TLR ligands, act as DAMPs, and provoke an inflammatory response [36]. For example, host nucleic acids can under certain circumstances bind to TLR7, TLR8, and TLR9 and induce inflammation [37]. For these reasons, TLR inhibition has been adopted as a strategy to reduce excessive inflammation, and molecular modulators that target TLR signalling have been developed to control the progression of inflammatory and autoimmune diseases.

1.3.3 RIG-I-like receptors and other PRRs

Recently, a cytosolic network of signalling receptors that detects PAMPs located in the cytosol was discovered (Figure 5).

Retinoic acid-inducible gene I (RIG-I-like receptors (RLRs) are RNA helicases that sense the viral genomic dsRNA, viral replication intermediates, and viral transcription products, and can thus recognise recognises a variety of viruses [38]. The family comprises RIG-I and melanoma differentiation-associated gene 5 (MDA-5) receptors, which control viral infections. In addition, IFN-inducible, dsRNA-dependent protein kinase R (PKR) is a cytoplasmic serine-threonine kinase that also binds dsRNA and mediates the inhibition of viral replication and the apoptosis of virus-infected cells [39].

AIM2-like receptors (ALRs) are part of a recently discovered family of cytoplasmic DNA sensors. The representative interferon gamma-inducible protein 16 (IFI-16) is a dsDNA receptor that acts in response to DNA viruses [40] and also apparently to bacterial DNA during bacterial infection [41].

These receptors, which are found in most tissues and cell types, signal *via* NF-κB, IRF-3 or IRF-7 to activate the expression of the gene for IFN-I, as well as other genes involved in host defence.

1.3.4 Why have several danger-signalling systems?

Considering the various ligand specificities of TLRs, RLRs, and ALRs, it is clear that an individual PAMP can be detected by more than one PRR. As example, viral or synthetic dsRNA is recognised by both TLR3 and MDA-5/RIG-I, exogenous DNA is recognised by TLR9 and IFI-16, and flagellin is bound by TLR5 and a NOD-like receptor (NLR) family member. Given this overlap of binding agonists, it might seem at first glance that there is a degree of redundancy in PAMP recognition. One possible explanation for this is that TLRs are more suited to sensing pathogens that are present extracellularly. However, once inside the cell, RLRs and NLRs are adept at detecting actively replicating pathogens. An alternative explanation is that RLRs act as initial viral sensors and triggers of IFN, which enhances TLR function and promotes development of the TLR-mediated adaptive response [42, 43]. Furthermore, since many viruses target these signalling pathways as a part of their immune evasion strategies [44], the inhibition of one system can be compensated for by the activity of another system. Therefore, TLRs and other PRRs seem to engage in crosstalk to control viral replication [43]. The beauty of this system is that a microbe can be detected by the immune system irrespective of its location.

In this thesis, I have studied TLR3 and TLR7 in particular. Polyinosinic-polycytidilic acid [poly(I:C)], which is a synthetic analogue of dsRNA, serves as a prototypic TLR3 ligand for studying the interactions of TLR3 with dsRNA. dsRNA is produced during the replication of most viruses, and its presence stimulates TLR3-mediated responses. Studies performed in mice have shown that the administration of poly(I:C) in an influenza vaccine induces a strong anti-viral effect that is not seen in the absence of the synthetic ligand [45]. Poly(I:C) also improves peptide-based cancer vaccines by eliciting tumour-specific CTLs. In contrast to its successful use in mice, poly(I:C) use in humans has proven to be problematic due to its degradation by serum nucleases [46] and safety issues associated with the administration of higher doses. Safer derivatives of poly(I:C) have been used in preclinical studies to generate mature MDDCs with strong IL-12 production and Th1-polarisation potential [47]. Importantly, poly(I:C) is also a ligand for RIG-I and MDA-5, and is thus not involved solely in TLR3 signalling.

1.4 Immunosuppression

1.4.1 DC-induced T-cell tolerance

Induction of immunity is not the only effector function of DCs. Equally important is the induction of tolerance, which is defined as host unresponsiveness to antigens. This lack of responsiveness prevents undesirable immune responses to harmless substances and to self-antigens, which would otherwise lead to autoreactive lymphocyte activation and detrimental attacks on tissues of the body (autoimmunity). In the thymus, DCs are involved in the elimination of self-reactive immature T cells, and thus establish so-called "central tolerance". In the peripheral tissues, the steady-state DCs are also likely to be tolerogenic; endocytosis of antigens by itself does not induce DC activation, and MHC-linked antigen presentation alone does not stimulate naive T cells in the absence of pathogen- or inflammatory signal-initiated DC maturation [48]. Instead, these immature DCs do not provide T-cell co-stimulation but cause activation-induced T-cell death, or anergy (inertness) [49]. Thus, in the absence of Signals 2 and 3, DCs provoke T-cell tolerance rather than T-cell activation, and this "peripheral tolerance" that is mediated by low-level migratory DCs contributes to immune homeostasis.

1.4.2 Indoleamine 2,3-dioxygenase (IDO)

DCs employ a range of mechanisms to establish tolerance. Indoleamine 2,3-dioxygenase (IDO) is a tryptophan-metabolising enzyme that can inhibit T-cell proliferation and suppress immune responses. The history of how IDO was discovered is intriguing. Isolated in 1963 from rabbit intestine as an enzyme implicated in the kynurenine pathway [50] (Figure 6), IDO had been known for decades for its ability to deplete the environment of the essential amino acid tryptophan, thereby inhibiting the growth of bacteria, viruses, and parasites. In 1998, IDO was re-discovered in a rather peculiar way through the seminal work of Munn and colleagues, who found that IDO inhibited maternal T-cell activation and prevented the rejection of allogeneic foetuses in mice [51]. This has placed IDO in a completely different biological context and opened up a new avenue of research into the immunoregulatory properties of this metabolic enzyme.

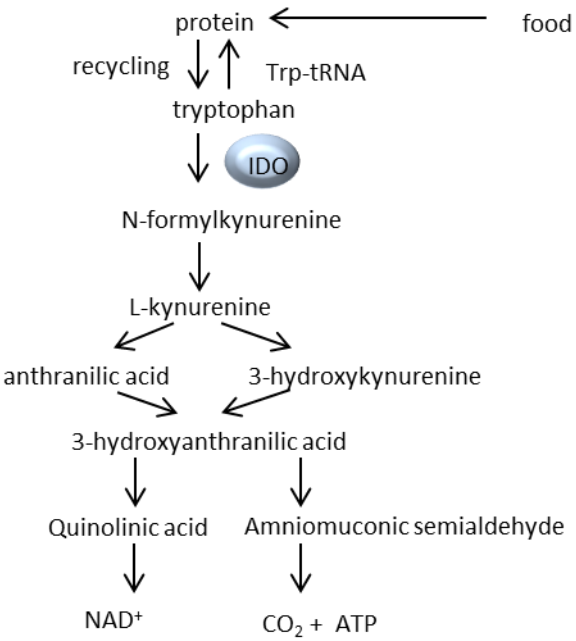

Figure 6. Schematic of the kynurenine pathway.

There are several possible mechanisms through which IDO blocks T-cell activation. First, the absence of tryptophan during T-cell activation leads to cell cycle arrest and subsequent inhibition of T-cell proliferation [52], or it induces apoptosis [53]. Second, tryptophan metabolites *per se* are toxic for T cells, thereby preventing their expansion [54, 55]. Interestingly, these metabolites induce selective apoptosis of Th1 cells over Th2 cells [56]. Third, IDO can activate regulatory T cells, which suppress even further the effector T-cell responses [57].

IDO is expressed in tissues with immune privilege, such as the placenta, gut, and the central nervous system. It is induced in APCs during inflammation through exposure to pro-inflammatory cytokines, especially IFN-γ. The T cells themselves participate in IDO stimulation, in that ligation of the CTLA-4 molecules on activated $CD4^+$ T cells to the CD80/CD86 co-stimulatory molecules on DCs represents a strong signal for IDO induction and suppression of T-cell mediated immunity [58].

Under normal physiological conditions, the activity of IDO favours the maintenance of homeostasis and the prevention of autoimmune disorders. However, IDO is abundantly expressed in the tumours and tumour-draining lymph nodes of mice, and since it prevents attacks by tumour-specific CTLs, this represents a tumour evasion mechanism [59]. In humans, IDO expression and the consequent IDO-mediated activation of Tregs may be the basis for therapeutic DC vaccination failure [60].

Interestingly, while IDO shows adverse effects on T cells, as well as on B cells and NK cells [55], it does not exert these activities on DCs. IDO can have a stimulating role in DC activation, manifested as the up-regulation of co-stimulatory and chemotactic molecules and, in apparent contradiction to what was previously postulated [61], increased T-cell-stimulatory capacity [62]. The mechanisms that determine whether IDO has a negative or positive outcome on the T-cell-stimulatory capacity of DCs remain elusive, although complex spatiotemporal regulation is likely to be involved.

1.4.3 Innate immune system in newborns

The immune system of newborns is very different from that of children or adults, in that it is characterised by functional immaturity and as a consequence, increased susceptibility to infections. This peculiarity stems from the challenges faced during life *in utero*, i.e., the avoidance of harmful pro-inflammatory responses and alloreactions between the mother and foetus, which could result in spontaneous abortion or pre-term delivery [63]. These conditions shape an immunological forma that circumvents the inflammatory Th1-type polarising reactions and that is skewed towards Th2-type responses. This is a double-edged sword in that it reduces the ability of the neonate to fight intracellular pathogens, the clearance of which demands a strong Th1 cellular immune response. It is also the reason why neonates are poor responders to many vaccines (e.g., vaccines against polio, measles, and mumps) [64-66].

The immaturity of the neonate immune system is usually ascribed to the sterile uterine environment and the consequent lack of antigen-experienced T and B lymphocytes, which account for the lack of pre-existing immunological memory [67]. However, inadequate T-cell activation is a consequence of the weak antigen-presenting properties of the APCs. In neonates, the DCs are immature and this immaturity is characterised by decreased expression of MHC and co-stimulatory molecules and reduced level of IL-12 secretion, which result in an inability to stimulate efficient Th1 responses [68]. Apart from IL-12, neonatal DCs and monocytes produce lower levels of the pro-inflammatory cytokines TNF-α and IFN type I (IFN-α, IFN-β) and higher levels of IL-6, IL-10, and IL-23, as compared with their adult counterparts [69], thus reflecting the above-mentioned Th2 bias (which is more suitable to defence against extracellular pathogens). The lack of IL-12 affects the production of IFN-γ by neonatal NK cells [70], which also have decreased killing ability; their cytotoxicity for tumour cells is less than 50% that of adult NK cells [71, 72]. The innate immune impairment in neonates also includes quantitative defects, such as altered levels of blood complement proteins, which can be 10%–70% lower than in adults [73, 74], and fewer neutrophil progenitor cells. The neutrophils of neonates show impairments in chemotaxis, phagocytosis, and microbicidal capacity [75, 76].

Neonatal NK cells have several functional defects that may render newborns more prone to viral infections. In this respect, they are deficient in killing HSV-infected cells [77] and have a reduced level of HSV-specific IFN-α secretion [78]. The impaired function can be attributed to the intrinsic features of neonatal NK cells, as their content of cytoplasmic granules, which

mediate cytotoxicity, is decreased and the expression of inhibitory receptors is increased [71]. In addition, the malfunction may be exacerbated by the lower levels of secretion of the NK-stimulating cytokines IL-12 and IFN-α by other cord blood cells. Taken together, these phenotypic and functional NK cell defects correspond to the impaired ability of neonates to control HSV infections.

Maturation of the immune system is age-dependent and is acquired during infancy when newborns, in an antigen-rich environment, encounter microbe-derived TLR agonists. Although TLRs are at the basis of this process, their ontology is not well-characterised. TLR expression may be lower in newborns than in adults, e.g., in blood-derived DCs [79]. In contrast, TLR expression may be fully developed during gestation, e.g., umbilical cord blood monocytes from full-term newborns express as high levels of TLR4 as do adults, a phenomenon that is not seen in pre-term newborns [80]. However, even when neonatal cells express TLRs to the same degree as adult cells, they still have impaired responses to certain TLR ligands, such as diminished TNF-α production by neonatal monocytes after stimulation of TLR2, TLR4 or TLR7 [81]. The mechanistic explanation for this is incomplete but involves differences in the levels of cytokine transcripts and neonatal plasma factors [81, 82]. Therefore, impairments in the maturation of TLR expression and TLR responsiveness predispose newborns to certain infections during early life, such as infections with HSV, respiratory syncytial virus, and *Mycobacterium tuberculosis* [83].

1.5 Cholera toxin (CT)

Cholera toxin (CT), which is produced by *Vibrio cholerae* and is a causative agent of cholera in humans, is a potent immunogen and adjuvant [84]. CT is composed of a catalytic A subunit (CTA) and a pentameric cell-binding B subunit (CTB). The adenosine-diphosphate (ADP)-ribosylating activity of CTA leads to an increase in cAMP and chloride channel protein activation, leading to excessive loss of ions and water. In humans, even small amounts of CT provoke drastic diarrhoea and dehydration, so the adjuvant effect of CT cannot be observed in humans. In experimental animals, CT is far less toxic and can be used both in immunisation studies and as an adjuvant.

In mice, the adjuvant effects of CT are: 1) increased permeability of the intestinal epithelium, resulting in increased uptake of antigens; and 2) stimulation of mucosal IgA antibody production. The most important adjuvant effect of CT is thought to be on antigen presentation exerted by DCs, macrophages, and B cells. CT provokes the up-regulation of MHC and co-stimulatory molecules, as well as increased expression of the CCR7 and CXCR4 chemokine receptors on both murine and human DCs [85, 86]. An important adjuvant mechanism of CT is mediated through the secretion of IL-1β, which induces DC maturation and acts as a mucosal adjuvant for co-administered protein antigens [87]. In mice, the adjuvant effect of CT is exerted on splenic DCs [88].

Although CT is probably one of the most intensively investigated microbial toxins and one of the most powerful adjuvants known, being widely used in experimental animals as a mucosal adjuvant or as a carrier molecule for covalently linked antigens [85, 89], the mechanism underlying its immunomodulatory effects is not completely known.

2 AIM

The overall goal of this thesis was to study the cellular intracellular signalling events of the innate immune system.

2.1 Paper I - Immune regulation by IDO in CT-exposed DCs

We developed a combined DC vaccine that has proven successful in the eradication of experimental tumours in mice. It is based on the use of CT both as an adjuvant and an antigen carrier. The antigen conjugated to CT enhanced >1000-fold the antigen-presenting capacity of the DCs *in vitro*, amplified T-cell responses *in vivo* after DC vaccination, and induced tumour-specific CTLs that eradicated an already established experimental tumour in mice [85, 90-92]. The conjugation of CT with the E7 protein of human papillomavirus (HPV) also gave rise to an efficient DC vaccine and induced the elimination of E7-expressing tumours in mice, provided that the vaccination was combined with the injection of the TLR9 agonist CpG ODN [93]. In addition, CT conjugation to HPV 16 E7 has been shown to enhance significantly E7-specific T-cell responses *ex vivo* in women with cervical dysplasia [94].

In several studies published a decade ago, it was shown that IDO has strong T-cell-suppressive characteristics that have negative impacts on the immune system, e.g., in cancer cases. Therefore, we assumed that IDO would not be expressed in immunogenic DCs, which drive a powerful cell-mediated immunity against tumours (this was *before* the traditional view of IDO was modified based on the findings that IDO may contribute to DC activation and have a stimulatory effect on immune responses). We wanted to investigate whether the adjuvanticity of CT would bypass IDO in the activation of DCs and subsequent expansion of T cells (Figure 7).

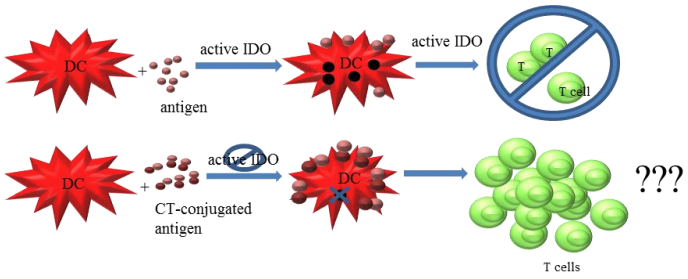

Figure 7. Hypothetical basis for the study.

The hypothesis underlying the work in Paper I was that:

♪ CT activates DCs without inducing the immunosuppressive enzyme IDO

2.2 Paper II - TLR3 in neonatal NK cells

The defective responses of neonatal NK cells to HSV are linked to the increased susceptibility of newborns to HSV infections. Newborns are extremely sensitive to HSV, and infection with HSV-1 or HSV-2 can lead to disseminated disease with multiple organ failure, including brain inflammation (encephalitis). HSV-2 causes a more frequent and severe form of neonatal herpes infection [95, 96] and is the etiological agent in 80% of cases of neonatal encephalitis [97].

Several seminal studies conducted by the group of Jean-Laurent Casanova reveal that many children with severe HSV-1 encephalitis have a primary deficiency in TLR3 signalling. In particular, autosomal mutations in four proteins of the TLR3 signalling pathway have been identified, as follows: dominant-negative mutations of the TLR3 protein [98, 99] and TRAF3 adaptor molecule [100]; a recessive mutation of UNC93B1, which is involved in endosomal TLR signalling [101, 102]; and both dominant and recessive mutations of the TRIF adaptor molecule [103]. As a consequence of these mutations, IFN I production was abolished. It was concluded that impairment of the TLR3 pathway predisposes for HSV-induced encephalitis.

This means that TLR3 signalling is crucial for controlling HSV replication, providing resistance to HSV in the central nervous system.

We investigated whether a lack of TLR3 or of another viral-sensing receptor determined the poor responses of neonates to viruses and their higher susceptibility to viral infections. The expression and function of signalling PRRs in neonates have not been systematically scrutinised. TLR expression was analysed in neonatal monocytes and T lymphocytes, and they showed the same levels of expression of TLRs 1-4, 8, and 9 as did adult monocytes and T lymphocytes [104]. As studies of RLRs and ALRs are missing, we examined the expression levels of viral-sensing receptors (i.e., TLR3, TLR7, TLR8, TLR9, RIG-I, MDA-5, PKR, and IFI-16) in cord blood cells.

The pilot analysis showed a lack of TLR3 expression in cord blood mononuclear cells (CBMC), whereas other nucleic acid-sensing receptors were expressed normally. This provided us with the basis for further TLR3 analyses in newborns (Figure 8).

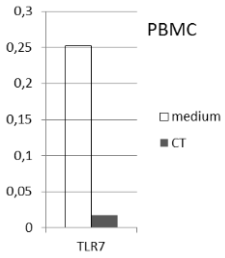

Figure 8. Results from a pilot study of PRR expression in cord blood cells.

The hypothesis underlying the work in Paper II was:

♪ Newborns have impaired expression of PRRs

2.3 Paper III - TLR7 in CT-exposed PBMCs

Taking into account the adjuvant characteristics of CT stated above, and addressing the mechanisms of CT activity, we asked if the adjuvant properties of CT could be associated with CT-induced up-regulation of PRRs. Adjuvants can be classified into two major functional groups: i) TLR-dependent adjuvants, i.e., TLR agonists, which act directly on DCs through TLR activation and the induction of the expression of MHC II and co-stimulatory molecules, cytokine secretion, and DC migration to the lymph nodes; and ii) TLR-independent adjuvants, e.g., alum, which act mainly to increase antigen uptake by APCs, although they also have immunostimulatory properties, which include leukocyte recruitment at the injection site and indirect DC activation through the induction of DAMP. To date, CT adjuvanticity has only been compared to that of TLR ligands, and there are no studies in the literature looking at whether CT induces/promotes TLR signalling. We surmised that CT might induce the expression of PRRs on innate immune cells.

In a pilot experiment with two donors, we examined the expression levels of viral-sensing TLRs (i.e., TLR3, TLR7, TLR8, TLR9), RLGs (RIG-I, MDA-5), PKR, and ALR (IFI-16) in peripheral blood mononuclear cells (PBMCs) after treatment with CT. A selective effect of CT on TLR7 mRNA expression was observed, so we investigated further the possible mechanisms behind this unexpected mode of modulation.

The hypothesis underlying the work in Paper III was:

♪ CT up-regulates PRR expression in innate immune cells

3 DISCUSSION

3.1 Paper I

In Paper I, we tested our hypothesis that CT activates DCs without inducing the immunoregulatory enzyme IDO. Initially, we showed that CT induces DC maturation, as the expression levels of MHC II molecule HLA-DR, the CD80 and CD83 co-stimulatory molecules, and the CD83 maturation marker was up-regulated in CT-pulsed DCs. Although the CT-pulsing of DC led to a marked increase in IDO mRNA expression, measurements of the biological activity of IDO showed no measurable IDO-mediated degradation of tryptophan in the CT-pulsed DCs. Interestingly, although CT-pulsing alone did not promote the production of active IDO, CD40L stimulation of CT-pulsed DCs resulted in increases in IDO mRNA expression and tryptophan-degrading IDO activity. From these results it is concluded that:1) CT-pulsed DCs express IDO following the CD40-CD40L engagement that occurs when DCs and T cells interact; and 2) their ability to induce T-cell responses in DC-T cell co-cultures is retained even in the presence of IDO, since CT-pulsed DCs were found to be highly efficient in stimulating both primary (allogeneic) and memory (autologous) $CD4^+$ T-cell responses. Moreover, the addition of an inhibitor of IDO did not affect T-cell expansion, which indicates that IDO expression in CT-pulsed DCs is not causally related to their T-cell stimulatory ability.

We compared CT-pulsed DCs with DCs that were matured in the presence of PGE_2, TNF-α, and IL-1. These cytokines, together with IL-6, are considered the Gold standard for the *ex vivo* maturation of MDDCs used in clinical trials. PGE_2, which is a lipid messenger that is released during inflammation, facilitates CCR7-mediated DC migration towards the lymph nodes in response to chemokines CCL19 and CCL21 and enhances their T-cell-stimulatory capacity [105, 106]. PGE_2 synergises with TNF-α in inducing DC maturation and greatly reduces the concentration of TNF-α required for IL-12 production [107]. The use of PGE_2 in clinical practice was called into question when it was discovered that PGE_2 up-regulates IDO [108]. However, it was shown soon thereafter that the T-cell-priming ability of PGE_2-matured, IDO-expressing DCs was not impaired, and that these cells stimulated even stronger T-cell proliferation than did mature MDDCs without active IDO [109]. It is clear that PGE_2, in combination with other stimuli, can generate mature DCs that express IDO and that are strongly immunostimulatory. Similarly, LPS-matured MDDCs that express IDO are

more effective at stimulating T cells than immature DCs without active IDO [110]. This is not surprising, since mature DCs are known to be more efficient APCs than immature DCs, both *in vitro* and in DC-immunisation trials with healthy volunteers [111], and mature DCs are proven to be superior in inducing tumour-specific CTL responses in the treatment of patients with metastatic melanoma [112, 113]. This has led to the notion that IDO is not associated exclusively with immunosuppression but can participate in stimulating immunity. New light has been shed on the concordant results in the early years (mainly represented by the work of Munn and Mellor; [114]) on the immunoregulatory role of IDO. Over time, the portrait of a DC-maturation process characterised by transition from an immature status that induces tolerance to a mature stage that directs immunity was replaced with a more "plastic" view, whereby mature DCs can present antigens in both immunogenic and tolerogenic manners (Figure 9).

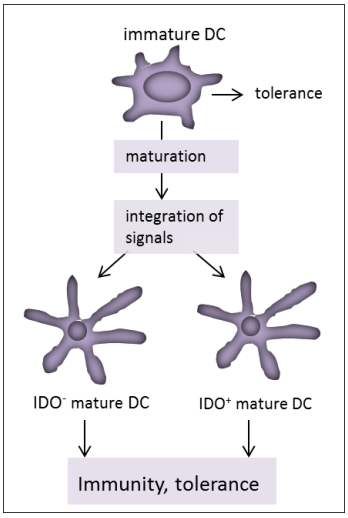

Figure 9. Overview of dendritic cell maturation and function in relation to IDO.

Initially, we concurred with traditional opinion, and we surmised that immunostimulatory agents or adjuvants, such as CT, would not promote the expression of a tolerogenic factor, such as IDO. Although this was certainly true for CT activity alone, as we showed that CT is not a crucial stimulus for

IDO, we were surprised to find that CT-pulsing primed for CD40L-induced IDO expression. In the *in vivo* context, this suggests that CT-pulsed DCs express IDO after the CD40-CD40L interaction with T cells in the lymph nodes. The strong T-cell expansion in response to CT-pulsed DC that we observed (which *in vivo* also occurs in the lymph nodes/organs) implies that the potent immunostimulatory capacity and IDO expression coincide in CT-matured DC. The same was valid for the mature DCs that we used as a positive control for IDO and strong T-cell-activating function. Nevertheless, the inhibition of IDO activity by the inhibitor 1-methyltryptophan (1-MT) in the assay did not influence T-cell proliferation, which indicates that, under our experimental conditions, T-cell activation was not under the control of IDO. Intriguingly, the IL-12p40 production that we detected upon CD40L stimulation in immature DCs, mature DCs, and even in CT-pulsed DCs (CT is not primarily a stimulator of IL-12) was blocked by 1-MT, which clearly indicates IDO-dependent IL-12p40 production in MDDCs. While we did not detect IL-12p70, as we did not provide a second signal (e.g., IFN-γ, LPS) required for its production, IL-12p40 itself has T-cell-stimulatory activity and potential to promote immune responses. Overall, our results support a positive effect of IDO on DC-effector functions, and are in accordance with existing knowledge regarding the contributions of IDO to DC maturation and the enhanced T-cell-stimulatory capacity of DCs. Several studies have identified critical roles for IDO in: a) eliciting the chemotactic and migratory activities of DCs [62]; b) increasing the expression of co-stimulatory molecules CD40, CD80, and CD86 [115]; c) stimulating the proliferation of naive T-cells [116].

However, I must elaborate on some issues that prevent the drawing of straightforward conclusions. First, the levels of active IDO that we detected may have been too low to influence T-cell proliferation in response to CT-pulsed DCs and cytokine-matured DCs. There are surprisingly contradictory data in the literature regarding the tryptophan/tryptophan metabolite concentrations required for any effect. Studies using human cells have shown that for T-cell proliferation to be inhibited, the tryptophan concentration must be 0.5–1.0 μM [52]. Of note, one group did not achieve inhibition of T-cell proliferation even when using medium without tryptophan [54]. As for tryptophan metabolites, concentrations of L-kynurenine and 3-hydroxyanthranilic acid in the range of 5–50 μM were shown to be non-toxic for purified $CD8^+$ T cells, while for apoptosis induction, a very high concentration of metabolites (500 μM) was needed over a longer period (5 days of culture) [110]. The concentration of tryptophan metabolites required to inhibit T-cell proliferation is reported in some studies to be as high as 100 μM [54, 55], although in other studies it is reported as being <10 μM [114,

117]. Therefore there is uncertainty as to the degree of IDO activity that is needed to disrupt T-cell function, regardless of whether this is mediated by tryptophan starvation or toxic metabolites. Although the decrease in tryptophan levels and increase in kynurenine concentrations observed in our experiments were comparable to those reported in previous studies, it seems likely that the MDDCs in our culture conditions did not generate sufficient levels of IDO activity to interfere with T-cell activation. Conversely, the T-cell proliferation measured in our assay was very high, perhaps due in part to the high concentration of the T-cell-stimulatory cytokine IL-23, which was abundantly secreted by all the MDDC groups (immature, CT-pulsed, and cytokine-matured DCs).

Second, if we regard IDO function from as a regulatory mechanism through which DCs dampen an immune response once the desired outcome has been achieved, then IDO is expected to be produced at a relatively late time-point during the immune reaction. We could not know whether IDO was produced during the T-cell proliferation assay, as it was not possible to analyse the co-culture supernatants because of the conditioning medium that we used. In these experiments, Iscove´s medium was used, which prevents measurements of IDO activity by high performance liquid chromatography due to background interference with kynurenine /tryptophan detection. This leaves open the question as to whether the functional IDO was produced at later time-points during the assay. Another point should also be considered here. The tryptophan/kynurenine ratio is considered to be a reliable measure of IDO activity. However, other products formed along the kynurenine pathway, especially 3-OH-kynurenine and 3-OH-anthranilic acid, are also immunosuppressive. Thus, in the absence of any measurements of other tryptophan metabolites we cannot make definitive conclusions as to the contributions of IDO to DC maturation and T-cell-activating capacity.

Third, the time for retrieval of the IDO-inducing signal is probably crucial for the cells. In the case of DCs, it is conceivable that the same factor(s) that signal for IDO have different outcomes depending on the time-point during DC maturation when they are present. In this respect, our experimental set-up has one major drawback. In our experiments, we supplied soluble CD40L to the MDDCs concomitantly with a 24-h period of stimulation with CT or a cocktail of cytokines, i.e., *during* DC maturation, and we then measured IDO activity. In contrast, in the DC-T cell experiments, the starting co-cultures contained DCs that had already matured for 24 h, and these cultures *subsequently* received the T cell-derived CD40L. Therefore, we based our inference regarding the T-cell-stimulatory capacity of IDO-expressing

MDDCs on IDO measurements that time-wisely do not correspond with each other. This is an issue that should be reconsidered.

Fourth, even if a sufficient level of IDO activity was accomplished in our DC-T cell co-cultures, it remains a possibility that a negative impact of IDO on T-cell proliferation was overcome by other mechanisms. For instance, the tryptophan levels in the culture medium are adequate to neutralise the IDO-mediated tryptophan starvation. Moreover, elevated levels of tryptophanyl-tRNA-synthetase, the enzyme responsible for linking tryptophan with its cognate tRNA, were found in proliferating T cells that were stimulated with IDO-producing MDDCs [108]. This process "rescues" tryptophan from being degraded by IDO and is thought to represent an additional mechanism to counteract the negative effect of IDO on proliferating T cells.

Investigations of IDO face several challenges. For example, 1-MT is used as a standard inhibitor of the enzymatic activity of IDO and to study immunoregulation mediated by IDO [58, 117]. However, two isoforms of 1-MT exist, the L- and D-stereoisomers, and it is still not certain which isoform is the more effective at inhibiting IDO. 1-D-MT has been used extensively due to its strong ability to reverse IDO-mediated suppression of anti-tumour immunity *in vivo*, whereas in biochemical assays, 1-L-MT is more efficient at inhibiting IDO. We used both isoforms but did not observe any major differences in terms of effectiveness. Similarly, several other groups saw no improvement in T-cell proliferation when using 1-MT [118, 119]. Even though they generated mature MDDCs with the characteristic $CD123^{+}CCR6^{+}$ phenotype, which according to Munn represent a human DC subset with constitutive expression of IDO and T-cell-inhibition capacity [117], these mature DCs did not express IDO. When stimulated to degrade tryptophan, these IDO-competent MDDCs did not inhibit T-cell proliferation [118, 119]. Of note, 1-MT seems to have an additional IDO-independent function, as evidenced by 1-MT enhancement of T-cell proliferation induced by cells defective in the production of IDO [120].

Given the above findings, a reasonable dilemma is the reliability of the *in vitro* system, as well is its correlation with the *in vivo* situation. DCs are known to be sensitive to the conditions under which they are cultured, and the same is true with regard to their production of IDO. Human studies of IDO are mainly performed with MDDCs, although it is known that certain factors in the methods used to obtain MDDCs, i.e., the nature of the culture medium or maturation cocktail, contribute to the functional expression of IDO [116]. Thus, *in vitro* studies may represent an artificial system in which IDO competence (expression and activity) is predominantly determined by

culturing factors rather than reflecting the intrinsic features of the studied cells. Nonetheless, the *in vivo* immunosuppressive role of IDO has been proven in mice [59, 61], and IDO expression has been detected in many human tumour biopsies [121, 122], implying that IDO functions in the same manner in humans. Furthermore, skin biopsies from patients with metastatic melanoma have shown $FoxP3^{+}$ regulatory T cells infiltrating the site of vaccination with IDO-positive mature DCs, which suggests IDO-promoted recruitment of Tregs [123]. Taken together, these studies support the notion that IDO is not just an *in vitro* artefact, but rather that it behaves as in the organism. In mice, the IDO-producing cell population seems to be restricted to a specific DC subset in the spleen [124] or to a small fraction of pDCs, which drive IDO-mediated suppression in the tumour-draining lymph node [61]. It is not yet clear whether a distinct DC subset or certain fractions of many subsets of DCs with peculiar characteristics function as IDO-relevant DCs *in vivo*.

Over the years, as different studies have confirmed or failed to confirm the initial results indicating the T-cell-suppressive role of IDO generated by IDO-expressing DCs, a new picture of the function of IDO-expressing human DCs emerged (Figure 10): i) without an appropriate signal received, the DCs do not express IDO; ii) DCs express IDO but the protein is not active and consequently, it has no influence on the T-cell responses; iii) DCs express IDO that actively degrades tryptophan but that does not have negative effects on T cells or T-cell activation; this is an area in which our results make a valuable contribution; and iv) DCs express functional IDO, which drives T-cell suppression.

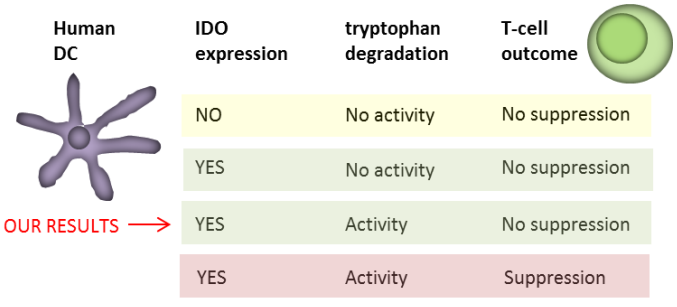

Figure 10. Differential IDO expression profiles and the consequent functions of human DCs (adapted from Terness, Trends Immunol, 2006; 27(2):68)

In summary, we show that CT-induced maturation and the induction of competent IDO in DCs are events that are not separated in time. Thus, our original hypothesis was incorrect. Investigations of IDO are problematic in that the functionality of the enzyme is very susceptible to different environmental signals, the type of maturation stimulus, and the state of DC maturation We have yet to comprehend how the immune system harmonises the multiple biological roles of IDO, and under which circumstances identical signals determine whether the same IDO mechanism will carry out antimicrobial defence or immunosuppression. The roles of IDO under other normal physiological conditions, such as the maintenance of homeostatic tolerance or pregnancy, or under pathological conditions, such as the evasion of tumour immune surveillance, also await our clarification.

3.2 Paper II

In Paper II, we analysed the levels of basal expression and induced expression of transcripts for the viral nucleic acid-sensing receptors TLR3, TLR7, TLR8, TLR9, RIG-I, MDA-5, PKR, and IFI-16 in umbilical cord and adult blood cells. As presented in the *Introduction* section, in a pilot experiment we noticed striking impairment of TLR3 mRNA expression in CBMCs, as compared with PBMCs. To define the mononuclear cell type responsible for this deficiency of TLR3 mRNA, we first showed that adult NK cells of the $CD56^{dim}$ subset contain the highest levels of TLR3 mRNA, and thereafter we focused our analyses on NK cells from cord and adult blood samples. The analyses of the same receptors on NK cells showed a similar selective impairment: the neonatal NK cells had an impaired TLR3 mRNA expression and the TLR3 protein was absent.

While unravelling the functional repercussions of TLR3 being absent, we found that neonatal NK cells were not able to respond to the TLR3 agonist poly(I:C) in terms of IFN-γ production, as compared with the abundant production of this cytokine by adult NK cells. Albeit to a lesser extent, the cytotoxic functions of neonatal NK cells induced upon TLR3 engagement were also affected; these cells were less competent at killing tumour cells after poly(I:C) stimulation, as compared with adult NK cells treated with poly(I:C). This phenomenon could not be attributed to an intrinsic defect of neonatal NK cells that resulted in inadequate cytotoxicity, since these cells attained adult levels of cytotoxicity upon activation with the NK cell-

stimulatory cytokine IL-12. Neonatal NK cells were weakly cytotoxic towards HSV-2, possibly due to the impaired up-regulation of TLR3 mRNA triggered by HSV-2.

Although it has been reported previously that neonatal NK cells are impaired in the secretion of IFN-γ [125], our results for the first time propose that the lack of TLR3 mRNA and protein expression in the NK cells from newborns is responsible for the aberrant TLR3-triggered IFN-γ production and cytotoxicity. A previous finding that a higher content of TLR3 mRNA in human NK cell clones correlated with higher levels of IFN-γ and cytotoxicity in response to TLR3 stimulation [126] suggested that TLR3 expression in NK cells is a prerequisite for a competent TLR3-mediated response. Compelling evidence comes from studies of children with HSV-1 encephalitis who are deficient in TLR3 and consequently cannot exert a TLR3-mediated response [98], underlining a key role for TLR3 in the defence against HSV-1. TLR3 signalling has also been proposed to be involved in the immune response against HSV-2 [127-129]. We found that neonatal NK cells could not lyse HSV-1-infected cells and had impaired cytotoxicity towards HSV-2, in agreement with previous findings [77]. Viewed together, these results prompt us to speculate that the lack of TLR3 underlies the hypersensitivity of newborns to HSV.

Regarding how TLR3 expression is regulated, the detection of similar levels of TLR3 mRNA in the NK cells of men and women, as well as in pregnant women indicates that the systemic regulation of TLR3 expression is not influenced by either sex hormones or pregnancy. Instead, the marked difference in TLR3 mRNA levels noted between the NK cells from pregnant women and the NK cells from cord blood suggest that TLR3 expression is regulated in the uterus during pregnancy. Decidual NK cells that we obtained from the placenta at full-term delivery contained low levels of TLR3 mRNA expression as that of cord NK cells. This is not completely surprising given their $CD56^{bright}$ phenotypes, since we revealed that the adult blood $CD56^{bright}$ NK cell subset expresses substantially less TLR3 mRNA than $CD56^{dim}$ NK cells. To appreciate the significance of these findings, one needs to take into account the developmental relationship between the two subsets of blood NK cells. Although it is a matter of debate, the following lines of evidence support the idea that $CD56^{bright}$ NK cells are the immature precursors of the more highly differentiated $CD56^{dim}$ NK cells: i) $CD56^{bright}$ NK cells can be differentiated *in vitro* into $CD56^{dim}$ cells [130], while the conversion in the opposite directions has not been seen; ii) after allogeneic or autologous bone marrow transplantation, NK cells are the main lymphocytes that populate the peripheral blood, and $CD56^{bright}$ NK cells are the first to appear [131, 132];

iii) the percentage of $CD56^{bright}$ NK cells is higher in young adults and declines with age, while $CD56^{dim}$ NK cells have the opposite pattern [133]; and iv) the shorter telomeres of $CD56^{dim}$ NK cells denote a more mature stage [134]. Thus, if $CD56^{dim}$ NK cells represent a more mature, TLR3-expressing subset, then the lower level of TLR3 expression on $CD56^{bright}$ NK cells is associated with immaturity. The immaturity of cord NK cells is characterised by a higher inhibitory/activating receptor ratio and low or null expression of the terminal differentiation marker CD57, which is associated with higher cytotoxicity and lower cytokine production [71, 135, 136]. However, the percentage of $CD56^{bright}$ NK cells is not higher in neonatal blood. It is conceivable that during pregnancy, a uterine environment rich in cytokines and hormones creates conditions that prevent the differentiation from $CD56^{bright}$ to $CD56^{dim}$. Disruption of the prevailing $CD56^{bright}$ phenotype of the uterine NK cells has been linked to decreased fertility in women suffering from infertility of unknown cause; a higher percentage of $CD56^{dim}CD16^{+}$ NK cells was found in the follicular fluid, which may not be beneficial for oocyte maturation [137].

Decidual NK cells are formed during pregnancy in the decidua layer that lines the placenta, most probably through the recruitment and further tissue-specific differentiation of the blood $CD56^{bright}$ NK cells [138]. Apart from a role in immunity, decidual NK cells control trophoblast growth, differentiation, and invasion during the implantation process. Although decidual NK cells contain abundant cytotoxic granules with high concentrations of perforin and granzyme (despite the $CD16^{-}$ phenotype) [139], they exhibit reduced cytotoxicity due to the immature formation of activating synapses [140]. Despite that their profile of activating receptors is complete and similar to that of other NK cells, some of the receptors on decidual NKs can transmit inhibitory signals due to the lack of an activating accessory protein [141]. Decidual NK cells do not kill trophoblast cells [142], although they lack the MHC I molecules HLA-A, HLA-B, and also HLA-C, which represents a deviation from the standard MHC-based NK-cell recognition concept. Instead, expression of the less polymorphic HLA-E and HLA-G as inhibitory receptor ligands is seen as a mechanism for immune unresponsiveness to trophoblasts (acting presumably by immobilising the decidual NK cells in the decidua and preventing their entry into the placenta) [143, 144]. Therefore, the normally poorly cytotoxic decidual NK cells evidently contribute to maintaining maternal immune tolerance towards the foetus, and increased cytotoxicity has been associated with harmful pregnancy outcomes, such as miscarriage [145, 146]. A recent study has revealed that the latter may be related to increased TLR3 expression in decidual NK cells, as women with unexplained recurrent spontaneous

miscarriage (range, 2–8 times) in the first trimester of gestation were found to have significantly higher levels of TLR3 mRNA and protein, as compared with women who electively terminated a normal pregnancy in the same gestation period [147]. Decidual NK cells from these patients showed significantly higher cytotoxicity against K562 tumour cells, implying a detrimental role of TLR3 in decidual NK cell activation and immune reactions to the foetus that might have caused miscarriage.

This finding ties in with our results and proposal that weak expression of TLR3 in the pregnant uterus and the developing foetus, i.e., on maternal decidual NK cells and cord blood NK cells, respectively, represents a mechanism for tolerance at the maternal-foetal interface. The activation of TLR3, which elicits a strong Th1-type immune response, would be unfavourable in the context of inflammatory and allogeneic reactions, and might compromise the integrity of the foetus. Although we noted with interest the reduced TLR3 expression detected on neonatal $CD8^+$ T cells, unfortunately we did not analyse the decidual $CD8^+$ T cells. The decidua is not rich in lymphocytes, and those maternal $CD8^+$ T lymphocytes located around the trophoblast are maintained in a tolerant status by HLA-G and inhibitory receptor interactions, which result in T-cell anergy [144]. It remains to be explored whether reduced TLR3 expression on cytotoxic cells, i.e., NK cells and T cells, prevents their over-activation and contributes to maternal-foetal tolerance.

A possible mechanism for local regulation of TLR3 involves hormones. The TLR3 mRNA and protein are expressed in the uterine endometrial NK cells of non-pregnant women [148], as well as in the epithelial cells of the non-pregnant endometrium. In these epithelial cells, the expression of TLR3 is subject to the cyclic, hormone-mediated regulation. Furthermore, hormone treatment of an endometrial cell line with estradiol abrogated the cytokine production induced by poly(I:C) [149].

Besides its role in viral recognition, TLR3 has emerged as a receptor for DAMPs, recognizing self-RNA released from damaged tissues or during injuries associated with exposure to solar irradiation. TLR3 also drives an inflammatory response during tissue necrosis. The human placenta contains areas of intense necrosis, particularly in the decidua during trophoblast invasion in the course of implantation. Since decidual NK cells play roles in trophoblast invasion and angiogenesis, it can be speculated that the suppressed TLR3 expression in decidual NK cells prevents the onset of inappropriate TLR3-triggered inflammatory events. Indeed, $CD56^+$ NK cells have been identified around the zone of decidual necrosis, and it has been

proposed that this "physiological necrosis" serves as a mechanism to divert the different maternal decidual inflammatory cells away from the arterioles where the trophoblast invasion and angiogenesis processes take place [150].

It is certain that foetuses, in a normally sterile uterus, constantly face the threat of intrauterine infections. The challenge is to achieve a balance between protection and pathological inflammation. TLRs, as the fundamental mediators of immune responses, are presumed to evoke adequate inflammatory reactions to clear the pathogen while avoiding foetal tissue damage. An association between viral infections of the genital tract and preterm labour has been reported [151-153], and intrauterine infections account for a high percentage of pre-term deliveries [154]. Pro-inflammatory cytokines (IL-1β, IL-6, TNF-α, IFN-γ) are suggested to have a causal role in early pregnancy abortion [155], and have been found at high concentrations in the amniotic fluids of women with intra-amniotic bacterial infection and pre-term labour [156, 157]. This supports the notion that up-regulation of TLR expression and TLR-triggered inflammation in the pregnant uterus signal for undesirable premature labour and pre-term delivery. In contrast, the inhibition of such TLR signalling systems would negatively affect the immune responses to viral infections. Our results confirm our hypothesis and show that the NK cells from cord blood do not express TLR3 and cannot harness the TLR3-triggered responses, as evidenced by impairments in IFN-γ production and cytotoxicity. These attributes of neonatal NK cells corroborate the overall picture of deficient cellular and Th1- type responses in newborns, and may predispose the baby to aberrant defence against HSV.

3.3 Paper III

In Paper III, we aimed to ascertain if CT could modulate PRR expression in adult PBMCs. Many adjuvants originate from microbial products and activate immune responses through engagement of PRRs. TLR ligands themselves are potent adjuvants for enhancing the APC functions of DCs, and CpG ODN stimulates the expression of its receptor, TLR9 [158]. However, some strong adjuvants are TLR-independent, e.g., aluminium-based adjuvants [159]. As already presented in the *Aims* section of the thesis, in a pilot study that analysed the effects of CT on the expression in PBMCs of transcripts for viral nuclear acid-sensing receptors TLR3, TLR7, TLR8, TLR9, RIG-I, MDA-5, PKR, and IFI-16, we uncovered a selective impact of CT on TLR7

expression. Overnight incubation of the PBMCs with CT resulted in the down-regulation of TLR7 expression, an effect that was seen already after 4 h and that increased further over time. When we examined the influence of CT on the cell types that dominantly express TLR7, i.e., pDCs and B cells, the same CT-induced decrease in TLR7 mRNA transcripts was observed.

CT binds with high affinity to its specific receptor, the monosialoganglioside GM1, which is expressed on the cell membrane of all nucleated cells. GM1 may be evenly expressed on the cell surface or may be grouped in specialised membrane invaginations (caveolae) that are rich in cholesterol, caveolin, and glycolipids. The binding of CTB to GM1 is used as the Gold standard to visualise these lipid rafts on different cells and to detect caveolae-mediated endocytosis [160, 161]. We aimed to examine the binding patterns of CT to different PBMC subpopulations, and to determine whether CT-mediated inhibition of TLR7 is preferentially exerted on TLR7 expressing cells owing to the higher level of binding of CT to these cells. Although all nucleated cells express GM1, the flow cytometry analysis revealed that neither the pDCs nor the B cells bound CTB on their surfaces. This was somewhat surprising as CTB binding to GM1 and the subsequent GM1 clustering and association with lipid rafts are crucial for CT trafficking into the cells, and thus represent a prerequisite for the activity of the toxin. Recently, it was shown that CT binding to DCs through GM1 is required for CT adjuvant activity [162]. However, another recent study has suggested that massive CTB binding to multiple GM1 receptors is not a strict precondition for CT-mediated toxicity. CT holotoxin that contained chimeric B pentamers with only one single native GM1-binding site was able to attach to one GM1 molecule, but this was enough for the CT-intoxication process [163]. This means that the level of CTB binding required for CT-mediated inhibition of TLR7 may have been below the detection limit of our flow cytometry analysis. The protein-receptor interactions are studied using more sensitive assays, e.g., a "flow cytometry protein-protein interaction assay".

Upon CTB binding to GM1, the CT-GM1 complex is internalised and transported into the endoplasmic reticulum *via* a retrograde trafficking pathway [164]. The CTA interacts non-covalently with the B subunit and is cleaved into the A1 and A2 chains. The carboxyl-terminus of the A2-chain stabilises the interactions with CTB and contains a sequence motif that is important for A1-chain trafficking (CT-A1). CT-A1 dissociates from the holotoxin and enters the cytosol. Here, it evades degradation by the proteasome due to its paucity of lysines residues, which are the sites of ubiquitination for misfolded host proteins. By virtue of its ADP-ribosyltransferase activity, CT-A1 ADP-ribosylates the α-subunit of the

stimulatory G protein, resulting in constitutive activation of adenylate cyclase. The consequent increase in cAMP concentration leads to hyperactivation of protein kinase A (PKA).

CT exerts most of its actions through the increasing the concentration of cAMP, and it has been shown that increasing cAMP levels by CT or other cAMP inducers is crucial in promoting the maturation of DCs [86, 165] and accounts in part for the adjuvant activity of CT [166]. Forskolin, which has the same cAMP-inducing property, is often used to determine the involvement in cellular processes of cAMP-mediated activities. Although we could show that CTA was required for the down-regulation of TLR7 mRNA (as this could not be achieved by CTB alone), the outcome was not a consequence of the CTA-induced increase in cAMP concentration or PKA involvement. Neither forskolin nor a cAMP analogue inhibited TLR7 mRNA expression. In addition, concomitant addition of PKA inhibitors together with CT did not influence the down-regulation of TLR7 mRNA expression. An increase in the level of cAMP does not explain all the observed effects of CT-induced maturation of DCs [86, 167], and other pathways must be involved. For example, cAMP-independent regulation by CT is involved in the activation of T cells and B cells [168] and cytokine secretion by PBMCs [169]. It has also been proposed that the presence of CTA itself could account for the specific properties of CT, since in experiments using CT mutants with non-toxin CTA, adjuvant activity *in vivo* was preserved [170].

PKC is a family of kinases that regulate the function of different proteins by phosphorylation of their serine and threonine residues. CT controls cytokine production by PBMCs through PKC [169]. There is evidence that PKC signalling interacts with TLR signalling, and this association is linked to TLR4-mediated cytokine secretion in macrophages [171]. In our experiments, the use of a PKC activator induced the same down-modulation of TLR7 mRNA as was seen with CT, which implies that CT acts through PKC. However, when we used PKC inhibitors, the disruption of the PKC pathway did not prevent CT-mediated TLR7 inhibition. These seemingly contradictory results suggest that CT regulates TLR7 transcription through not only the PKC signalling pathway, but also additional pathways.

CT can act through the induction of soluble factors, such as PGE_2 and nitric oxide (NO). These factors participate in the maturation of DCs induced by CT but also of bystander toxin-free DCs [165]. NO and superoxide anions directly stimulate DC maturation both phenotypically and functionally [172], and can act synergistically with other stimuli, such as LPS and TNF-α [173]. Among the many effects of NO, the best known is the activation of guanylate

cyclise, which converts GTP to cyclic GMP (cGMP), followed by the activation of cGMP-dependent protein kinase [174], a mechanism that we have not yet included in our analysis.

Many of the effects of CT are accomplished through the induction of cytokine secretion. CT has been reported to induce in PBMCs the production of the immunostimulatory cytokines IL-1β and IL-6 [169], and these cytokines are, together with IL-8, secreted by epithelial cells of the intestine where they promote the *in vivo* immune response to CT. We detected abundant production of both IL-6 and IL-8 in CT-stimulated PBMCs and pDCs. In general, Th1 cytokine levels are proposed to correlate with enhanced TLR expression. In patients with intestinal inflammation, the mucosal Th1 cytokines correlate with enhanced intestinal TLR expression and signalling (i.e., TLR2, TLR3, and TLR4) [175, 176]. In contrast, Th2 cytokines are known to down-regulate TLR signalling (TLR3 and TLR4) [177]. We attempted to ascertain whether the production of IL-6 and IL-8 that we observed is involved in the regulatory effect of CT on TLR7 expression. In a pilot experiment, we observed IL-6- and IL-8-induced down-regulation of TLR7 mRNA, mirroring the effect of CT. However, additional experiments are needed to confirm this potential regulatory mechanism.

These results were rather surprising to us since we surmised that CT, which exerts an immunostimulatory adjuvant role on APC, would have the opposite effect. We have not fully managed to understand the implication of this unexpected finding, or the underlying mechanism(s). The CT-induced TLR7 downregulation was not due to the toxic effects of CT, since CT seemed to prevent apoptosis in PBMC. CT can inhibit the cell-growth or induce apoptosis in some normal or tumour cells, mediated by either cAMP-dependent [178] or independent mechanisms [179, 180]. Although in our experiments the observed inhibition of TLR7 mRNA expression required the CTA subunit, the consequent increase in cAMP concentration did not exert toxic effects on PBMC. In addition, other adjuvants or mitogens can enhance TLR7 expression (or have no effect), as we showed for the TLR9 ligand CpG ODN and the pokeweed mitogen. We also confirmed that *S.aureus* gives rise to a strong TLR7 upregulation, as shown previously [181]. These stimuli, similar to CT, also induce IL-6 and IL-8 production, which according to our preliminary data contributes to TLR7 mRNA downregulation; however, in contrast to CT, they also promote INF-α and/or IFN-γ responses [182, 183]. IFN-α/β is the main cytokine that positively regulates the expression of many TLR, including TLR7, and IFN-γ is also associated with the increased TLR expression in a variety of cell types [184, 185]. It is thus possible that the combination of several cytokines creates a tight regulatory mechanism that

overcomes the negative effects of some cytokines (i.e. IL-6) and positively controls TLR7 expression. We speculate that in the case of CT, the lack of IFN (IFN-α/β and/or IFN-γ) might contribute to TLR7 downregulation. We have yet to perform these experiments.

TLR7 has recently been ascribed an important role in the development of autoimmune diseases in humans and mice [186]. In autoimmune-prone mice, duplication of the *tlr7* gene promotes the development of a systemic lupus erythematosus (SLE)-like disease [187], while deletion of the gene confers disease resistance [188]. In contrast, autoimmune-resistant mice with *tlr7* gene copy number amplification develop spontaneous inflammation and autoimmunity [189]. Patients with SLE have increased serum levels of IFN-α, secreted presumably by pDCs, which correspond to their levels of disease activity [190]. PBMCs from patients with SLE express higher levels of TLR7 [191], and in some patients, *tlr7* gene duplications have been detected and correlate with disease susceptibility [192]. The molecular basis for SLE and other autoimmune diseases, such as rheumatoid arthritis, is believed to stem from immune complexes, which are composed of autoantigens and self-RNA or self-DNA, which are endogenous ligands for TLR7 and TLR9, respectively. Studies that show that these autoimmune complexes can activate TLR9 signalling on pDCs and induce IFN-α production have suggested that the same mechanism may lead to TLR7 activation and the triggering of disease development [193]. RNA-containing immune complexes from patients with SLE induce Th1 cytokine production, and the released apoptotic material, which is rich in self nucleic acids, stimulates IFN-α secretion, a response that is terminated by RNases, which implies the involvement of TLR7 [186]. Taken together, these findings emphasise the deleterious impacts of TLR7 activation on inflammatory and autoimmune conditions. It seems that CT, conceivably in the course of infection with *V. cholera* or when used as an adjuvant, does not induce TLR7 up-regulation and is thus less likely to promote unwanted autoimmunity. Moreover, we postulate that CT, an adjuvant not associated with inflammation, may in the absence of TLR7 ligand engagement and activation, lead to TLR7 down-regulation and thereby exert one of its anti-inflammatory adjuvant activities.

In summary, our hypothesis that CT induces TLR expression was wrong. On the contrary, CT reduces TLR7 expression by pDCs and B cells, in a process that is cAMP- and PKA-independent but that involves PKC, and perhaps also IL-6. The relevance of this phenomenon, and whether it occurs *in vivo*, remains to be unravelled. However, our data imply that the transcription of the gene that encodes TLR7 is negatively regulated in a fashion different from that of other nucleic acid-sensing receptors.

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♫

REFERENCES

1. *Thucydides (1934) The Peloponnesian War (Crawley, trans.), p.112. Modern Library, New York.*
2. *Lucan (1928) The civil war (Duff, trans.), book IX. Loeb Classic Library, London.*
3. *Steinman, R. M. and Cohn, Z. A. (1973) Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. J Exp Med. **137**, 1142-62.*
4. *Dzionek, A., Fuchs, A., Schmidt, P., Cremer, S., Zysk, M., Miltenyi, S., Buck, D. W., Schmitz, J. (2000) BDCA-2, BDCA-3, and BDCA-4: three markers for distinct subsets of dendritic cells in human peripheral blood. J Immunol. **165**, 6037-46.*
5. *Randolph, G. J., Inaba, K., Robbiani, D. F., Steinman, R. M., Muller, W. A. (1999) Differentiation of phagocytic monocytes into lymph node dendritic cells in vivo. Immunity. **11**, 753-61.*
6. *Dominguez, P. M. and Ardavin, C. (2010) Differentiation and function of mouse monocyte-derived dendritic cells in steady state and inflammation. Immunol Rev. **234**, 90-104.*
7. *Shortman, K. and Naik, S. H. (2007) Steady-state and inflammatory dendritic-cell development. Nat Rev Immunol. **7**, 19-30.*
8. *Villadangos, J. A. and Shortman, K. (2010) Found in translation: the human equivalent of mouse CD8+ dendritic cells. J Exp Med. **207**, 1131-4.*
9. *De Smedt, T., Pajak, B., Muraille, E., Lespagnard, L., Heinen, E., De Baetselier, P., Urbain, J., Leo, O., Moser, M. (1996) Regulation of dendritic cell numbers and maturation by lipopolysaccharide in vivo. J Exp Med. **184**, 1413-24.*
10. *Cumberbatch, M., Dearman, R. J., Kimber, I. (1997) Langerhans cells require signals from both tumour necrosis factor-alpha and interleukin-1 beta for migration. Immunology. **92**, 388-95.*
11. *Cella, M., Scheidegger, D., Palmer-Lehmann, K., Lane, P., Lanzavecchia, A., Alber, G. (1996) Ligation of CD40 on dendritic cells triggers production of high levels of interleukin-12 and enhances T cell stimulatory capacity: T-T help via APC activation. J Exp Med. **184**, 747-52.*
12. *Kalinski, P., Hilkens, C. M., Wierenga, E. A., Kapsenberg, M. L. (1999) T-cell priming by type-1 and type-2 polarized dendritic cells: the concept of a third signal. Immunol Today. **20**, 561-7.*
13. *Delespesse, G., Ohshima, Y., Yang, L. P., Demeure, C., Sarfati, M. (1999) OX40-Mediated cosignal enhances the maturation of naive*

human CD4+ T cells into high IL-4-producing effectors. Int Arch Allergy Immunol. ***118****, 384-6.*

14. *Kalinski, P., Schuitemaker, J. H., Hilkens, C. M., Wierenga, E. A., Kapsenberg, M. L. (1999) Final maturation of dendritic cells is associated with impaired responsiveness to IFN-gamma and to bacterial IL-12 inducers: decreased ability of mature dendritic cells to produce IL-12 during the interaction with Th cells. J Immunol.* ***162****, 3231-6.*
15. *Langenkamp, A., Messi, M., Lanzavecchia, A., Sallusto, F. (2000) Kinetics of dendritic cell activation: impact on priming of TH1, TH2 and nonpolarized T cells. Nat Immunol.* ***1****, 311-6.*
16. *Mauri, C. and Bosma, A. (2012) Immune regulatory function of B cells. Annu Rev Immunol.* ***30****, 221-41.*
17. *Carson, W. E., Giri, J. G., Lindemann, M. J., Linett, M. L., Ahdieh, M., Paxton, R., Anderson, D., Eisenmann, J., Grabstein, K., Caligiuri, M. A. (1994) Interleukin (IL) 15 is a novel cytokine that activates human natural killer cells via components of the IL-2 receptor. J Exp Med.* ***180****, 1395-403.*
18. *Aste-Amezaga, M., D'Andrea, A., Kubin, M., Trinchieri, G. (1994) Cooperation of natural killer cell stimulatory factor/interleukin-12 with other stimuli in the induction of cytokines and cytotoxic cell-associated molecules in human T and NK cells. Cell Immunol.* ***156****, 480-92.*
19. *Robertson, M. J. and Ritz, J. (1990) Biology and clinical relevance of human natural killer cells. Blood.* ***76****, 2421-38.*
20. *Lanier, L. L., Phillips, J. H., Hackett, J., Jr., Tutt, M., Kumar, V. (1986) Natural killer cells: definition of a cell type rather than a function. J Immunol.* ***137****, 2735-9.*
21. *Karre, K., Ljunggren, H. G., Piontek, G., Kiessling, R. (1986) Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. Nature.* ***319****, 675-8.*
22. *Bukowski, J. F., Warner, J. F., Dennert, G., Welsh, R. M. (1985) Adoptive transfer studies demonstrating the antiviral effect of natural killer cells in vivo. J Exp Med.* ***161****, 40-52.*
23. *Orange, J. S. (2002) Human natural killer cell deficiencies and susceptibility to infection. Microbes Infect.* ***4****, 1545-58.*
24. *Biron, C. A., Byron, K. S., Sullivan, J. L. (1989) Severe herpesvirus infections in an adolescent without natural killer cells. N Engl J Med.* ***320****, 1731-5.*
25. *Yasukawa, M. and Kobayashi, Y. (1985) Inhibition of herpes simplex virus replication in vitro by human cytotoxic T cell clones and natural killer cell clones. J Gen Virol.* ***66 (Pt 10)****, 2225-9.*
26. *Hayakawa, Y. and Smyth, M. J. (2006) CD27 dissects mature NK cells into two subsets with distinct responsiveness and migratory capacity. J Immunol.* ***176****, 1517-24.*

27. *Hayakawa, Y., Huntington, N. D., Nutt, S. L., Smyth, M. J. (2006) Functional subsets of mouse natural killer cells. Immunol Rev.* ***214****, 47-55.*
28. *Beg, A. A. (2002) Endogenous ligands of Toll-like receptors: implications for regulating inflammatory and immune responses. Trends Immunol.* ***23****, 509-12.*
29. *Janeway, C. A., Jr. (1989) Approaching the asymptote? Evolution and revolution in immunology. Cold Spring Harb Symp Quant Biol.* ***54 Pt 1****, 1-13.*
30. *Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J. M., Hoffmann, J. A. (1996) The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. Cell.* ***86****, 973-83.*
31. *Medzhitov, R., Preston-Hurlburt, P., Janeway, C. A., Jr. (1997) A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. Nature.* ***388****, 394-7.*
32. *Poltorak, A., He, X., Smirnova, I., Liu, M. Y., Van Huffel, C., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C., Freudenberg, M., Ricciardi-Castagnoli, P., Layton, B., Beutler, B. (1998) Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science.* ***282****, 2085-8.*
33. *Zarember, K. A. and Godowski, P. J. (2002) Tissue expression of human Toll-like receptors and differential regulation of Toll-like receptor mRNAs in leukocytes in response to microbes, their products, and cytokines. J Immunol.* ***168****, 554-61.*
34. *Miyake, K. (2007) Innate immune sensing of pathogens and danger signals by cell surface Toll-like receptors. Semin Immunol.* ***19****, 3-10.*
35. *Yasuda, K., Rutz, M., Schlatter, B., Metzger, J., Luppa, P. B., Schmitz, F., Haas, T., Heit, A., Bauer, S., Wagner, H. (2006) CpG motif-independent activation of TLR9 upon endosomal translocation of "natural" phosphodiester DNA. Eur J Immunol.* ***36****, 431-6.*
36. *Barton, G. M., Kagan, J. C., Medzhitov, R. (2006) Intracellular localization of Toll-like receptor 9 prevents recognition of self DNA but facilitates access to viral DNA. Nat Immunol.* ***7****, 49-56.*
37. *Means, T. K., Latz, E., Hayashi, F., Murali, M. R., Golenbock, D. T., Luster, A. D. (2005) Human lupus autoantibody-DNA complexes activate DCs through cooperation of CD32 and TLR9. J Clin Invest.* ***115****, 407-17.*
38. *Pichlmair, A. and Reis e Sousa, C. (2007) Innate recognition of viruses. Immunity.* ***27****, 370-83.*
39. *Lee, S. B. and Esteban, M. (1994) The interferon-induced double-stranded RNA-activated protein kinase induces apoptosis. Virology.* ***199****, 491-6.*
40. *Unterholzner, L., Keating, S. E., Baran, M., Horan, K. A., Jensen, S. B., Sharma, S., Sirois, C. M., Jin, T., Latz, E., Xiao, T. S., Fitzgerald,*

K. A., Paludan, S. R., Bowie, A. G. (2010) IFI16 is an innate immune sensor for intracellular DNA. Nat Immunol. ***11****, 997-1004.*

41. Prantner, D., Darville, T., Nagarajan, U. M. (2010) Stimulator of IFN gene is critical for induction of IFN-beta during Chlamydia muridarum infection. J Immunol. ***184****, 2551-60.*

42. Daffis, S., Suthar, M. S., Szretter, K. J., Gale, M., Jr., Diamond, M. S. (2009) Induction of IFN-beta and the innate antiviral response in myeloid cells occurs through an IPS-1-dependent signal that does not require IRF-3 and IRF-7. PLoS Pathog. ***5****, e1000607.*

43. Loo, Y. M. and Gale, M., Jr. (2011) Immune signaling by RIG-I-like receptors. Immunity. ***34****, 680-92.*

44. Johnston, J. B., Barrett, J. W., Nazarian, S. H., Goodwin, M., Ricciuto, D., Wang, G., McFadden, G. (2005) A poxvirus-encoded pyrin domain protein interacts with ASC-1 to inhibit host inflammatory and apoptotic responses to infection. Immunity. ***23****, 587-98.*

45. Ichinohe, T., Watanabe, I., Ito, S., Fujii, H., Moriyama, M., Tamura, S., Takahashi, H., Sawa, H., Chiba, J., Kurata, T., Sata, T., Hasegawa, H. (2005) Synthetic double-stranded RNA poly(I:C) combined with mucosal vaccine protects against influenza virus infection. J Virol. ***79****, 2910-9.*

46. Salem, M. L., Kadima, A. N., Cole, D. J., Gillanders, W. E. (2005) Defining the antigen-specific T-cell response to vaccination and poly(I:C)/TLR3 signaling: evidence of enhanced primary and memory CD8 T-cell responses and antitumor immunity. J Immunother. ***28****, 220-8.*

47. Adams, M., Navabi, H., Jasani, B., Man, S., Fiander, A., Evans, A. S., Donninger, C., Mason, M. (2003) Dendritic cell (DC) based therapy for cervical cancer: use of DC pulsed with tumour lysate and matured with a novel synthetic clinically non-toxic double stranded RNA analogue poly [I]:poly [C(12)U] (Ampligen R). Vaccine. ***21****, 787-90.*

48. Hawiger, D., Inaba, K., Dorsett, Y., Guo, M., Mahnke, K., Rivera, M., Ravetch, J. V., Steinman, R. M., Nussenzweig, M. C. (2001) Dendritic cells induce peripheral T cell unresponsiveness under steady state conditions in vivo. J Exp Med. ***194****, 769-79.*

49. Lutz, M. B. and Schuler, G. (2002) Immature, semi-mature and fully mature dendritic cells: which signals induce tolerance or immunity? Trends Immunol. ***23****, 445-9.*

50. Higuchi (1963) Enzymatic formation of D-kynurenine. Federation Proceedings. ***22****, 243.*

51. Munn, D. H., Zhou, M., Attwood, J. T., Bondarev, I., Conway, S. J., Marshall, B., Brown, C., Mellor, A. L. (1998) Prevention of allogeneic fetal rejection by tryptophan catabolism. Science. ***281****, 1191-3.*

52. *Munn, D. H., Shafizadeh, E., Attwood, J. T., Bondarev, I., Pashine, A., Mellor, A. L. (1999) Inhibition of T cell proliferation by macrophage tryptophan catabolism. J Exp Med.* ***189****, 1363-72.*
53. *Lee, G. K., Park, H. J., Macleod, M., Chandler, P., Munn, D. H., Mellor, A. L. (2002) Tryptophan deprivation sensitizes activated T cells to apoptosis prior to cell division. Immunology.* ***107****, 452-60.*
54. *Frumento, G., Rotondo, R., Tonetti, M., Damonte, G., Benatti, U., Ferrara, G. B. (2002) Tryptophan-derived catabolites are responsible for inhibition of T and natural killer cell proliferation induced by indoleamine 2,3-dioxygenase. J Exp Med.* ***196****, 459-68.*
55. *Terness, P., Bauer, T. M., Rose, L., Dufter, C., Watzlik, A., Simon, H., Opelz, G. (2002) Inhibition of allogeneic T cell proliferation by indoleamine 2,3-dioxygenase-expressing dendritic cells: mediation of suppression by tryptophan metabolites. J Exp Med.* ***196****, 447-57.*
56. *Fallarino, F., Grohmann, U., Vacca, C., Bianchi, R., Orabona, C., Spreca, A., Fioretti, M. C., Puccetti, P. (2002) T cell apoptosis by tryptophan catabolism. Cell Death Differ.* ***9****, 1069-77.*
57. *Hill, M., Tanguy-Royer, S., Royer, P., Chauveau, C., Asghar, K., Tesson, L., Lavainne, F., Remy, S., Brion, R., Hubert, F. X., Heslan, M., Rimbert, M., Berthelot, L., Moffett, J. R., Josien, R., Gregoire, M., Anegon, I. (2007) IDO expands human CD4+CD25high regulatory T cells by promoting maturation of LPS-treated dendritic cells. Eur J Immunol.* ***37****, 3054-62.*
58. *Munn, D. H., Sharma, M. D., Mellor, A. L. (2004) Ligation of B7-1/B7-2 by human CD4+ T cells triggers indoleamine 2,3-dioxygenase activity in dendritic cells. J Immunol.* ***172****, 4100-10.*
59. *Katz, J. B., Muller, A. J., Prendergast, G. C. (2008) Indoleamine 2,3-dioxygenase in T-cell tolerance and tumoral immune escape. Immunol Rev.* ***222****, 206-21.*
60. *Chung, D. J., Rossi, M., Romano, E., Ghith, J., Yuan, J., Munn, D. H., Young, J. W. (2009) Indoleamine 2,3-dioxygenase-expressing mature human monocyte-derived dendritic cells expand potent autologous regulatory T cells. Blood.* ***114****, 555-63.*
61. *Munn, D. H., Sharma, M. D., Hou, D., Baban, B., Lee, J. R., Antonia, S. J., Messina, J. L., Chandler, P., Koni, P. A., Mellor, A. L. (2004) Expression of indoleamine 2,3-dioxygenase by plasmacytoid dendritic cells in tumor-draining lymph nodes. J Clin Invest.* ***114****, 280-90.*
62. *Hwang, S. L., Chung, N. P., Chan, J. K., Lin, C. L. (2005) Indoleamine 2, 3-dioxygenase (IDO) is essential for dendritic cell activation and chemotactic responsiveness to chemokines. Cell Res.* ***15****, 167-75.*
63. *Makrigiannakis, A., Petsas, G., Toth, B., Relakis, K., Jeschke, U. (2011) Recent advances in understanding immunology of reproductive failure. J Reprod Immunol.* ***90****, 96-104.*

64. *Vekemans, J., Ota, M. O., Wang, E. C., Kidd, M., Borysiewicz, L. K., Whittle, H., McAdam, K. P., Morgan, G., Marchant, A. (2002) T cell responses to vaccines in infants: defective IFNgamma production after oral polio vaccination. Clin Exp Immunol.* ***127****, 495-8.*

65. *Gans, H., Yasukawa, L., Rinki, M., DeHovitz, R., Forghani, B., Beeler, J., Audet, S., Maldonado, Y., Arvin, A. M. (2001) Immune responses to measles and mumps vaccination of infants at 6, 9, and 12 months. J Infect Dis.* ***184****, 817-26.*

66. *Ota, M. O., Vekemans, J., Schlegel-Haueter, S. E., Fielding, K., Whittle, H., Lambert, P. H., McAdam, K. P., Siegrist, C. A., Marchant, A. (2004) Hepatitis B immunisation induces higher antibody and memory Th2 responses in new-borns than in adults. Vaccine.* ***22****, 511-9.*

67. *Rabian-Herzog, C., Lesage, S., Gluckman, E. (1992) Characterization of lymphocyte subpopulations in cord blood. Bone Marrow Transplant.* ***9 Suppl 1****, 64-7.*

68. *Langrish, C. L., Buddle, J. C., Thrasher, A. J., Goldblatt, D. (2002) Neonatal dendritic cells are intrinsically biased against Th-1 immune responses. Clin Exp Immunol.* ***128****, 118-23.*

69. *Kollmann, T. R., Crabtree, J., Rein-Weston, A., Blimkie, D., Thommai, F., Wang, X. Y., Lavoie, P. M., Furlong, J., Fortuno, E. S., 3rd, Hajjar, A. M., Hawkins, N. R., Self, S. G., Wilson, C. B. (2009) Neonatal innate TLR-mediated responses are distinct from those of adults. J Immunol.* ***183****, 7150-60.*

70. *Wilson, C. B., Westall, J., Johnston, L., Lewis, D. B., Dower, S. K., Alpert, A. R. (1986) Decreased production of interferon-gamma by human neonatal cells. Intrinsic and regulatory deficiencies. J Clin Invest.* ***77****, 860-7.*

71. *Luevano, M., Daryouzeh, M., Alnabhan, R., Querol, S., Khakoo, S., Madrigal, A., Saudemont, A. (2012) The unique profile of cord blood natural killer cells balances incomplete maturation and effective killing function upon activation. Hum Immunol.* ***73****, 248-57.*

72. *Yabuhara, A., Kawai, H., Komiyama, A. (1990) Development of natural killer cytotoxicity during childhood: marked increases in number of natural killer cells with adequate cytotoxic abilities during infancy to early childhood. Pediatr Res.* ***28****, 316-22.*

73. *Wynn, J. L. and Levy, O. (2010) Role of innate host defenses in susceptibility to early-onset neonatal sepsis. Clin Perinatol.* ***37****, 307-37.*

74. *Firth, M. A., Shewen, P. E., Hodgins, D. C. (2005) Passive and active components of neonatal innate immune defenses. Anim Health Res Rev.* ***6****, 143-58.*

75. *Mariscalco, M. M., Tcharmtchi, M. H., Smith, C. W. (1998) P-Selectin support of neonatal neutrophil adherence under flow:*

contribution of L-selectin, LFA-1, and ligand(s) for P-selectin. Blood. ***91****, 4776-85.*

76. Klein, R. B., Fischer, T. J., Gard, S. E., Biberstein, M., Rich, K. C., Stiehm, E. R. (1977) Decreased mononuclear and polymorphonuclear chemotaxis in human newborns, infants, and young children. Pediatrics. ***60****, 467-72.*

77. Leibson, P. J., Hunter-Laszlo, M., Douvas, G. S., Hayward, A. R. (1986) Impaired neonatal natural killer-cell activity to herpes simplex virus: decreased inhibition of viral replication and altered response to lymphokines. J Clin Immunol. ***6****, 216-24.*

78. Kohl, S., Frazier, J. J., Greenberg, S. B., Pickering, L. K., Loo, L. S. (1981) Interferon induction of natural killer cytotoxicity in human neonates. J Pediatr. ***98****, 379-84.*

79. Porras, A., Kozar, S., Russanova, V., Salpea, P., Hirai, T., Sammons, N., Mittal, P., Kim, J. Y., Ozato, K., Romero, R., Howard, B. H. (2008) Developmental and epigenetic regulation of the human TLR3 gene. Mol Immunol. ***46****, 27-36.*

80. Forster-Waldl, E., Sadeghi, K., Tamandl, D., Gerhold, B., Hallwirth, U., Rohrmeister, K., Hayde, M., Prusa, A. R., Herkner, K., Boltz-Nitulescu, G., Pollak, A., Spittler, A. (2005) Monocyte toll-like receptor 4 expression and LPS-induced cytokine production increase during gestational aging. Pediatr Res. ***58****, 121-4.*

81. Levy, O., Zarember, K. A., Roy, R. M., Cywes, C., Godowski, P. J., Wessels, M. R. (2004) Selective impairment of TLR-mediated innate immunity in human newborns: neonatal blood plasma reduces monocyte TNF-alpha induction by bacterial lipopeptides, lipopolysaccharide, and imiquimod, but preserves the response to R-848. J Immunol. ***173****, 4627-34.*

82. Belderbos, M. E., van Bleek, G. M., Levy, O., Blanken, M. O., Houben, M. L., Schuijff, L., Kimpen, J. L., Bont, L. (2009) Skewed pattern of Toll-like receptor 4-mediated cytokine production in human neonatal blood: low LPS-induced IL-12p70 and high IL-10 persist throughout the first month of life. Clin Immunol. ***133****, 228-37.*

83. Siegrist, C. A. (2001) Neonatal and early life vaccinology. Vaccine. ***19****, 3331-46.*

84. Finkelstein, R. A. and Dorner, F. (1985) Cholera enterotoxin (choleragen). Pharmacol Ther. ***27****, 37-47.*

85. George-Chandy, A., Eriksson, K., Lebens, M., Nordstrom, I., Schon, E., Holmgren, J. (2001) Cholera toxin B subunit as a carrier molecule promotes antigen presentation and increases CD40 and CD86 expression on antigen-presenting cells. Infect Immun. ***69****, 5716-25.*

86. Gagliardi, M. C., Sallusto, F., Marinaro, M., Langenkamp, A., Lanzavecchia, A., De Magistris, M. T. (2000) Cholera toxin induces

maturation of human dendritic cells and licences them for Th2 priming. Eur J Immunol. ***30****, 2394-403.*

87. *Staats, H. F. and Ennis, F. A., Jr. (1999) IL-1 is an effective adjuvant for mucosal and systemic immune responses when coadministered with protein immunogens. J Immunol.* ***162****, 6141-7.*

88. *Grdic, D., Ekman, L., Schon, K., Lindgren, K., Mattsson, J., Magnusson, K. E., Ricciardi-Castagnoli, P., Lycke, N. (2005) Splenic marginal zone dendritic cells mediate the cholera toxin adjuvant effect: dependence on the ADP-ribosyltransferase activity of the holotoxin. J Immunol.* ***175****, 5192-202.*

89. *Lycke, N. and Holmgren, J. (1986) Strong adjuvant properties of cholera toxin on gut mucosal immune responses to orally presented antigens. Immunology.* ***59****, 301-8.*

90. *Sun, J. B., Eriksson, K., Li, B. L., Lindblad, M., Azem, J., Holmgren, J. (2004) Vaccination with dendritic cells pulsed in vitro with tumor antigen conjugated to cholera toxin efficiently induces specific tumoricidal CD8+ cytotoxic lymphocytes dependent on cyclic AMP activation of dendritic cells. Clin Immunol.* ***112****, 35-44.*

91. *Eriksson, K., Fredriksson, M., Nordstrom, I., Holmgren, J. (2003) Cholera toxin and its B subunit promote dendritic cell vaccination with different influences on Th1 and Th2 development. Infect Immun.* ***71****, 1740-7.*

92. *Eriksson, K., Sun, J. B., Nordstrom, I., Fredriksson, M., Lindblad, M., Li, B. L., Holmgren, J. (2004) Coupling of antigen to cholera toxin for dendritic cell vaccination promotes the induction of MHC class I-restricted cytotoxic T cells and the rejection of a cognate antigen-expressing model tumor. Eur J Immunol.* ***34****, 1272-81.*

93. *Chandy, A. G., Nurkkala, M., Josefsson, A., Eriksson, K. (2007) Therapeutic dendritic cell vaccination with Ag coupled to cholera toxin in combination with intratumoural CpG injection leads to complete tumour eradication in mice bearing HPV 16 expressing tumours. Vaccine.* ***25****, 6037-46.*

94. *Nurkkala, M., Wassen, L., Nordstrom, I., Gustavsson, I., Slavica, L., Josefsson, A., Eriksson, K. (2010) Conjugation of HPV16 E7 to cholera toxin enhances the HPV-specific T-cell recall responses to pulsed dendritic cells in vitro in women with cervical dysplasia. Vaccine.* ***28****, 5828-36.*

95. *Rudnick, C. M. and Hoekzema, G. S. (2002) Neonatal herpes simplex virus infections. Am Fam Physician.* ***65****, 1138-42.*

96. *Kimberlin, D. W. (2004) Neonatal herpes simplex infection. Clin Microbiol Rev.* ***17****, 1-13.*

97. *Aurelius, E., Johansson, B., Skoldenberg, B., Forsgren, M. (1993) Encephalitis in immunocompetent patients due to herpes simplex virus type 1 or 2 as determined by type-specific polymerase chain*

reaction and antibody assays of cerebrospinal fluid. J Med Virol. ***39****, 179-86.*

98. Zhang, S. Y., Jouanguy, E., Ugolini, S., Smahi, A., Elain, G., Romero, P., Segal, D., Sancho-Shimizu, V., Lorenzo, L., Puel, A., Picard, C., Chapgier, A., Plancoulaine, S., Titeux, M., Cognet, C., von Bernuth, H., Ku, C. L., Casrouge, A., Zhang, X. X., Barreiro, L., Leonard, J., Hamilton, C., Lebon, P., Heron, B., Vallee, L., Quintana-Murci, L., Hovnanian, A., Rozenberg, F., Vivier, E., Geissmann, F., Tardieu, M., Abel, L., Casanova, J. L. (2007) TLR3 deficiency in patients with herpes simplex encephalitis. Science. ***317****, 1522-7.*

99. Guo, Y., Audry, M., Ciancanelli, M., Alsina, L., Azevedo, J., Herman, M., Anguiano, E., Sancho-Shimizu, V., Lorenzo, L., Pauwels, E., Philippe, P. B., Perez de Diego, R., Cardon, A., Vogt, G., Picard, C., Andrianirina, Z. Z., Rozenberg, F., Lebon, P., Plancoulaine, S., Tardieu, M., Valerie, D., Jouanguy, E., Chaussabel, D., Geissmann, F., Abel, L., Casanova, J. L., Zhang, S. Y. (2011) Herpes simplex virus encephalitis in a patient with complete TLR3 deficiency: TLR3 is otherwise redundant in protective immunity. J Exp Med. ***208****, 2083-98.*

100. Perez de Diego, R., Sancho-Shimizu, V., Lorenzo, L., Puel, A., Plancoulaine, S., Picard, C., Herman, M., Cardon, A., Durandy, A., Bustamante, J., Vallabhapurapu, S., Bravo, J., Warnatz, K., Chaix, Y., Cascarrigny, F., Lebon, P., Rozenberg, F., Karin, M., Tardieu, M., Al-Muhsen, S., Jouanguy, E., Zhang, S. Y., Abel, L., Casanova, J. L. (2010) Human TRAF3 adaptor molecule deficiency leads to impaired Toll-like receptor 3 response and susceptibility to herpes simplex encephalitis. Immunity. ***33****, 400-11.*

101. Casrouge, A., Zhang, S. Y., Eidenschenk, C., Jouanguy, E., Puel, A., Yang, K., Alcais, A., Picard, C., Mahfoufi, N., Nicolas, N., Lorenzo, L., Plancoulaine, S., Senechal, B., Geissmann, F., Tabeta, K., Hoebe, K., Du, X., Miller, R. L., Heron, B., Mignot, C., de Villemeur, T. B., Lebon, P., Dulac, O., Rozenberg, F., Beutler, B., Tardieu, M., Abel, L., Casanova, J. L. (2006) Herpes simplex virus encephalitis in human UNC-93B deficiency. Science. ***314****, 308-12.*

102. Kim, Y. M., Brinkmann, M. M., Paquet, M. E., Ploegh, H. L. (2008) UNC93B1 delivers nucleotide-sensing toll-like receptors to endolysosomes. Nature. ***452****, 234-8.*

103. Sancho-Shimizu, V., Perez de Diego, R., Lorenzo, L., Halwani, R., Alangari, A., Israelsson, E., Fabrega, S., Cardon, A., Maluenda, J., Tatematsu, M., Mahvelati, F., Herman, M., Ciancanelli, M., Guo, Y., AlSum, Z., Alkhamis, N., Al-Makadma, A. S., Ghadiri, A., Boucherit, S., Plancoulaine, S., Picard, C., Rozenberg, F., Tardieu, M., Lebon, P., Jouanguy, E., Rezaei, N., Seya, T., Matsumoto, M., Chaussabel, D., Puel, A., Zhang, S. Y., Abel, L., Al-Muhsen, S., Casanova, J. L. (2011) Herpes simplex encephalitis in children with autosomal

recessive and dominant TRIF deficiency. J Clin Invest. ***121****, 4889-902.*

104. Dasari, P., Zola, H., Nicholson, I. C. (2011) Expression of Toll-like receptors by neonatal leukocytes. Pediatr Allergy Immunol. ***22****, 221-8.*

105. Luft, T., Jefford, M., Luetjens, P., Toy, T., Hochrein, H., Masterman, K. A., Maliszewski, C., Shortman, K., Cebon, J., Maraskovsky, E. (2002) Functionally distinct dendritic cell (DC) populations induced by physiologic stimuli: prostaglandin E(2) regulates the migratory capacity of specific DC subsets. Blood. ***100****, 1362-72.*

106. Scandella, E., Men, Y., Gillessen, S., Forster, R., Groettrup, M. (2002) Prostaglandin E2 is a key factor for CCR7 surface expression and migration of monocyte-derived dendritic cells. Blood. ***100****, 1354-61.*

107. Rieser, C., Bock, G., Klocker, H., Bartsch, G., Thurnher, M. (1997) Prostaglandin E2 and tumor necrosis factor alpha cooperate to activate human dendritic cells: synergistic activation of interleukin 12 production. J Exp Med. ***186****, 1603-8.*

108. Braun, D., Longman, R. S., Albert, M. L. (2005) A two-step induction of indoleamine 2,3 dioxygenase (IDO) activity during dendritic-cell maturation. Blood. ***106****, 2375-81.*

109. Krause, P., Singer, E., Darley, P. I., Klebensberger, J., Groettrup, M., Legler, D. F. (2007) Prostaglandin E2 is a key factor for monocyte-derived dendritic cell maturation: enhanced T cell stimulatory capacity despite IDO. J Leukoc Biol. ***82****, 1106-14.*

110. Weber, W. P., Feder-Mengus, C., Chiarugi, A., Rosenthal, R., Reschner, A., Schumacher, R., Zajac, P., Misteli, H., Frey, D. M., Oertli, D., Heberer, M., Spagnoli, G. C. (2006) Differential effects of the tryptophan metabolite 3-hydroxyanthranilic acid on the proliferation of human CD8+ T cells induced by TCR triggering or homeostatic cytokines. Eur J Immunol. ***36****, 296-304.*

111. Dhodapkar, M. V., Steinman, R. M., Sapp, M., Desai, H., Fossella, C., Krasovsky, J., Donahoe, S. M., Dunbar, P. R., Cerundolo, V., Nixon, D. F., Bhardwaj, N. (1999) Rapid generation of broad T-cell immunity in humans after a single injection of mature dendritic cells. J Clin Invest. ***104****, 173-80.*

112. Salio, M., Shepherd, D., Dunbar, P. R., Palmowski, M., Murphy, K., Wu, L., Cerundolo, V. (2001) Mature dendritic cells prime functionally superior melan-A-specific CD8+ lymphocytes as compared with nonprofessional APC. J Immunol. ***167****, 1188-97.*

113. Thurner, B., Haendle, I., Roder, C., Dieckmann, D., Keikavoussi, P., Jonuleit, H., Bender, A., Maczek, C., Schreiner, D., von den Driesch, P., Brocker, E. B., Steinman, R. M., Enk, A., Kampgen, E., Schuler, G. (1999) Vaccination with mage-3A1 peptide-pulsed mature, monocyte-derived dendritic cells expands specific cytotoxic T cells

and induces regression of some metastases in advanced stage IV melanoma. J Exp Med. ***190****, 1669-78.*

114. Mellor, A. L. and Munn, D. H. (1999) Tryptophan catabolism and T-cell tolerance: immunosuppression by starvation? Immunol Today. ***20****, 469-73.*

115. Von Bubnoff, D., Scheler, M., Wilms, H., Fimmers, R., Bieber, T. (2011) Identification of IDO-positive and IDO-negative human dendritic cells after activation by various proinflammatory stimuli. J Immunol. ***186****, 6701-9.*

116. Munn, D. H., Mellor, A. L., Rossi, M., Young, J. W. (2005) Dendritic cells have the option to express IDO-mediated suppression or not. Blood. ***105****, 2618.*

117. Munn, D. H., Sharma, M. D., Lee, J. R., Jhaver, K. G., Johnson, T. S., Keskin, D. B., Marshall, B., Chandler, P., Antonia, S. J., Burgess, R., Slingluff, C. L., Jr., Mellor, A. L. (2002) Potential regulatory function of human dendritic cells expressing indoleamine 2,3-dioxygenase. Science. ***297****, 1867-70.*

118. Terness, P., Chuang, J. J., Bauer, T., Jiga, L., Opelz, G. (2005) Regulation of human auto- and alloreactive T cells by indoleamine 2,3-dioxygenase (IDO)-producing dendritic cells: too much ado about IDO? Blood. ***105****, 2480-6.*

119. Lob, S., Ebner, S., Wagner, S., Weinreich, J., Schafer, R., Konigsrainer, A. (2007) Are indoleamine-2,3-dioxygenase producing human dendritic cells a tool for suppression of allogeneic T-cell responses? Transplantation. ***83****, 468-73.*

120. Terness, P., Chuang, J. J., Opelz, G. (2006) The immunoregulatory role of IDO-producing human dendritic cells revisited. Trends Immunol. ***27****, 68-73.*

121. Uyttenhove, C., Pilotte, L., Theate, I., Stroobant, V., Colau, D., Parmentier, N., Boon, T., Van den Eynde, B. J. (2003) Evidence for a tumoral immune resistance mechanism based on tryptophan degradation by indoleamine 2,3-dioxygenase. Nat Med. ***9****, 1269-74.*

122. Pietra, G., Manzini, C., Rivara, S., Vitale, M., Cantoni, C., Petretto, A., Balsamo, M., Conte, R., Benelli, R., Minghelli, S., Solari, N., Gualco, M., Queirolo, P., Moretta, L., Mingari, M. C. (2012) Melanoma cells inhibit natural killer cell function by modulating the expression of activating receptors and cytolytic activity. Cancer Res. ***72****, 1407-15.*

123. Wobser, M., Voigt, H., Houben, R., Eggert, A. O., Freiwald, M., Kaemmerer, U., Kaempgen, E., Schrama, D., Becker, J. C. (2007) Dendritic cell based antitumor vaccination: impact of functional indoleamine 2,3-dioxygenase expression. Cancer Immunol Immunother. ***56****, 1017-24.*

124. Mellor, A. L., Baban, B., Chandler, P., Marshall, B., Jhaver, K., Hansen, A., Koni, P. A., Iwashima, M., Munn, D. H. (2003) Cutting

edge: induced indoleamine 2,3 dioxygenase expression in dendritic cell subsets suppresses T cell clonal expansion. J Immunol. ***171****, 1652-5.*

125. Eljaafari, F. M., Takada, H., Tanaka, T., Doi, T., Ohga, S., Hara, T. (2011) Potent induction of IFN-gamma production from cord blood NK cells by the stimulation with single-stranded RNA. J Clin Immunol. ***31****, 728-35.*

126. Sivori, S., Falco, M., Carlomagno, S., Romeo, E., Moretta, L., Moretta, A. (2007) Heterogeneity of TLR3 mRNA transcripts and responsiveness to poly (I:C) in human NK cells derived from different donors. Int Immunol. ***19****, 1341-8.*

127. Ashkar, A. A., Yao, X. D., Gill, N., Sajic, D., Patrick, A. J., Rosenthal, K. L. (2004) Toll-like receptor (TLR)-3, but not TLR4, agonist protects against genital herpes infection in the absence of inflammation seen with CpG DNA. J Infect Dis. ***190****, 1841-9.*

128. MacDonald, E. M., Savoy, A., Gillgrass, A., Fernandez, S., Smieja, M., Rosenthal, K. L., Ashkar, A. A., Kaushic, C. (2007) Susceptibility of human female primary genital epithelial cells to herpes simplex virus, type-2 and the effect of TLR3 ligand and sex hormones on infection. Biol Reprod. ***77****, 1049-59.*

129. Yao, X. D. and Rosenthal, K. L. (2011) Herpes simplex virus type 2 virion host shutoff protein suppresses innate dsRNA antiviral pathways in human vaginal epithelial cells. J Gen Virol. ***92****, 1981-93.*

130. Chan, A., Hong, D. L., Atzberger, A., Kollnberger, S., Filer, A. D., Buckley, C. D., McMichael, A., Enver, T., Bowness, P. (2007) CD56bright human NK cells differentiate into CD56dim cells: role of contact with peripheral fibroblasts. J Immunol. ***179****, 89-94.*

131. Gottschalk, L. R., Bray, R. A., Kaizer, H., Gebel, H. M. (1990) Two populations of CD56 (Leu-19)+/CD16+ cells in bone marrow transplant recipients. Bone Marrow Transplant. ***5****, 259-64.*

132. Jacobs, R., Stoll, M., Stratmann, G., Leo, R., Link, H., Schmidt, R. E. (1992) CD16- CD56+ natural killer cells after bone marrow transplantation. Blood. ***79****, 3239-44.*

133. Lutz, C. T., Karapetyan, A., Al-Attar, A., Shelton, B. J., Holt, K. J., Tucker, J. H., Presnell, S. R. (2011) Human NK cells proliferate and die in vivo more rapidly than T cells in healthy young and elderly adults. J Immunol. ***186****, 4590-8.*

134. Romagnani, C., Juelke, K., Falco, M., Morandi, B., D'Agostino, A., Costa, R., Ratto, G., Forte, G., Carrega, P., Lui, G., Conte, R., Strowig, T., Moretta, A., Munz, C., Thiel, A., Moretta, L., Ferlazzo, G. (2007) CD56brightCD16- killer Ig-like receptor- NK cells display longer telomeres and acquire features of CD56dim NK cells upon activation. J Immunol. ***178****, 4947-55.*

135. Tanaka, H., Kai, S., Yamaguchi, M., Misawa, M., Fujimori, Y., Yamamoto, M., Hara, H. (2003) Analysis of natural killer (NK) cell

activity and adhesion molecules on NK cells from umbilical cord blood. Eur J Haematol. ***71****, 29-38.*

136. Lopez-Verges, S., Milush, J. M., Pandey, S., York, V. A., Arakawa-Hoyt, J., Pircher, H., Norris, P. J., Nixon, D. F., Lanier, L. L. (2010) CD57 defines a functionally distinct population of mature NK cells in the human CD56dimCD16+ NK-cell subset. Blood. ***116****, 3865-74.*

137. Lukassen, H. G., van der Meer, A., van Lierop, M. J., Lindeman, E. J., Joosten, I., Braat, D. D. (2003) The proportion of follicular fluid CD16+CD56DIM NK cells is increased in IVF patients with idiopathic infertility. J Reprod Immunol. ***60****, 71-84.*

138. Moffett-King, A. (2002) Natural killer cells and pregnancy. Nat Rev Immunol. ***2****, 656-63.*

139. Koopman, L. A., Kopcow, H. D., Rybalov, B., Boyson, J. E., Orange, J. S., Schatz, F., Masch, R., Lockwood, C. J., Schachter, A. D., Park, P. J., Strominger, J. L. (2003) Human decidual natural killer cells are a unique NK cell subset with immunomodulatory potential. J Exp Med. ***198****, 1201-12.*

140. Kopcow, H. D., Allan, D. S., Chen, X., Rybalov, B., Andzelm, M. M., Ge, B., Strominger, J. L. (2005) Human decidual NK cells form immature activating synapses and are not cytotoxic. Proc Natl Acad Sci U S A. ***102****, 15563-8.*

141. Vacca, P., Pietra, G., Falco, M., Romeo, E., Bottino, C., Bellora, F., Prefumo, F., Fulcheri, E., Venturini, P. L., Costa, M., Moretta, A., Moretta, L., Mingari, M. C. (2006) Analysis of natural killer cells isolated from human decidua: Evidence that 2B4 (CD244) functions as an inhibitory receptor and blocks NK-cell function. Blood. ***108****, 4078-85.*

142. King, A., Birkby, C., Loke, Y. W. (1989) Early human decidual cells exhibit NK activity against the K562 cell line but not against first trimester trophoblast. Cell Immunol. ***118****, 337-44.*

143. Rouas-Freiss, N., Goncalves, R. M., Menier, C., Dausset, J., Carosella, E. D. (1997) Direct evidence to support the role of HLA-G in protecting the fetus from maternal uterine natural killer cytolysis. Proc Natl Acad Sci U S A. ***94****, 11520-5.*

144. Hunt, J. S., Petroff, M. G., McIntire, R. H., Ober, C. (2005) HLA-G and immune tolerance in pregnancy. FASEB J. ***19****, 681-93.*

145. Wold, A. S. and Arici, A. (2005) Natural killer cells and reproductive failure. Curr Opin Obstet Gynecol. ***17****, 237-41.*

146. Aoki, K., Kajiura, S., Matsumoto, Y., Ogasawara, M., Okada, S., Yagami, Y., Gleicher, N. (1995) Preconceptional natural-killer-cell activity as a predictor of miscarriage. Lancet. ***345****, 1340-2.*

147. Bao, S. H., Shuai, W., Tong, J., Wang, L., Chen, P., Sun, J. (2012) Increased expression of Toll-like receptor 3 in decidual natural killer cells of patients with unexplained recurrent spontaneous miscarriage. Eur J Obstet Gynecol Reprod Biol. ***165****, 326-30.*

148. *Eriksson, M., Meadows, S. K., Basu, S., Mselle, T. F., Wira, C. R., Sentman, C. L. (2006) TLRs mediate IFN-gamma production by human uterine NK cells in endometrium. J Immunol.* ***176****, 6219-24.*

149. *Lesmeister, M. J., Jorgenson, R. L., Young, S. L., Misfeldt, M. L. (2005) 17Beta-estradiol suppresses TLR3-induced cytokine and chemokine production in endometrial epithelial cells. Reprod Biol Endocrinol.* ***3****, 74.*

150. *Kliman, H. J., Sammar, M., Grimpel, Y. I., Lynch, S. K., Milano, K. M., Pick, E., Bejar, J., Arad, A., Lee, J. J., Meiri, H., Gonen, R. (2012) Placental protein 13 and decidual zones of necrosis: an immunologic diversion that may be linked to preeclampsia. Reprod Sci.* ***19****, 16-30.*

151. *Gomez, L. M., Ma, Y., Ho, C., McGrath, C. M., Nelson, D. B., Parry, S. (2008) Placental infection with human papillomavirus is associated with spontaneous preterm delivery. Hum Reprod.* ***23****, 709-15.*

152. *Arechavaleta-Velasco, F., Gomez, L., Ma, Y., Zhao, J., McGrath, C. M., Sammel, M. D., Nelson, D. B., Parry, S. (2008) Adverse reproductive outcomes in urban women with adeno-associated virus-2 infections in early pregnancy. Hum Reprod.* ***23****, 29-36.*

153. *Eskild, A., Bruu, A. L., Stray-Pedersen, B., Jenum, P. (2005) Epstein-Barr virus infection during pregnancy and the risk of adverse pregnancy outcome. BJOG.* ***112****, 1620-4.*

154. *Goldenberg, R. L., Hauth, J. C., Andrews, W. W. (2000) Intrauterine infection and preterm delivery. N Engl J Med.* ***342****, 1500-7.*

155. *Vitoratos, N., Papadias, C., Economou, E., Makrakis, E., Panoulis, C., Creatsas, G. (2006) Elevated circulating IL-1beta and TNF-alpha, and unaltered IL-6 in first-trimester pregnancies complicated by threatened abortion with an adverse outcome. Mediators Inflamm.* ***2006****, 30485.*

156. *Romero, R., Manogue, K. R., Mitchell, M. D., Wu, Y. K., Oyarzun, E., Hobbins, J. C., Cerami, A. (1989) Infection and labor. IV. Cachectin-tumor necrosis factor in the amniotic fluid of women with intraamniotic infection and preterm labor. Am J Obstet Gynecol.* ***161****, 336-41.*

157. *El-Bastawissi, A. Y., Williams, M. A., Riley, D. E., Hitti, J., Krieger, J. N. (2000) Amniotic fluid interleukin-6 and preterm delivery: a review. Obstet Gynecol.* ***95****, 1056-64.*

158. *Krieg, A. M. (2006) Therapeutic potential of Toll-like receptor 9 activation. Nat Rev Drug Discov.* ***5****, 471-84.*

159. *De Gregorio, E., D'Oro, U., Wack, A. (2009) Immunology of TLR-independent vaccine adjuvants. Curr Opin Immunol.* ***21****, 339-45.*

160. *Gupta, N. and DeFranco, A. L. (2003) Visualizing lipid raft dynamics and early signaling events during antigen receptor-mediated B-lymphocyte activation. Mol Biol Cell.* ***14****, 432-44.*

161. *Wolf, A. A., Fujinaga, Y., Lencer, W. I. (2002) Uncoupling of the cholera toxin-G(M1) ganglioside receptor complex from endocytosis, retrograde Golgi trafficking, and downstream signal transduction by depletion of membrane cholesterol. J Biol Chem.* ***277****, 16249-56.*
162. *Gustafsson, T., Hua, Y. J., Dahlgren, M. W., Livingston, M., Johansson-Lindbom, B., Yrlid, U. (2013) Direct interaction between cholera toxin and dendritic cells is required for oral adjuvant activity. Eur J Immunol.* ***43****, 1779-88.*
163. *Jobling, M. G., Yang, Z., Kam, W. R., Lencer, W. I., Holmes, R. K. (2012) A single native ganglioside GM1-binding site is sufficient for cholera toxin to bind to cells and complete the intoxication pathway. MBio.* ***3****.*
164. *Lencer, W. I. and Tsai, B. (2003) The intracellular voyage of cholera toxin: going retro. Trends Biochem Sci.* ***28****, 639-45.*
165. *Bagley, K. C., Abdelwahab, S. F., Tuskan, R. G., Lewis, G. K. (2006) Cholera toxin indirectly activates human monocyte-derived dendritic cells in vitro through the production of soluble factors, including prostaglandin E(2) and nitric oxide. Clin Vaccine Immunol.* ***13****, 106-15.*
166. *Lycke, N., Tsuji, T., Holmgren, J. (1992) The adjuvant effect of Vibrio cholerae and Escherichia coli heat-labile enterotoxins is linked to their ADP-ribosyltransferase activity. Eur J Immunol.* ***22****, 2277-81.*
167. *Gagliardi, M. C., Sallusto, F., Marinaro, M., Vendetti, S., Riccomi, A., De Magistris, M. T. (2002) Effects of the adjuvant cholera toxin on dendritic cells: stimulatory and inhibitory signals that result in the amplification of immune responses. Int J Med Microbiol.* ***291****, 571-5.*
168. *Staples, K. J., Bergmann, M., Tomita, K., Houslay, M. D., McPhee, I., Barnes, P. J., Giembycz, M. A., Newton, R. (2001) Adenosine 3',5'-cyclic monophosphate (cAMP)-dependent inhibition of IL-5 from human T lymphocytes is not mediated by the cAMP-dependent protein kinase A. J Immunol.* ***167****, 2074-80.*
169. *Krakauer, T. (1996) Evidence for protein kinase C pathway in the response of human peripheral blood mononuclear cells to cholera toxin. Cell Immunol.* ***172****, 224-8.*
170. *Yamamoto, M., Kiyono, H., Yamamoto, S., Batanero, E., Kweon, M. N., Otake, S., Azuma, M., Takeda, Y., McGhee, J. R. (1999) Direct effects on antigen-presenting cells and T lymphocytes explain the adjuvanticity of a nontoxic cholera toxin mutant. J Immunol.* ***162****, 7015-21.*
171. *Loegering, D. J. and Lennartz, M. R. (2011) Protein kinase C and toll-like receptor signaling. Enzyme Res.* ***2011****, 537821.*

*172. Kantengwa, S., Jornot, L., Devenoges, C., Nicod, L. P. (2003) Superoxide anions induce the maturation of human dendritic cells. Am J Respir Crit Care Med. **167**, 431-7.*

*173. Paolucci, C., Burastero, S. E., Rovere-Querini, P., De Palma, C., Falcone, S., Perrotta, C., Capobianco, A., Manfredi, A. A., Clementi, E. (2003) Synergism of nitric oxide and maturation signals on human dendritic cells occurs through a cyclic GMP-dependent pathway. J Leukoc Biol. **73**, 253-62.*

*174. Denninger, J. W. and Marletta, M. A. (1999) Guanylate cyclase and the .NO/cGMP signaling pathway. Biochim Biophys Acta. **1411**, 334-50.*

*175. Hausmann, M., Kiessling, S., Mestermann, S., Webb, G., Spottl, T., Andus, T., Scholmerich, J., Herfarth, H., Ray, K., Falk, W., Rogler, G. (2002) Toll-like receptors 2 and 4 are up-regulated during intestinal inflammation. Gastroenterology. **122**, 1987-2000.*

*176. Cario, E. and Podolsky, D. K. (2000) Differential alteration in intestinal epithelial cell expression of toll-like receptor 3 (TLR3) and TLR4 in inflammatory bowel disease. Infect Immun. **68**, 7010-7.*

*177. Mueller, T., Terada, T., Rosenberg, I. M., Shibolet, O., Podolsky, D. K. (2006) Th2 cytokines down-regulate TLR expression and function in human intestinal epithelial cells. J Immunol. **176**, 5805-14.*

*178. Smets, L. A. and Van Rooy, H. (1987) Mitogenic and antimitogenic effects of cholera toxin-mediated cyclic AMP levels in 3T3 cells. J Cell Physiol. **133**, 395-9.*

*179. Anderson, D. L. and Tsoukas, C. D. (1989) Cholera toxin inhibits resting human T cell activation via a cAMP-independent pathway. J Immunol. **143**, 3647-52.*

*180. Viallet, J., Sharoni, Y., Frucht, H., Jensen, R. T., Minna, J. D., Sausville, E. A. (1990) Cholera toxin inhibits signal transduction by several mitogens and the in vitro growth of human small-cell lung cancer. J Clin Invest. **86**, 1904-12.*

*181. Hadley, J. S., Wang, J. E., Foster, S. J., Thiemermann, C., Hinds, C. J. (2005) Peptidoglycan of Staphylococcus aureus upregulates monocyte expression of CD14, Toll-like receptor 2 (TLR2), and TLR4 in human blood: possible implications for priming of lipopolysaccharide signaling. Infect Immun. **73**, 7613-9.*

*182. Pichereau, S., Moran, J. J., Hayney, M. S., Shukla, S. K., Sakoulas, G., Rose, W. E. (2012) Concentration-dependent effects of antimicrobials on Staphylococcus aureus toxin-mediated cytokine production from peripheral blood mononuclear cells. J Antimicrob Chemother. **67**, 123-9.*

*183. Parcina, M., Wendt, C., Goetz, F., Zawatzky, R., Zahringer, U., Heeg, K., Bekeredjian-Ding, I. (2008) Staphylococcus aureus-induced plasmacytoid dendritic cell activation is based on an IgG-mediated memory response. J Immunol. **181**, 3823-33.*

184. *Miettinen, M., Sareneva, T., Julkunen, I., Matikainen, S. (2001) IFNs activate toll-like receptor gene expression in viral infections. Genes Immun.* ***2****, 349-55.*
185. *Bosisio, D., Polentarutti, N., Sironi, M., Bernasconi, S., Miyake, K., Webb, G. R., Martin, M. U., Mantovani, A., Muzio, M. (2002) Stimulation of toll-like receptor 4 expression in human mononuclear phagocytes by interferon-gamma: a molecular basis for priming and synergism with bacterial lipopolysaccharide. Blood.* ***99****, 3427-31.*
186. *Krieg, A. M. and Vollmer, J. (2007) Toll-like receptors 7, 8, and 9: linking innate immunity to autoimmunity. Immunol Rev.* ***220****, 251-69.*
187. *Pisitkun, P., Deane, J. A., Difilippantonio, M. J., Tarasenko, T., Satterthwaite, A. B., Bolland, S. (2006) Autoreactive B cell responses to RNA-related antigens due to TLR7 gene duplication. Science.* ***312****, 1669-72.*
188. *Christensen, S. R., Shupe, J., Nickerson, K., Kashgarian, M., Flavell, R. A., Shlomchik, M. J. (2006) Toll-like receptor 7 and TLR9 dictate autoantibody specificity and have opposing inflammatory and regulatory roles in a murine model of lupus. Immunity.* ***25****, 417-28.*
189. *Deane, J. A., Pisitkun, P., Barrett, R. S., Feigenbaum, L., Town, T., Ward, J. M., Flavell, R. A., Bolland, S. (2007) Control of toll-like receptor 7 expression is essential to restrict autoimmunity and dendritic cell proliferation. Immunity.* ***27****, 801-10.*
190. *Niewold, T. B., Hua, J., Lehman, T. J., Harley, J. B., Crow, M. K. (2007) High serum IFN-alpha activity is a heritable risk factor for systemic lupus erythematosus. Genes Immun.* ***8****, 492-502.*
191. *Komatsuda, A., Wakui, H., Iwamoto, K., Ozawa, M., Togashi, M., Masai, R., Maki, N., Hatakeyama, T., Sawada, K. (2008) Up-regulated expression of Toll-like receptors mRNAs in peripheral blood mononuclear cells from patients with systemic lupus erythematosus. Clin Exp Immunol.* ***152****, 482-7.*
192. *Garcia-Ortiz, H., Velazquez-Cruz, R., Espinosa-Rosales, F., Jimenez-Morales, S., Baca, V., Orozco, L. (2010) Association of TLR7 copy number variation with susceptibility to childhood-onset systemic lupus erythematosus in Mexican population. Ann Rheum Dis.* ***69****, 1861-5.*
193. *Vallin, H., Perers, A., Alm, G. V., Ronnblom, L. (1999) Anti-double-stranded DNA antibodies and immunostimulatory plasmid DNA in combination mimic the endogenous IFN-alpha inducer in systemic lupus erythematosus. J Immunol.* ***163****, 6306-13.*