Role and Modulation of Dendritic Cells in Organ Transplantation

PhD Thesis

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Abbreviations

AA-DC	Alternatively-activated Dendritic cells
Ab	Antibody
Ag	Antigen
APC	Antigen presenting cell(s)
CD	Cluster of Differentiation
CpG	Cytosine phosphatidyl Guanine
CLR	C-type lectin receptor
CR	Complement receptor
DAF	Decay Accelerating Factor
DAMP	Damage Associated Molecular Pattern(s)
DC	Dendritic cell(s)
DC-SIGN	Dendritic cell specific ICAM3 grabbing non-integrin
DXS	Low molecular weight dextran sulfate (MW 5000)
DNA	Deoxyribonucleic acid
EC	Endothelial cell(s)
ELISA	Enzyme-linked immunosorbent assay
ET-1	Endothelin-1
FACS	Fluorescence activated cell scanning
HA	Hyaluronic acid
HIF	Hypoxia inducible factor
HLA	Human leukocyte antigen
HMGB-1	High mobility group box 1
HS	Heparan sulfate
HSPG	Heparan sulfate proteoglycans
Ig	Immunoglobulin
IL	Interleukin
I/R	Ischemia/Reperfusion
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
mAb	monoclonal Antibody
MCP	Membrane Cofactor Protein
MHC	Major histocompatibility complex
MMR	Macrophage mannose receptor

MoDC	monocyte-derived Dendritic cells
MyD88	Myeloid differentiation primary response gene 88
NF-κB	Nuclear factor kappa B
NLR	NOD like receptor
PAMP	Pathogen Associated Molecular Pattern(s)
PAR	Protease activated receptor
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
RAGE	Receptor of advanced glycation endproducts
RBC	Red blood cell(s)
rHDL	reconstituted high density lipoprotein
TCR	T cell receptor
TF	Tissue factor
TLR	Toll-like receptor
TNF	Tumor necrosis factor

Abstract

Modern immunosuppressive regimens have improved the short-term success of transplantation over the last decades. Nevertheless severe side-effects can be the consequence of long-term use of immunosuppressive drugs. The most suitable solution to overcome these problems would be the establishment of graft specific tolerance. In this context, the role of the innate immunity in organ transplantation cannot be neglected. Failure of tolerance induction in clinical organ transplantation has been attributed to a strong activation of innate immunity, particularly the dendritic cells (DC), mainly due to ischemia/reperfusion (I/R) injury of the donor organ.

The working hypothesis, upon which this thesis is based, was to further study the role played by DC in the context of organ transplantation and to identify candidate substances for modulating DC maturation in context of tolerance induction.

Low molecular weight dextran sulfate (DXS), a known complement inhibitor and endothelial cell protectant, was further studied within this thesis. Our results demonstrate a novel inhibitory effect of DXS on TLR4-mediated maturation of DC, resulting in reduced upregulation of costimulatory molecules, cytokine secretion and prevention of T cell proliferation. Additionally, an inhibitory effect of DXS on TLR2-mediated activation of NK cells has been demonstrated. The second substance considered in this thesis was reconstituted high density lipoprotein (rHDL) because of its beneficial effects on the endothelium. Treatment of DC with rHDL prevented their TLR-induced maturation.

Follow-up studies on DC maturation mechanisms provided new evidence of involvement of the transcription factor hypoxia-inducible factor 1α (HIF-1α), a molecule connected to inflammation and ischemia, in TLR-induced maturation of human DC. In addition, we showed that stimulation of human DC with TLR agonists induce the production of endothelin-1 (ET-1), a vasoactive peptide involved in the pathogenesis of I/R injury.

In summary, our data demonstrate a novel inhibitory effect of DXS and rHDL on DC maturation and the involvement of HIF-1α in TLR-mediated DC maturation. In addition, TLR2-agonist mediated activation of NK cells was also prevented by DXS.

1 Introduction

1-1 Barriers in Clinical Organ Transplantation

Modern immunosuppressive regimens for organ transplantation have shown good short-term results, but much less dramatic improvements in long-term outcomes. Severe side-effects such as infections, nephrotoxicity, cancer and liver diseases could be the consequences of long-term immunosuppression. One solution to overcome these problems would be the establishment of transplantation tolerance. Transplantation tolerance is a state of permanent acceptance of the graft in absence of continuous immunosuppression. Successful tolerance induction was demonstrated in rodent models as early as 1953 by the group of Sir Peter B. Medawar (1). However, the transfer of such protocols to large animals and to humans has turned out to be very difficult. There are various reasons why most of the experimental tolerance protocols fail in clinical transplantation. Prolonged ischemia time of the organ, brain death of the donor as well as immunological memory are considered to be important factors. The innate immune system has been shown to be activated in ischemia/reperfusion (I/R) injury as well as brain death and other factors (Table 1) (2-4). Therefore, the development of methods to blunt or modulate innate immune response s is likely to have a significant impact on organ transplantation.

TABLE 1. STIMULATION FACTORS OF INNATE IMMUNITY IN ORGAN TRANSPLANTATION

DONOR	TRANSPORT	RECIPIENT
Mortal injury (Severe trauma, Hemorrage or Cerebral Ischemia)	Cold Ischemia	Warm Ischemia (Cardiac by-pass)
Emergency interventions	Duration (Time)	Infection
Brain Death		Drug Therapy
Postmortem support		Alloimmunity
Organ procurement		

1-2 Innate Immunity and Organ Transplantation

Historically the immune system has always been divided into the innate and the adaptive immune system. The non-adaptive defenses form the earliest barrier to infection. All the many microorganisms which a human being encounters every day in life only occasionally cause disease. Most of them are detected and destroyed within minutes or hours by immune mechanisms that do not rely on the clonal

expansion of antigen-specific lymphocytes and thus do not require prolonged periods of induction (5). These are the mechanisms of innate immunity. In organ transplantation, the adaptive immune system has been considered to be the main player in graft rejection for a long time. Advances in recent years have revealed that the innate immune system is crucially involved in shaping the adaptive immune response. According to these new findings, there is increasing evidence that innate immunity play an important role in the outcome of solid organ transplantation, namely in graft rejection or tolerance induction.

1-3 Humoral Innate Immunity

1-3-1 The Complement System

The complement system was discovered by Jules Bordet as a heat-labile component of normal plasma. The activity was said to "complement" the antibacterial activity of antibodies. Nowadays we know that the complement system is consisting of more than 30 proteins in plasma and on cell surfaces. The amount of complement proteins in plasma is approximately 3 mg/ml. There are three main pathways of activation: the classical, alternative and the mannan-binding lectin (MBL) pathway. Complement plays an important role in the immune system and has been demonstrated to be crucially involved in various diseases as summarized in Table 2.

The first complement pathway discovered was the classical pathway. The proteins of this pathway are designated from C1 to C9. The proteins of the alternative pathway are called factors, followed by a letter as e.g. factor B. Several complement proteins are cleaved during activation. Cleaved fragments are designated with lower suffixes (e.g. the complement protein C5 is cleaved into C5a and C5b). The large fragment is usually called "b" and the small "a". All the pathways converge in the formation of the C3 convertase. The terminal complement pathway, leading to the formation of the membrane-attacking complex (MAC, or C5b-9), is a unique system that builds up a lipophilic complex into cell membranes forming a pore that ultimately cause the lysis of the cell (6, 7). The detailed complement activation pathways are shown in Figure 1.

TABLE 2. PHYSIOLOGICAL ROLES OF FLUID-PHASE COMPLEMENT PROTEINS

ACTIVITY	RESPONSIBLE COMPLEMENT PROTEIN
Host Defense against Infections	
Opsonization	Covalently bound fragements of C3 and C4
Chemotaxis and activation of leukocytes	Anaphylotoxins (C5a, C3a and C4a) and their corresponding receptors (C5aR and C3aR)
Lysis of bacteria and cells	Membrane-attack complex (C5b-9)
Interface between innate and adaptive immunity	
Augmentation of antibody responses	C3b and C4b bound to immune complexes and to antigen; C3 receptors on B cells and antigen-presenting cells
Enhancement of immunological memory	C3b and C4b bound to immune complexes and to antigen; C3 receptors on follicular dendritic cells
Disposal of waste	
Clearance of immune complexes from tissues	C1q; covalently bound fragments C3 and C4
Clearance of apoptotic cells	C1q; covalently bound fragments C3 and C4
Ischemia/Reperfusion injury	
Tissue Injury and Inflammation	C1q; covalently bound fragments C3 and C4; Anaphylotoxins C5a and C3a; Membrane-attack complex (C5b-9)

The classical pathway is initiated by binding of the C1 complex (C1q bound to two C1r and C1s) to IgG or IgM bound to an antigen. Conformational changes lead to activation of C1r which subsequently cleaves its associated C1s to generate an active serine protease. C1s cleaves then C4 and then C2 to generate C4b and C2a, which together form the C3 convertase (C4b2a) bound to the cell or bacterial surface. The C3 convertase cleaves C3 into C3a and C3b molecules, the latter coating the cell surface and therefore acting as opsonins. Cell surfaces coated with C3b promote further C3 cleavage by the amplification loop. The next step is the formation of the C5 convertase. C5 is captured by the C5 convertase through binding of an acceptor site of C3b, and is thus rendered susceptible to cleavage by the serine protease activity of C2a or Bb. This reaction generates C5b and C5a, a very potent anaphylatoxin. C5b binds one molecule C6, and the C5b6 complex binds C7. Conformational change leads to exposure of a hydrophobic site on C7, which inserts into the lipid bilayer. C8 binds to the complex and induces the polymerization of C9 into a pore-forming structure, called membrane attacking complex (MAC) or C5b-9.

The lectin or MBL pathway uses similar proteins as the classical pathway. MBL forms a complex with mannan-binding lectin-associated proteases 1 and 2 (MASP-1 and MASP-2) and specifically binds to mannose residues, which are present on

pathogen surfaces. When the complex binds to the pathogen surface, MASP-2 is activated to cleave C4 and C2 in a similar way to C1s with a subsequently analogous pathway.

The alternative pathway can be initiated in the absence of antibodies by covalent binding of a small amount of C3b to hydroxyl groups on cell-surface carbohydrates or proteins and is activated by low-grade cleavage of C3 in plasma. It is initiated by spontaneous hydrolysis of the thioester bond of C3 to form C3(H2O). This molecule has an another conformation, allowing binding of factor B (fB) with subsequent cleavage of fB to Bb and Ba by factor D (fD). Bb remains associated with C3(H2O) forming the C3 convertase C3(H2O)Bb in the fluid phase. Following cleavage of C3 into C3a and C3b, C3b binds to the cell surface and fB forming the alternative pathway C3 convertase C3bBb.

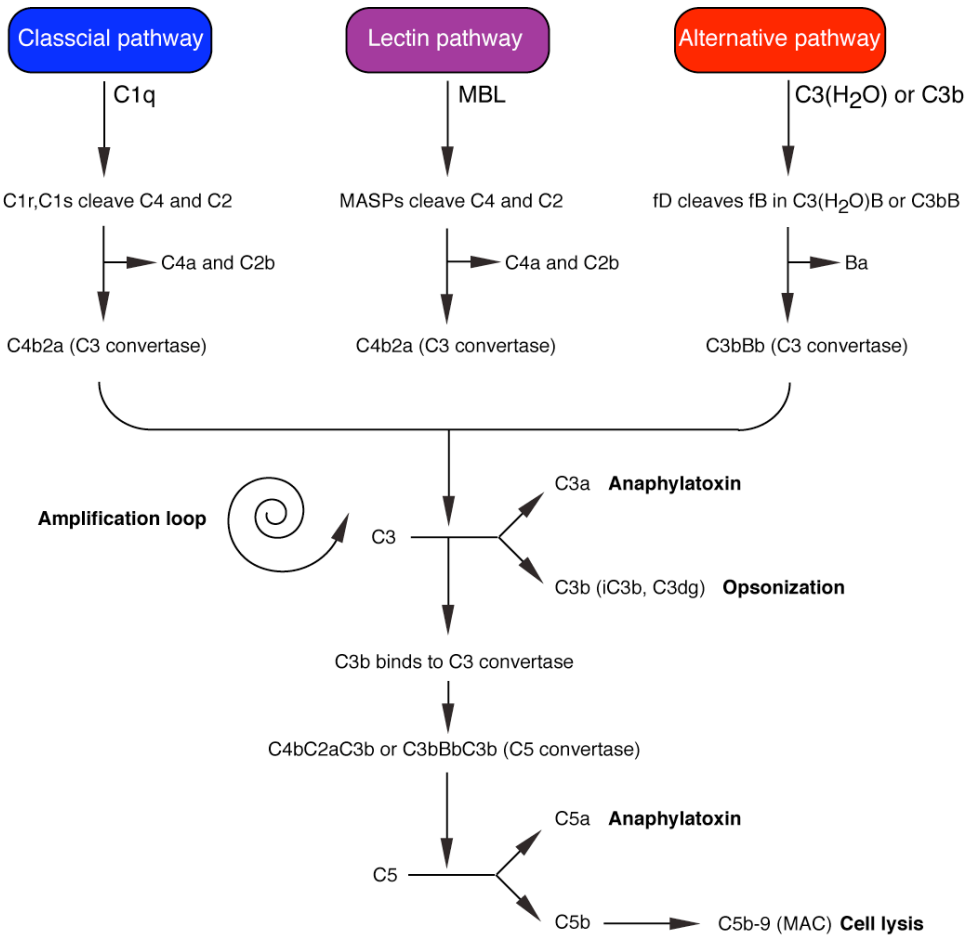

Figure 1: Three main pathways of complement activation

Recent findings revealed that several molecules are capable of circumventing these three major complement pathways. The coagulation cascade protein thrombin can act as a substitute for the C3-dependent C5 convertase and cleave C5 and generate C5a (8). MBL can induce C3 deposition by engagement of the alternative pathway to generate the C3 convertase C3bBb in the absence of C2 and C4 (9). Moreover, a study of Kang *et al.* has demonstrated that the receptor SIGN-R1 can directly bind C1q and assemble the C3 convertase without the help of antibodies and absence of factor B (10).

The complement system has to be tightly regulated in order to avoid unwanted activation. Nowadays we know several fluid-phase as well as membrane-bound complement regulators, which are summarized in Table 3.

TABLE 3. REGULATORY PROTEINS OF COMPLEMENT

REGULATOR	FUNCTION
C1-INH	Binds to activated C1r, C1s, removing them from C1q; binds to activated MASP-2, removing it from MBL
C4BP	Binds C4b, displacing C2a; cofactor for C4b cleavage by Factor I
Factor H	Binds C3b, displacing Bb; cofactor for Factor I
Factor I	Serine protease that cleaves C3b and C4b; aided by Factor H, MCP, C4BP or CR1
Factor P	Stabilize the alternative C3 convertase
S-protein	Binds to the fluid phase of C5b67
Clusterin	Binds to the fluid phase of C5b67
Heparin	Binds to the fluid phase of C5b67
Carboxypeptidase N (CPN)	Anaphylotoxin inactivator
CR1 (CD35)	Binds C4b, displacing C2a; or C3b displacing Bb; cofactor for Factor I
CR2 (CD21)	C3d receptor
DAF (CD55)	Displaces Bb from C3b and C2a from C4b
MCP (CD46)	Promotes C3b and C4b inactivation by Factor I
CD59	Prevents formation of MAC
Homologous Restriction Factor (HRF)	Reduces binding of C9 to the target

1-3-1-1 Complement in Organ Transplantation

That complement is involved in allograft rejection has been known since the seminal studies on graft-specific antibody responses by Paul Terasaki (11) almost 50 years ago. Still today, the presence of preformed, complement-activating antibodies in

the circulation of the recipient, like in ABO-incompatibility and HLA-presensitization, represents a formidable barrier to successful transplantation. The development of therapeutic options to allow for ABO-incompatible transplantation began as early as 25 years ago (12, 13) and today ABO-incompatible living-related kidney transplantation is increasingly being performed using specific anti-A/B immunoabsorption or plasmapheresis and anti-CD20 antibody treatment (14). However, while we have learned how to deal with anti-A/B blood group antibodies, no recipe has been found as yet to reliably prevent or treat rejection due to anti-HLA antibodies, and it is feared that there will be both acute and long-term effects of the presence of such antibodies in the recipient's circulation (15). Complement inhibitors have been widely applied to treat antibody-mediated rejection in animal transplantation models, notably in xenotransplantation, but with the exception of intravenous immunoglobulins (IVIG) and C1-inhibitor (16-20) they are currently not used in clinical transplantation.

In addition to activation by graft-specific antibodies circulating in the blood of the recipient, the deposition of complement on graft cells is also triggered by ischemia/reperfusion (I/R) injury. In clinical transplantation, donor organ procurement and preservation inevitably lead to I/R injury, which critically influences allograft survival. Indeed, approaches to inhibit complement activation in I/R injury showed beneficial effects in experimental models of transplantation and beyond. The use of soluble complement receptor 1 (sCR1) was shown to attenuate post-ischemic myocardial inflammation and necrosis (21) and attenuate I/R injury in experimental models of lung and liver transplantation (22-24). These findings have led to studies on the mechanism by which the complement system is activated in I/R injury. Taken together, these studies showed that all three major activation pathways of complement are involved in I/R injury. In a rabbit model of myocardial I/R injury participation of the classical complement pathway was demonstrated by use of a specific C1s inhibitor (25). Involvement of the alternative pathway was observed in a mouse model of renal I/R injury in which mice deficient of factor B had less deposition of C3 (26), and recently also an important contribution of the lectin pathway to I/R injury was reported (26-28). While the precise mechanisms of I/R injury are not yet fully understood it has been shown that neoepitopes are exposed on endothelial cells (EC) upon ischemia and recognized upon reperfusion by pattern recognition

molecules such as MBL, either directly or via binding of naturally occurring IgM, triggering complement activation and subsequent graft damage (29-31).

The importance of local complement synthesis in allotransplantation has recently been highlighted by Sacks and Zhou (32). The three most important differences of local versus systemic complement production are rapid action, tissue penetrability and site-specific regulation. Glomerular epithelial cells in the kidney spontaneously express the C3 gene and the latter plays a role in local, immune-mediated tissue injury (33). Some of the complement factors, such as C3, have a large molecular weight (180 kDa), which limits the availability of such molecules in the extracellular compartment, supporting the importance of local production for tissue penetrability (32). It was shown that expression of complement regulatory proteins was reduced in ischemic tissue, suggesting that not only production but also regulation of complement takes place site-specifically (34). Therapeutic approaches targeted at systemic complement depletion may therefore not be efficient and they are not desirable either, because complement contributes to microbial opsonization and immune complex clearing, and a lack of this function may render a graft recipient more prone to infections, especially when complement depletion is envisaged for a prolonged period of time and combined with immunosuppression. Indeed, a strategy targeted at systemic complement inhibition using the rodent C3 convertase inhibitor CR1-related protein y-Ig (Crry-Ig) did not reduced renal I/R injury (35). Strategies and/or substances, which lead to site-specific, local complement inhibition are therefore clearly needed to prevent I/R injury in transplantation.

1-3-2 The Coagulation System

The coagulation system has an important role in hemostasis, but there is also an intensive interaction with the complement system (36). Furthermore, thrombin or other coagulation proteases, besides their role in thrombosis, have the ability to activate protease-activated receptors (PAR). Four PAR (PAR_{1-4}) have been identified so far. They all belong to the G protein coupled receptors and are activated by serine proteases. PAR play a role in innate immunity (37, 38). For these reasons, the coagulation system has been added here as part of the innate immune system.

Similar to the complement system, the coagulation system consists of multiple plasma proteins, which interact with one another in complex cascades. Classically, the coagulation system has been divided into the intrinsic/contact activation pathway and

the extrinsic/tissue factor (TF) pathway, both pathway converge at the level of factor X to a final common pathway (Figure 2). The contact activation pathway is initiated by the activation of prekallikrein, high molecular weight kininogen (HMWK) and factor XII to activated FXIIa, which in turns activates FXI to FXIa and FXVIII to FXVIIIa. The extrinsic pathway is activated by exposed TF, which triggers activation of FVII to FVIIa. Both FVIIa and FVIIIa converge to activate FX to FXa, which sets off activation of FV to FVa and subsequently initiates the key step to activating prothrombin to thrombin, which itself converts fibrinogen to fibrin (39, 40). Thrombotic disorder may hence end up as bleeding disorder. Therefore, as with the complement system, unwanted, deleterious activation of procoagulatory pathways requires a tight regulation. Regulators of the coagulation cascade are C1-INH, tissue factor pathway inhibitor (TFPI), activated protein C, protein S (cofactor for activated protein C), thrombomodulin (TM, activates protein C), plasminogen activator inhibitor (PAI), protein Z-dependent protease inhibitor (ZPI), protein Z (cofactor for ZPI) and antithrombin III as shown in Figure 2. Members of the coagulation system cooperate closely with platelets. Activated platelets provide negatively charged phospholipids surfaces, where the process of coagulation can occur (39). Furthermore, release of chondroitin sulfate by activated platelets could trigger activation of the complement system (41).

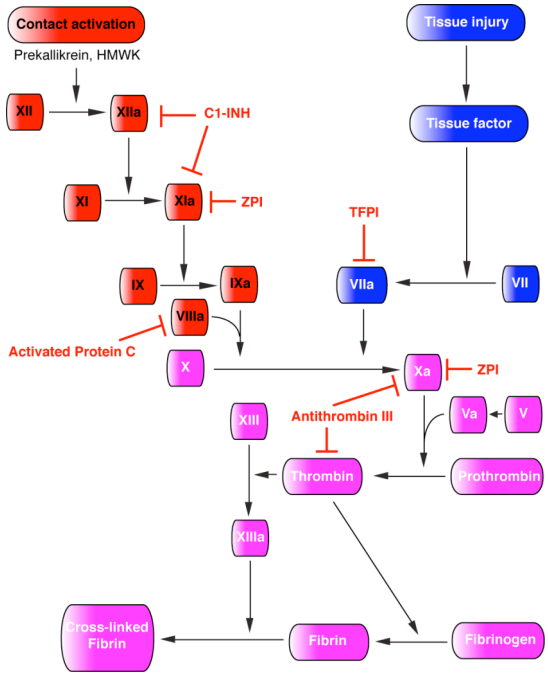

Figure 2: The coagulation cascade (contact activation pathway in red, tissue factor pathway in blue, common pathway in pink). Regulators are indicated in red.

1-3-2-1 The Coagulation System in Organ Transplantation

Activation of EC occurring during I/R injury leads to a pro-coagulant state of the endothelium. For example, induction of TF expression was observed during I/R injury of rabbit and pig heart (42-44), rat kidney (45) and following cold storage of organs (46). A recent study has shown the involvement of PAR_1 in a model of mouse heart-to-rat model. Donor PAR_1 expression and its activation by thrombin is required to generate local monocyte chemoattractant protein-1 (MCP-1), which is responsible to recruit natural killer (NK) cells and macrophages into the graft (47). Expression of PAR_{1-3} on the mRNA level have been shown for dendritic cells (DC) and they seem to be involved in the differentiation of DC (48). The precise role of PAR expression on DC remains to be clarified. In addition, platelets were found to promote allograft rejection by release of soluble CD40L (CD154), which activates antigen-presenting cells (APC) through CD40 (49).

1-4 Cellular Part of Innate Immunity

Traditionally, the following cells are categorized as members of the innate immune system: Neutrophils, eosinophils, basophils, mast cells, monocytes, macrophages, DC, NK cells, NK-T cells, γδ T cells and B-1 cells. Most of them interact with players of the adaptive immune system, namely the T lymphocytes. In this respect, another cell type should be considered in this category, namely the EC. There is growing evidence that microvascular EC not only play a role in leukocyte adhesion but also possess antigen-presenting capacities (50).

1-4-1 Pattern Recognition by the Innate Immune System

The hallmark of the innate immune system is the use of not clonally distributed, germline-encoded pattern recognition receptors (PRR) to sense invading organisms or tissue injury. PRR are expressed either on the cell surface or intracellularly. Some others are secreted into the bloodstream or tissue fluid. Principal functions of PRR include opsonization, activation of complement and coagulation, phagocytosis, activation of pro- or anti-inflammatory signaling pathways as well as apoptosis. Although microorganisms and viruses have tremendous variety, they possess common structural features, like lipopolysaccharide (LPS) from gram-negative bacteria, lipoteichoic acid (LTA) from gram-positive bacteria, lipoarabinomannan (LAM) from

mycobacteria, unmethylated DNA and several others. These molecules are called pathogen-associated molecular patterns (PAMP). In recent years, PRR have been shown to bind several endogenous molecules that are released in response to tissue injury and cellular stress (described in more detail in section 1-4-2-1). These molecules are therefore called damage-associated molecular patterns (DAMP). However, both PAMP and DAMP are sensed by PRR.

Collectins, ficolins, C-reactive protein (CRP) and serum amyloid A (SAP) are secreted PRR. The molecule MBL and surfactant proteins (SP) A and D are prominent members of the collectin family. They typically form oligomeric receptors and are characterized by a collagenous region, the C-type lectin domain. Ficolins, namely L-ficolin, M-ficolin and H-ficolin, are structurally similar to collectins, but contain a fibrinogenous instead of a C-type lectin domain. MBL and ficolins activate the lectin complement pathway, whereas SP-A and SP-D act as opsonins. CRP and SAP are members of the pentraxin family. They can act as opsonins by binding of phosphorylcholine on bacterial surfaces or apoptotic cells as well as to C1q and thus activate the classical complement pathway (5).

Several cell surface PRR have been described. The most prominent family of PRR are the toll-like receptors (TLR) which will be described in more detail in the next section. C-type lectin receptors (CLR) as e.g. macrophage mannose receptor (MMR; CD206), DEC-205 (CD205) or DC-SIGN (CD209) are mainly phagocytic receptors. They recognize glycoproteins, which lead to subsequent up-take of the antigen (Ag) and delivery to lysosomal compartments and destruction by lysosomal enzymes. A different phagocytic receptor is the macrophage scavenger receptor (MSR, CD204), which recognizes polyanionic structures on pathogens like LPS or LTA (5). An additional cell surface PRR is the receptor of advanced glycation end products (RAGE), first described as receptor for nonenzymatic glycation and protein products (51). Recent studies have shown that RAGE binds DAMP like the high-mobility group box 1 (HMGB-1) protein (52, 53).

In recent years, two other families of “non-toll” related PRR have received increasing attention, namely the NOD-like receptors (NLR) and RIG-like helicases (RLH). NLR and RLH are soluble proteins that survey the intracellular milieu. The family of NLR can be further subdivided into two subfamilies that consist of the NODs and the NALP family. The first NLRs reported were NOD1 and NOD2, which

recognize bacterial petidoglycans leading to a subsequent activation of NF-κB (54, 55). The NALP family consists of several members. Especially NALP3 seems to be involved in sensing of cellular stress. NALP3, together with adaptors proteins like ASC and CARDINAL, complexes with capase-1 to form the so-called NALP3-inflammasome, which is a crucial "molecular platform" controlling activation of the proinflammatory cytokines IL-1β and IL-18. RLH are cytoplasmic sensors of viral dsRNA, which activate the transcription factors NF-κB and Interferon regulatory factor (IRF) 3/7, leading to production of type I interferons (56).

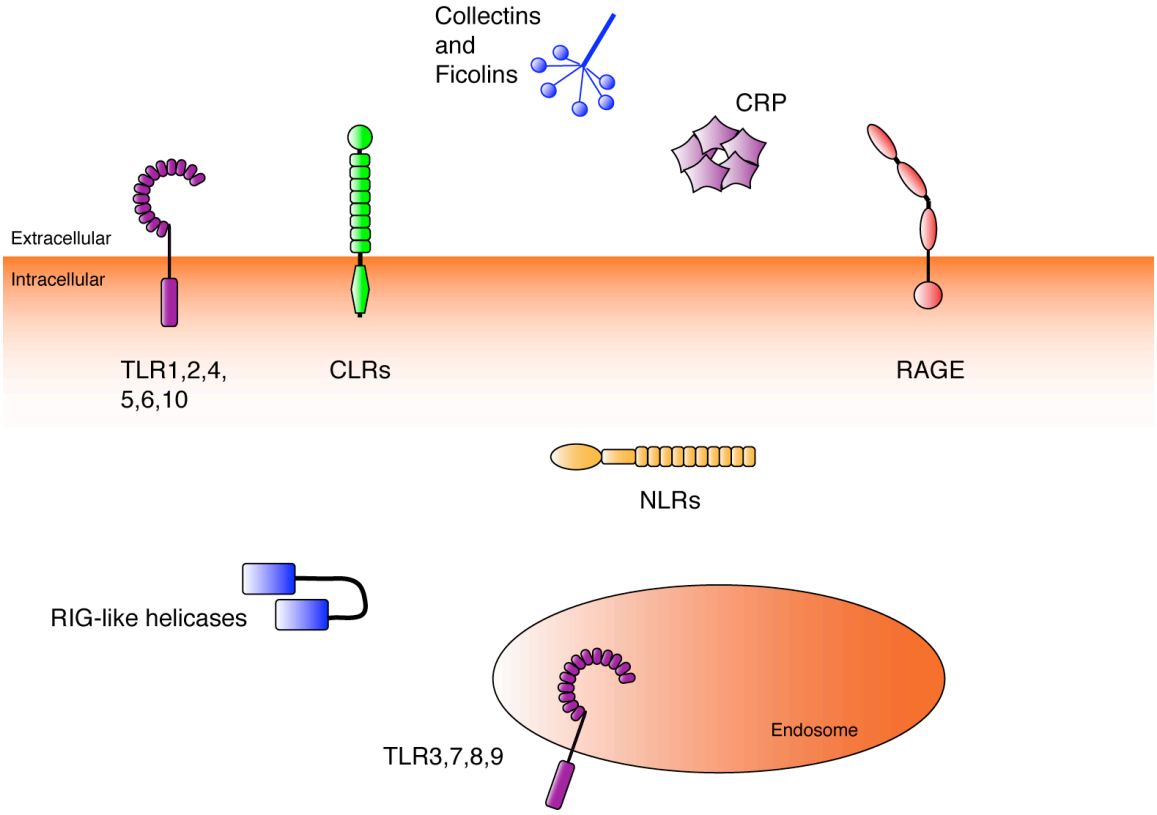

Figure 3: Pattern-recognition receptors of the innate immune system

1-4-2 The Family of the Toll-like receptors

A year after discovery of the role of drosophila Toll in the host defense against fungal infection (57), a mammalian homologue of the drosophila Toll was identified, referred to as the toll-like receptor 4 (58). So far, 13 members of the TLR family have been identified in mammals, ten in humans and twelve in mice. Mice do not express TLR10, but do express TLR11, TLR12 and TLR13 (59). TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11 are displayed on the cell surface while TLR3, TLR7, TLR8 and

TLR9 are localized intracellularly. TLR are distributed and differentially expressed in various cell types and tissues. They are present on polymorphonuclear cells, macrophages, mast cells, DC, NK cells, T cells and B cells. With respect to human DC, a different TLR expression on different subsets has been observed. DC from the myeloid lineage are reported to express TLR1, TLR2, TLR3, TLR4, TLR5, TLR6 and TLR8 whereas plasmacytoid DC express TLR1, TLR7, TLR9 and TLR10 (60, 61). Interestingly, TLR expression could also be detected on epithelial cells and EC. Moreover, mesenchymal and parenchymal cells of different organs and tissue like kidney, heart, lung, liver skin, brain and intestine express TLR, but their functional role and relevance is not yet fully understood (62).

The molecular weight of TLR ranges between 90-115 kDa. The extracellular region of Toll contains leucine-rich repeats (LRR) motifs whereas the cytoplasmic domain possess similarity with that of the mammalian Interleukin-1 receptor (IL-1R) family and is designated as Toll/IL-1R (TIR) homology domain, which has around 200 amino acids. Within this domain, the regions of homology comprise three conserved boxes, which are crucial for signaling.

After ligand binding, TLRs dimerize and undergo the conformational change required for recruitment of downstream signaling molecules. In general, these include the adaptor molecule myeloid differentiation primary-response protein 88 (MyD88), TIR-domain-containing adaptor protein (TIRAP; also known as MyD88-adaptor-like protein or Mal), IL-1R-associated kinases (IRAKs), transforming growth factor-β (TGF-β)-activated kinase (TAK1), TAK1-binding protein 1 (TAB1), TAB2 and tumor-necrosis factor (TNF)-receptor-associated factor 6 (TRAF6) (63, 64). The TLR family signaling pathway shown in Figure 4 is highly homologous to that of the IL-1R family and represents the core pathway of all TLR, except for TLR3.

Studies in 2001 have revealed the existence of a MyD88-independent pathway. It has been shown that stimulation of MyD88-deficient DC with LPS still induced their maturation (65). Therefore, exposure to LPS could induce TLR4-signaling via a MyD88-dependent as well as MyD88-independent pathway, which subsequently activates IRF3 (66). Up to now, TLR3-signaling is considered to be MyD88-independent (64).

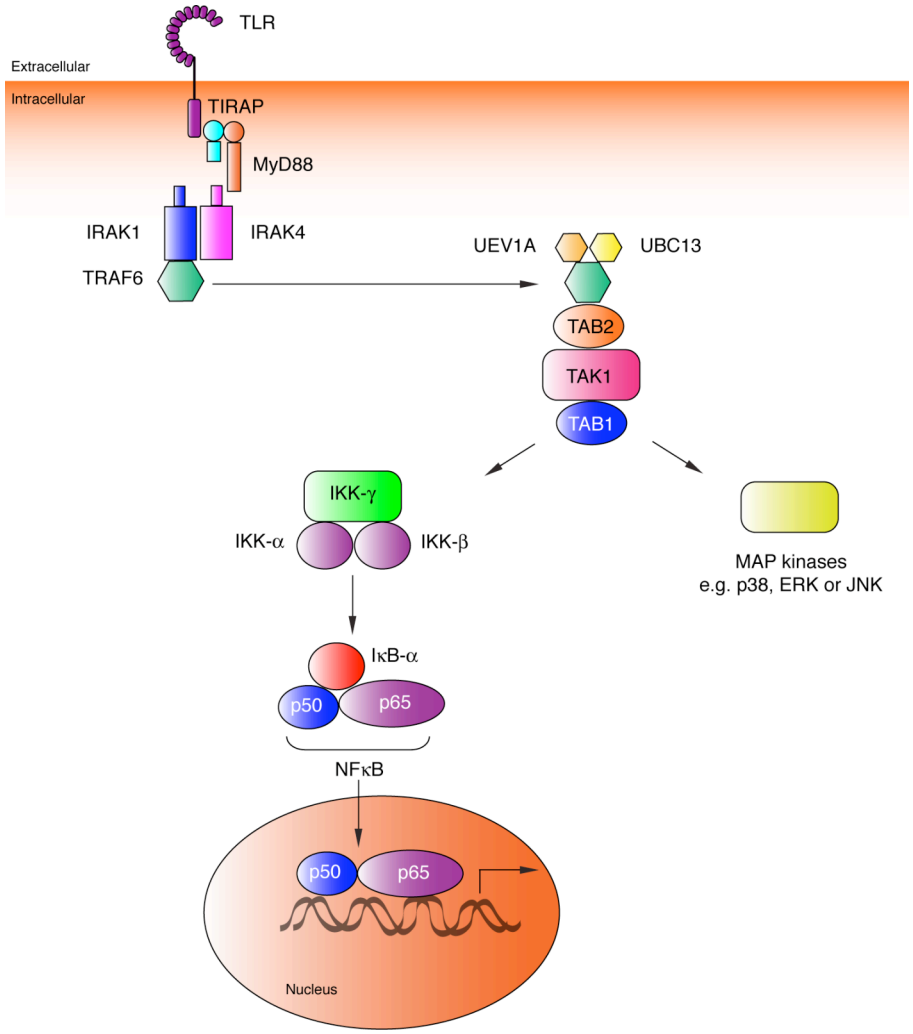

Figure 4: The TLR signaling cascade starts with recruitment of TIRAP and MyD88 to the cytoplasmic TIR domain of the TLR, where it facilitates the association of IRAK4 with the receptor complex through a homophilic death domain interaction. The binding of MyD88 to IRAK4 facilitates IRAK4 mediated phosphorylation of a crucial residue or residues in the kinase-activation loop of IRAK1. Activated IRAK1 then autophosphorylates residues in its N-Terminus, and this hyperphosphorylation of IRAK1 enables TRAF6 to bind to this complex. The complex IRAK1-TRAF6 complex then disengages from the receptor and interacts at the plasma membrane with another preformed complex consisting of TAK1, TAB1 and TAB2. This interaction induces phosphorylation of TAB2 and TAK1, which then translocates together with TRAF6 and TAB1 to the cytoplasm, where it associates with the ubiquitin ligases UBC13 (ubiquitin-conjugating enzyme 13) and UEV1A (ubiquitin-conjugating enzyme E2 variant 1). This leads to the ubiquitylation of TRAF6, which induces the activation of TAK1. Activation of TAK1 finally leads to phosphorylation the IKK complex (The IKK complex contains two catalytic subunits, IKKα and IKKβ and regulatory subunit IKKγ). This complex then phosphorylates IκB which leads to the degradation of IκB and consequently the release of the transcription factor NF-κB. Activation of TAK1 also results in the activation of mitogen-activated protein kinases (MAPK), such as p38, c-Jun NH_2-terminal kinase (JNK) and extracellular receptor kinase (ERK).

1-4-2-1 Danger Signals and Toll-like receptors in Organ Transplantation

In 1989, Charles Janeway proposed that the immune system has evolved to discriminate between self from infectious non-self (67). However, this hypothesis could not explain why we get a robust immune response in autoimmune diseases or against transplants. Five years later, in 1994, Polly Matzinger introduced the danger model (68). She postulated that the immune system has not evolved to recognize infection *per se* but to rather non-physiological cell death, damage or stress which could be a universal sign of danger. In 1999 she published a paper, in which she described that DC undergo maturation when they are co-cultured with necrotic cells (69). Subsequently, different endogenous danger molecules were identified as summarized in Table 4. Danger signals or DAMP and TLR need therefore also be discussed in the context of I/R injury and transplantation.

Beside the very important role of TLR during infections, there is growing evidence that these receptors are involved in various diseases such as autoimmune diseases and transplantation. Furthermore, TLR seem to be involved in regulation of antigen-processing and presentation (70). In transplantation, they were clearly shown to facilitate or even be responsible for graft rejection (71). Furthermore, agonists of TLR4, TLR8 and TLR9 were demonstrated to counteract the suppressive activity of regulatory T cells (Treg) (72, 73). In particular, it has been suggested that I/R injury leads to a release of danger signals, which trigger TLR. A recent study in mice provided evidence for the production of TLR4 agonists in liver I/R injury (74). TLR2 and TLR4-deficient transgenic mice showed less injury after reperfusion (75-77). Expression of epithelial- and leukocyte-associated TLR4 contributed similarly to injury (77). Kaczorowski *et al.* showed in a murine cardiac transplantation model that serum levels of TNF-α, IL-1β, IL-6, troponin I and MCP-1 were dramatically reduced in mice deficient in TLR4 signaling. Furthermore, these mice had reduced intragraft mRNA levels of TNF-α, IL-1β, IL-6, EGR-1, ICAM-1 and iNOS (78).

The research focus of our group lies especially on the EC of the graft. The endothelial surface layer, the glycocalyx, is composed of glycoproteins and glycolipids, among them heparan sulfate proteoglycans (HSPG) are very abundant. Major cell surface membrane HSPG can be subdivided into syndecans (four members) and glypicans (six members), as well as several minor membrane HSPG like epican or betaglycan (79). The HSPG layer of healthy EC is crucial for the

anticoagulant and anti-inflammatory properties of the endothelium, by enhancing the activity of TFPI and antithrombin III (80-82). HSPG are rapidly released from the EC surface under conditions of inflammation and tissue damage (83-85). Just recently this has also been shown in the plasma of vascular surgery patients (86), in which elevated levels of syndecan-1 and heparan sulfate (HS) was found just 15 minutes after reperfusion. HS release is supposed to be mediated by proteolytic cleavage of the protein core or by endoglycolytic cleavage of the HS chains (84, 87). Free HS has been shown to act as an endogenous danger signal by inducing maturation of macrophages and DC via TLR4 *in vitro* (88-91). Furthermore, injection of HS *in vivo* induced a systemic inflammatory response syndrome (SIRS) in mice (92).

TABLE 4. DAMAGE-ASSOCIATED MOLECULAR PATTERNS (DAMP)

MOLECULE	EFFECT	RECEPTOR
Heparan sulfate (HS)	In vitro: DC maturation, Macrophage activation In vivo: Induction of SIRS	TLR4
Hyaluronan (HA)	In vitro: DC maturation In vivo: Adjuvant activity	CD44, TLR2 and TLR4
Fibronogen	In vitro: DC maturation	Integrins and TLR4
Collagen-derived peptides	In vitro: DC maturation	CXCR2
Fibronectin	In vitro: DC maturation	Integrins
Elastin-derived peptides	In vitro: chemotaxis	Integrins
Laminin	In vitro: Neutrophil recruitment In vivo: chemotaxis	Integrins
Serum Amyloid A	In vitro: Macrophage activation	TLR2
HMGB1	In vitro: DC maturation In vivo: Adjuvant activity	RAGE, TLR2, TLR4
Uric acid (MSU)	In vitro: DC maturation In vivo: Adjuvant activity	TLR2, TLR4, CD14
Chromatin, nucleosomes and DNA	In vitro: DC maturation In vivo: DC maturation	TLR9 (with BCR or Fc receptor)
HSPs	In vitro: DC maturation In vivo: DC migration	CD14, CD91, TLR2, TLR4, CD40
Adenosine and ATP	In vitro: DC maturation In vivo: Exharbation of bronchial asthma	P1, P2X and P2Y; A1, A2A, A2B and A3
Galectins	In vitro: DC maturation	CD2
Thioredoxin	In vitro: chemotaxis In vivo: chemotaxis	n.d.
S100 proteins	In vivo: Neutrophil recruitment	RAGE
Cathelicindins	In vitro: DC maturation	FRPL1
Defensins	In vitro: DC maturation In vivo: Adjuvant activity	CCR6 and TLR4
N-formylated peptides	In vitro: DC chemotaxis	FRP and FPRL1

Recent publications also described a critical interplay between TLR and complement after co-activation *in vivo*. Hawlisch *et al.* demonstrated a regulatory role of C5a on TLR4 induced immune responses *in vitro* and *in vivo*. C5a has been shown to negatively regulate production of IL-12 family members such as IL-12, IL-23 and IL-27 in inflammatory macrophages (93). Mice deficient in the membrane complement inhibitor DAF have elevated levels of TNF-α, IL-1β and IL-6 in response to TLR agonists, whereas the levels of IL-12p40 are slightly decreased (94).

1-4-2-2 Interaction between HIF-1α, ET-1 and Toll-like receptors

Another interesting interaction of TLR, namely with the transcription factor hypoxia-inducible factor 1α (HIF-1α), has been demonstrated in recent years. HIF-1α has been described as a key regulator of a broad range of cellular and systemic responses to hypoxic conditions. Recent findings suggest that HIF-1α is also involved in myeloid cell-mediated inflammation (95). Furthermore, HIF-1α is a master regulator of the bactericidal capacity of phagocytes (96). LPS induces the expression of HIF-1α in murine macrophages (97) and DC (98). Additionally, HIF-1α knock-out mice develop less clinical signs of sepsis (99). These findings suggest that HIF-1α may also be an important mediator of inflammatory responses.

Interestingly, DC have angiogenic properties, i.e. they produce pro- as well as anti-angiogenic factors, e.g. VEGF and IL-12 respectively. Ehrenreich *et al.* demonstrated, that human macrophages produce endothelin-1 (ET-1) in response to the TLR agonist LPS (100). One study showed, that stimulation of human monocyte-derived DC (MoDC) with TNF-α or with gram-positive cocci *S. aureus* results in secretion of ET-1. Endothelins, including ET-1, ET-2 and ET-3 are widely distributed in tissues and mainly produced by endothelial and epithelial cells. ET-1 is a very potent vasoconstrictor (101) but has also been shown to exert pleiotropic effects in different biological processes of diseases such as cancer (102), I/R injury (103) or diabetes (104).

1-4-3 Dendritic Cells and their Role in the Immune System

DC constitute a heterogeneous population of bone-marrow derived APC. Different subsets of DC have been described in mice and humans. Subsets of DC in humans are conventional myeloid DC (mDC), plasmacytoid DC (pDC) and epidermal

Langerhans cells. The best-studied human DC are the MoDC, which belong to the myeloid lineage. B and T lymphocytes are mediators of the adaptive immune system, but they are mostly under the control of DC. T cells recognize a processed Ag bound to MHC class I or II molecules on the surface of DC via their T cell receptor (TCR). Recognition leads to the generation of cytotoxic $CD8^+$ T cells (CTL) or helper $CD4^+$ T cells. T helper cells can be further subdivided by their function and cytokine profile into Th1, Th2 and Th17. Furthermore, T lymphocytes can develop into regulatory T cells. DC can crucially influence the development of T cells. For efficient priming of T cells DC have to undergo maturation, which is characterized by upregulation costimulatory molecules, MHC molecules as well as adhesion molecules. These molecules build up the immunological synapse between T cells and DC. Activation of DC is mediated by danger signals, like PAMP or DAMP, or proinflammatory cytokines such as TNF-α or IL-1β as well as through ligation with CD40L (CD154) expressed on T cells.

Recent reports have highlighted an intensive cross-talk between DC and NK cells, EC, neutrophils and the complement system (see Figure 5). Reciprocal activation-pathways have been described to occur between NK cells and DC. Activation of NK cells by DC seems to be mediated via cell-contact interactions through e.g. NKp30 or IL-15Rα as well as cytokines like IL-12, IL-15 and IL-18 (105-107), whereas secretion of TNF-α and IL-12 by NK cells seems to contribute to DC maturation.

Interaction of DC with neutrophils, the most abundant leukocytes in the blood stream, has recently been investigated. Neutrophils are able to induce maturation of DC via interactions of CR3 and CEACAM-1 (CD66a) with DC-SIGN enhancing Th1 polarization. In addition, beside cell-contact mediated activation, secretion of TNF-α by activated neutrophils seems to enhance DC maturation (108, 109). Furthermore, it has been shown that neutrophils can transfer Ag to DC (110). Interestingly, another study has demonstrated, that the release of cell-surface microvesicles, so-called exosomes derived from activated neutrophils, inhibit DC maturation (111). DC are suggested to prolong neutrophil survival (110).

There is evidence in several cases of an intensive cross-talk between EC and DC. The endothelium serves as site for DC adhesion and migration into the inflamed

tissue. Contribution of EC to DC maturation has been already discussed in section 1-4-2-1.

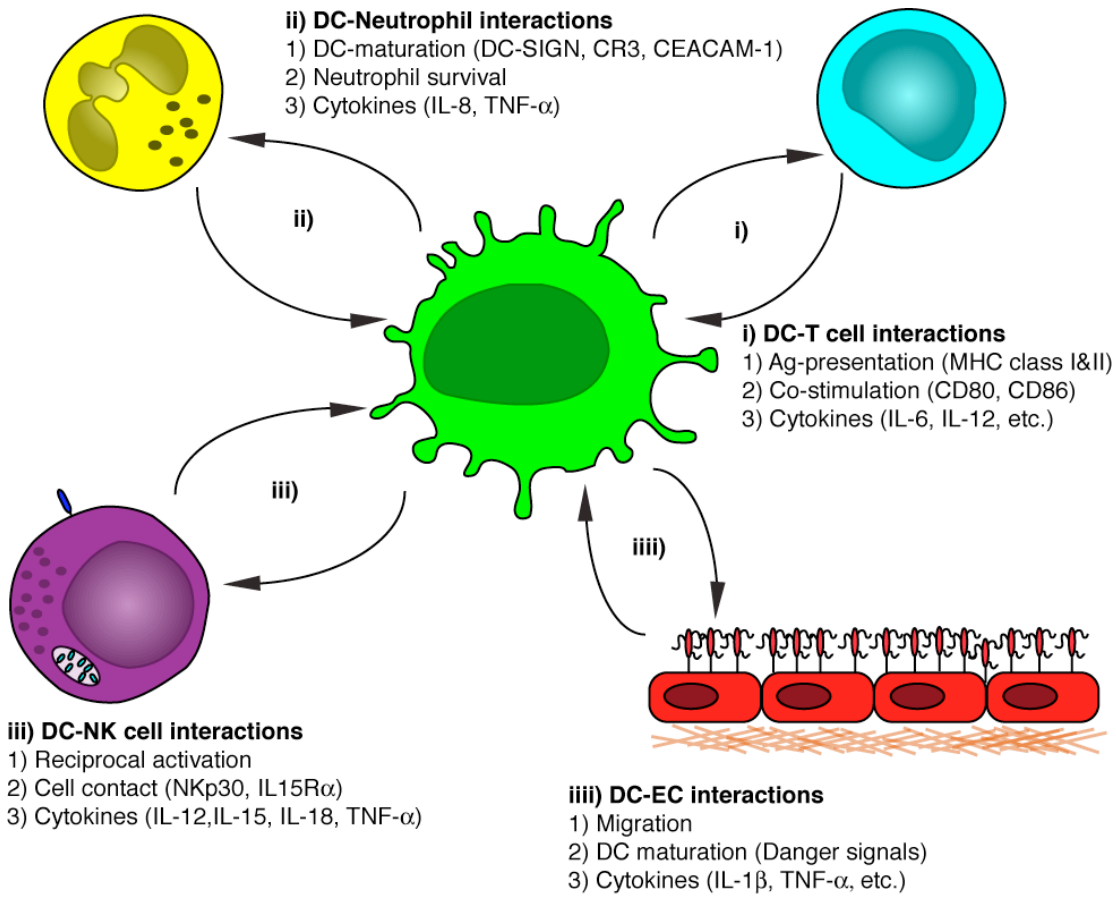

Figure 5: Interactions of DC with different cells of the immune system.

Similar to macrophages, immature DC have been described as a rich source of the complement proteins C1q (112, 113), C3 (114, 115), C4BP, C7, C8, Factor I and Factor B (115, 116) in mice and humans. Furthermore, they express DAF, MCP and CD59 (see Figure 6). Concerning pDC, there is no information available on the capacity of this subtype of DC to produce complement components or respond functionally to these molecules (117). C1q produced by immature DC is functionally active in complement activation and binding to apoptotic cells and is necessary for clearance of the latter (112, 118, 119). The production of C1q is down-regulated upon DC maturation *in vitro* and *in vivo* (112). Recently, the effect of C1q on differentiation of monocytes into immature DC was examined. DC differentiated in the presence of C1q secrete less TNF-α, IL-6 and IL-10 in response to LPS. In addition, upregulation of CD80, CD83 and CD86 was impaired along with a reduced capacity to stimulate alloreactive T cells (120). In contrast, immobilized C1q has been

demonstrated to induce maturation of DC including upregulation of costimulatory molecules, cytokine production and T cell stimulation (121). DC also express several complement receptors such as the CR3 (CD11b/CD18) and CR4 (CD11c/CD18) as well as the seven-transmembrane G-protein coupled receptors C3aR and C5aR. Binding of iC3b to CR3 on APC has been reported to be crucial for systemic tolerance induction to an antigen injected into the anterior chamber of the eye, as shown in CVF-treated rats and C3 deficient mice. In addition, Sohn *et al.* showed that binding of iC3b to CR3 on antigen-pulsed peritoneal exudate cells leads to an increase of IL-10 and TGF-β secretion *in vitro* (122). CR3 and CR4 are supposed to be apoptotic-cell receptors, and signaling through these receptors has been shown to render human MoDC tolerogenic (123). Complement activation leads to the release of the anaphylatoxins C3a and C5a. As DC express C3aR (124) as well as C5aR (125, 126) on their surface, generation of C3a and subsequent signaling through C3aR has an important impact on DC-T cell interactions (127).

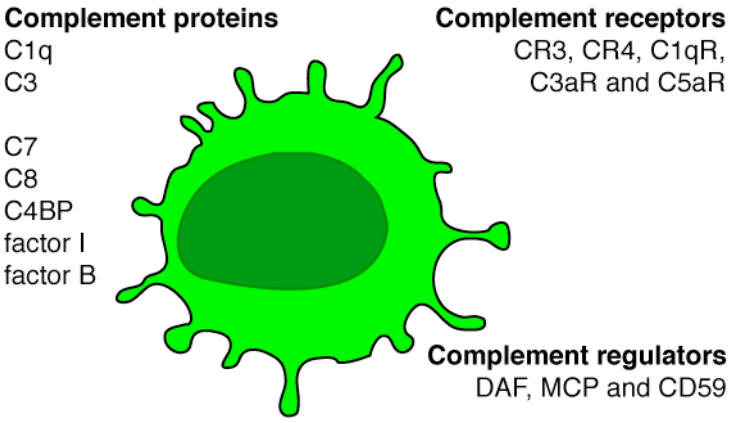

Figure 6: DC interact with complement proteins, but are also able to produce complement

1-4-3-1 Dendritic Cells in Organ Transplantation

DC are undoubtedly involved in organ transplantation (128). Their roles in organ transplantation are various. On one hand, DC in both the graft donor and the recipient can act as "foe", stimulating rejection. On the other hand, they have been described to act as "friend", promoting graft acceptance (129). In organ transplantation, T cell stimulation by donor DC, presenting an Ag by non-self MHC class molecules is called "the direct pathway". Priming of T cells by recipient DC, presenting peptides of donor origin, mostly processed donor MHC class molecules, is called "the indirect pathway" of allorecognition (130). Another, so called "semi-indirect pathway" has

been described recently, where recipient DC acquire donor MHC class molecules through cell-to-cell contact and induce T cell activation (131). Historically, the direct pathway has been linked to acute rejection. The indirect pathway predominates at a later stage and is the main mechanism of allorecognition in late graft rejection (130). In a recent *in vivo* study the distribution of DC during rejection as well as tolerization has been investigated. The data indicated, that direct and indirect allorecognition occur shortly after cardiac transplantation. Donor as well as recipient DC were detected in the spleen and lymph nodes during the first week. But only indirect presentation was persistent in the lymph nodes, not in the spleen, of tolerized recipients (132). Another study in mice demonstrated that recipient pDC are able to induce peripheral $CD4^+CD25^+FoxP3^+$ Treg in the lymph nodes *in vivo*, whereas they migrate into the spleen during rejection (133). Other subsets of tolerogenic DC than pDC have been considered to have similar functions in other settings (134).

1-5 Therapeutic Strategies in Organ Transplantation

1-5-1 Complement Inhibition in Organ transplantation

Therapeutic options for complement inhibition in transplantation are possible both on the side of the donor organ and the recipient, and inhibition of complement in the fluid phase and on cell surfaces can be distinguished. Systemic complement depletion can be achieved by the use of cobra venom Factor (CVF). Indeed, CVF therapy was successfully used in pig-to-primate transplantation in combination with immunosuppressive drugs (135, 136), but its effect on I/R injury is less clear: while a reduction of myocardial infarct size was already described 30 years ago (137), reports on its effect on cerebral I/R injury are contradictory (138, 139). Treatment of the recipient with sCR1 has been shown to prevent I/R injury and early graft failure in isogeneic (140) as well as allogeneic lung transplantation (141, 142). Similar results were achieved with the use of an C1-INH which was described to reduce early graft damage in a lung transplantation model in the dog (143). The beneficial effect of C1-INH was also seen in patients who suffered from acute graft injury after lung transplantation (17). C1-INH also binds to graft EC, while still maintaining its function as complement inhibitor (144), and could therefore potentially be used as an additive to organ preservation solutions in order to protect the graft from I/R injury. Attempts in the same direction, namely the development of a membrane-targeted

complement inhibitor, were made by Richard Smith and colleagues (145). This construct, termed 'Mirococept', consisting of a part of CR1 linked to a lipophilic tail to anchor it in cell membranes, was successfully used later on in rat kidney transplantation experiments and in perfusion of human kidneys (146).

Another approach to confer protection from complement-mediated damage to the graft is genetic manipulation of the donor organ. Donor pigs expressing human DAF (CD55), CD59, and/or MCP (CD46) have been produced for xenotransplantation (147). Research in this direction is being continued, but so far it is technically not feasible to express such genes on a human allograft prior to transplantation. On the other hand, novel fluid-phase complement inhibitors such as Pexelizumab, an anti-C5 antibody, are being tested in clinical trials in the field of cardiology (148), and start to enter the transplantation arena as shown by recent reports in mice, in which anti-C5 antibody treatment led to a complete block of terminal complement activity and prevented antibody-mediated rejection (149, 150).

1-5-2 Coagulation Inhibition in Organ Transplantation

Inhibition of the coagulation system has shown to have a beneficial effect in I/R injury. Treatment with anti-TF Ab or TF antisense oligonucleotides have been demonstrated to reduce inflammation and leukocyte recruitment (42, 45). In a similar manner, antithrombin III limited the leukocyte recruitment (151). In addition, treatment with heparin has shown to reduce skeletal muscle I/R injury (152). A study performed in our group has shown that DXS reduces expression of TF in a porcine model of myocardial I/R injury (44).

1-5-3 Modulation of Dendritic cells in Organ Transplantation

Transplantation tolerance is achieved through the control of T cells. Two main processes can be distinguished: central and peripheral tolerance. Central tolerance mechanisms take place in the thymus, whereas peripheral tolerance is generated at locations of antigen recognition and processing in secondary lymphoid tissue. Central tolerance involves elimination of self-reactive T lymphocytes and positive selection of those that are not. The key mechanisms in establishing peripheral tolerance are interactions of APC, particularly DC, with T cells are deletion, anergy, ignorance and/or regulation. During the past decade, numerous studies have unveiled mechanisms by which DC induce or maintain tolerance *in vivo*. Some of these studies

have investigated the generation of DC *in vitro*, which induce after transfer, donor-specific tolerance. These DC were then generally named tolerogenic DC. Tolerogenic DC are immature, maturation-resistant or alternatively-activated DC. They express low levels of costimulatory molecules, and have an impaired ability to synthesize Th1 cell driving cytokines. Furthermore, they are able to induce or expand Treg. In addition, tolerogenicity is not specific to the described DC subset or restricted to immature state (153, 154).

In general, different approaches are considered in transplantation procedures to directly manipulate DC. One method is to generate *in vitro* tolerogenic donor or recipient DC, which are then administered to the recipient to downregulate T cell responses. Another method is based on the *in situ* delivery of alloantigen to quiescent DC in the lymphoid organ of the recipient. Furthermore, combined treatment with immunoregulatory reagents have been shown to induce the generation of tolerogenic DC and Tregs *in vivo* (155).

TABLE 5. FEATURES OF TOLEROGENIC DENDRITIC CELLS

I) Expression of low levels of surface MHC molecules and costimulatory molecules (e.g. CD80 and CD86)
II) Low production of IL-12 and high IL-10, TGF-β and IDO production
III) Low production of proinflammatory cytokines (e.g. TNF-α, IL-6)
IV) Resistance to maturation in response to danger signals
V) The ability to generate or expand alloantigen-specific naturally occurring regulatory T cells
VI) The ability to promote apoptosis of effector T cells
VII) The ability to migrate to T cell areas in secondary lymphoid tissue (e.g. expression of CCR7)
VIII) In vivo longevity and resistance to NK cell or T cell medated killing
IX) The ability to respond to regulatory T cells by upregulating expression of e.g. IL-10 or IDO

There are various agents described in the literature, which modulate and generate tolerogenic DC such as anti-inflammatory cytokines like IL-10 and TGF-β, or inducers of cyclic AMP like prostaglandin E_2 (PGE_2) or 1α,25-dihydroxyvitamin D_3. Furthermore, immunosuppressive drugs as e.g. cyclosporine, corticosteroids, tacrolimus, rapamycin, mycophenolate mofetil (MMF) and sanglifehrin A have shown to modulate DC function. Inhibitors of NF-κB like deoxyspergualin (DSG) or its analogue LF15-0915 have shown to inhibit DC maturation (154, 156). Induction of

indoleamine 2,3-dioxygenase (IDO) expression by DC has been attributed to tolerogenic properties. IDO catalyses the depletion of the essential amino acid tryptophan and enhances the production of immunoregulatory kynurenine metabolites that inhibit T-cell proliferation and promote T-cell apoptosis (157).

1-5-4 Candidates for Dendritic cell modulation

1-5-4-1 Low Molecular Weight Dextran Sulfate (DXS)

Low molecular weight dextran sulfate, a highly sulfated poly-glucose, was shown by Wuillemin *et al*. to act as a soluble complement inhibitor by enhancing the activity of C1-INH (158). DXS also binds to exposed thioesters of activated C3 and C4 and thus inhibits their deposition (158). The exact mechanism leading to potentiation of C1-INH by DXS is unknown (159). However, sulfate groups seem to play a role since dextrin, an alpha 1-4 linked polyglucose similar to dextran, which is alpha 1-6 linked, has no anti-complement effect but dextrin bearing carboxylic and benzyl amide sulfonate groups inhibits complement activation *in vivo* and *in vitro* in experimental models (159, 160). Furthermore, DXS binds the complement regulatory factor H (161). DXS interacts with the factor H polyanion recognition site and by this enhances the binding of factor H to the C3b-target complex. The affinity of factor H to C3b is regulated at or near the 13th short consensus repeat (SCR) domain of factor H, which is a positively charged site. The polyanion DXS therefore inhibits all three complement pathways as shown by hemolytic tests (CH50, AP50 and LP50) (162). In addition, it inhibits the coagulation system by enhancing the anti-coagulatory activity of antithrombin III and C1-INH against activated Factor XI (163). Moreover, DXS interferes with platelet adhesion (164).

Work in our laboratory has shown that DXS acts as an EC protectant by protecting the EC from complement-mediated damage in allo- as well as xenotransplantation models and in myocardial I/R injury. This cytoprotective effect correlates with dose-dependent binding of DXS to porcine and human EC. Binding of DXS is considerably increased when EC are activated by complement or treated with heparinase. Binding of DXS to activated endothelium was also reported in a rat model of thrombotic microangiopathy (TMA) in the kidney. In this model, biotin-DXS staining was detected only on the surface of the glomerular endothelium with TMA but not in the kidney without TMA (165).

In addition, DXS, bound to activated EC, protects them from NK cell- as well as complement-mediated cytotoxicity (162). Added in solution to whole human blood, hyperacute xenograft rejection is attenuated in *ex-vivo* pig lung perfusion with whole human blood (166). In a pig model of acute myocardial I/R injury, DXS significantly reduced infarct size (44). DXS administrated intravenously *in vivo* in a heterotopic hamster-to-rat xenotransplantation model, in combination with cyclosporin, led to long-term xenograft survival (167). And, as detailed below, we recently showed that DXS facilitates anti-CD4 mAb induced long-term allograft survival in rats despite of prolonged cold graft ischemia (168). These data suggest that DXS bears a unique capacity to bind to activated EC, thus conferring local, site-specific protection from complement-mediated damage.

1-5-4-2 Reconstituted High Density Lipoprotein (rHDL)

A beneficial effect of treatment with reconstituted High Density Lipoprotein (rHDL), containing apolipoprotein A-I and phosphatidylcholine (PS), has been described in multiple conditions of diseases like arteriosclerosis, myocardial infarction, stroke and endotoxemia. rHDL has been described to inhibit up-regulation of inflammatory adhesion molecules like ICAM-1 (CD54), VCAM-1 (CD106) and E-selectin (CD62E) on endothelial cells (EC) (169) as well as reduced thrombin induced tissue-factor (TF) expression (170). Furthermore, a recently performed study in humans has shown that rHDL reduces plasma levels of TNF-α and expression of CD11b on monocytes (171). Protection against cardiac I/R injury has been demonstrated by a reduced cardiac content of TNF-α and enhanced secretion of prostaglandin (172). The effect of rHDL in gram-negative sepsis has been demonstrated via binding and neutralizing lipopolysaccharide (LPS) and reduction of CD14 expression on monocytes (173). Taken together rHDL can be described as a substance, which attenuates the pro-inflammatory effects of many mediators of innate immunity

2 Objectives of this Thesis

Activation of the innate immune system in organ transplantation due to I/R injury as well as brain death of the donor is considered as one of the main problems in translating tolerance-inducing protocols into the clinics. Modulation and inhibition of dendritic cell (DC) maturation has been shown to improve graft survival. This work was mainly supported and incorporated in the EU Project "RISET" (*R*eprogramming the *I*mmune *S*ystem for the *E*stablishment of *T*olerance; www.risetfp6.com). The specific aims of this thesis were the following:

1) To investigate the effect of the complement inhibitor and endothelial protectant low molecular weight dextran sulfate (DXS) on maturation of human DC.

2) To study the effect of DXS on the activation of NK cells.

3) To evaluate the influence of reconstituted high density lipoprotein (rHDL) on maturation of DC in order to examine the potency of this substance to impede the link between innate and adaptive immunity.

4) To analyze the interplay of toll-like receptor (TLR) stimulation in human DC and the transcription factor hypoxia-inducible factor 1α (HIF-1α) during the maturation process.

5) To evaluate if stimulation of human DC with different TLR agonists induces the production of the vasoactive peptide endothelin-1 (ET-1).

3. Publications

3-1 Paper I: Influence of DXS on maturation of human DC

Title: The complement inhibitor low molecular weight dextran sulfate inhibits TLR4-induced phenotypic and functional maturation of human DC

Authors: Rolf Spirig, Cees van Kooten, Carolina Obregon, Laurent Nicod, Mohamed Daha and Robert Rieben

published in *The Journal of Immunology*, 2008

Dendritic cells (DC) play an extremely important role in graft rejection as well as in tolerance induction. Low molecular weight dextran sulfate (DXS) has been shown to act as complement inhibitor and endothelial cell protectant in xeno- as well as allotransplantation. We therefore hypothesized that DXS could also influence maturation of DC.

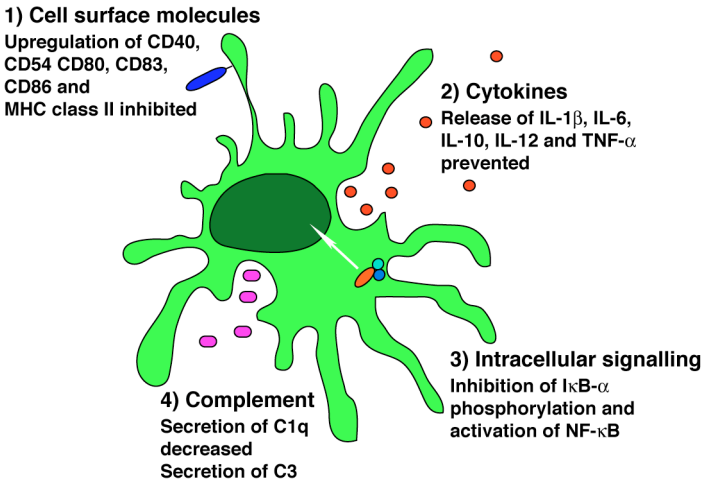

Figure 7: Influence of DXS on TLR induced maturation of human DC. Spirig *et al.*, 2008, Molecular Immunology

In this study we could show, that DXS is able to prevent phenotypic and functional maturation of human DC *in vitro*. Upregulation of costimulatory as well as antigen-presenting molecules is significantly inhibited by DXS in response to TLR4 triggered activation of the cells. The pro-inflammatory cytokine pattern of stimulated DC is significantly reduced. These data suggest that DXS might not only act as a local endothelial protectant by inhibiting complement mediated damage but also prevents activation of DC.

The Journal of Immunology

The Complement Inhibitor Low Molecular Weight Dextran Sulfate Prevents TLR4-Induced Phenotypic and Functional Maturation of Human Dendritic Cells[1]

Rolf Spirig,* Cees van Kooten,‡ Carolina Obregon,† Laurent Nicod,† Mohamed Daha,‡ and Robert Rieben[2]*

Low molecular weight dextran sulfate (DXS) has been reported to inhibit the classical, alternative pathway as well as the mannan-binding lectin pathway of the complement system. Furthermore, it acts as an endothelial cell protectant inhibiting complement-mediated endothelial cell damage. Endothelial cells are covered with a layer of heparan sulfate (HS), which is rapidly released under conditions of inflammation and tissue injury. Soluble HS induces maturation of dendritic cells (DC) via TLR4. In this study, we show the inhibitory effect of DXS on human DC maturation. DXS significantly prevents phenotypic maturation of monocyte-derived DC and peripheral myeloid DC by inhibiting the up-regulation of CD40, CD80, CD83, CD86, ICAM-1, and HLA-DR and down-regulates DC-SIGN in response to HS or exogenous TLR ligands. DXS also inhibits the functional maturation of DC as demonstrated by reduced T cell proliferation, and strongly impairs secretion of the proinflammatory mediators IL-1β, IL-6, IL-12p70, and TNF-α. Exposure to DXS leads to a reduced production of the complement component C1q and a decreased phagocytic activity, whereas C3 secretion is increased. Moreover, DXS was found to inhibit phosphorylation of IκB-α and activation of NF-κB. These findings suggest that DXS prevents TLR-induced maturation of human DC and may therefore be a useful reagent to impede the link between innate and adaptive immunity. ***The Journal of Immunology,*** **2008, 181: 878–890.**

Low molecular weight dextran sulfate (DXS),[3] a sulfated polysaccharide of m.w. 5000, is known to inhibit alternative, classical, and lectin complement pathways (1) as well as the coagulation cascade (2). In addition, DXS has been shown to act as an endothelial cell protectant (3, 4). It prevents human complement- and NK cell-mediated cytotoxicity in vitro (3) and, in combination with cyclosporin A, induces long-term graft survival in a hamster-to-rat cardiac xenotransplantation model in vivo (5). Moreover, DXS is very effective in protecting vasculature and tissue from ischemia/reperfusion injury, as recently shown in a porcine model of acute myocardial infarction (6). Taken together DXS can be described as a substance that attenuates the proinflammatory effects of many mediators of innate immunity. We hypothesized, therefore, that DXS might also influence the function of APCs, which are crucially involved in graft rejection and tolerance induction.

Dendritic cells (DC) are the most potent APC and play an important role in bridging innate and adaptive immunity (7). APC are pivotal for the initiation of T cell-mediated immune responses, as seen for example in allograft rejection as well as in tolerance induction (8, 9). The properties of DC essentially depend on maturation and migration of the cells. DC residing in the peripheral tissue are normally in a phenotypically and functionally immature state. Immature DC do not induce primary immune responses because they do not express the costimulatory molecules CD80, CD86, and CD40, nor do they express antigenic peptides as stable complexes with MHC molecules (7). The balance between the induction of immune reactivity and immune modulation, including tolerance induction, seems to be determined by the subtype of the DC, their state of maturation, and the secretion of soluble mediators including cytokines. Furthermore, similar to macrophages, immature DC have been described as a rich source of the complement proteins C1q (10, 11) and C3 (12). C1q produced by immature DC is functionally active in complement activation and binding to apoptotic cells (13). The production of C1q is down-regulated upon DC maturation in vitro and in vivo (10). C3 synthesis by DC is reported to be essential for T cell activation (12). Interestingly, the binding of the C3 activation product iC3b to the complement receptor (CR3, CD11b/CD18), or α_M on APC has been reported to be crucial for tolerance (14). DC also express TLR, which are pattern recognition receptors that distinguish conditions of well-being from conditions of disease (15). Activating signals such as pathogenic compounds derived from microorganisms, e.g., LPS, as well as endogenous molecules like heparan sulfates (HS) induce maturation of DC via TLR (16).

Healthy endothelial cell are covered by a layer of HS proteoglycans, which is crucial for the anticoagulant and anti-inflammatory properties of the endothelium. HS proteoglycans are rapidly released under conditions of inflammation and tissue damage (17–19). The release of HS is mediated by proteolytic cleavage of the protein core or by endoglycolytic cleavage of the HS chains

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[3] Abbreviations used in this paper: DXS, dextran sulfate; DC, dendritic cell; MoDC, monocyte-derived DC; HS, heparan sulfate; LTA, lipoteichoic acid.

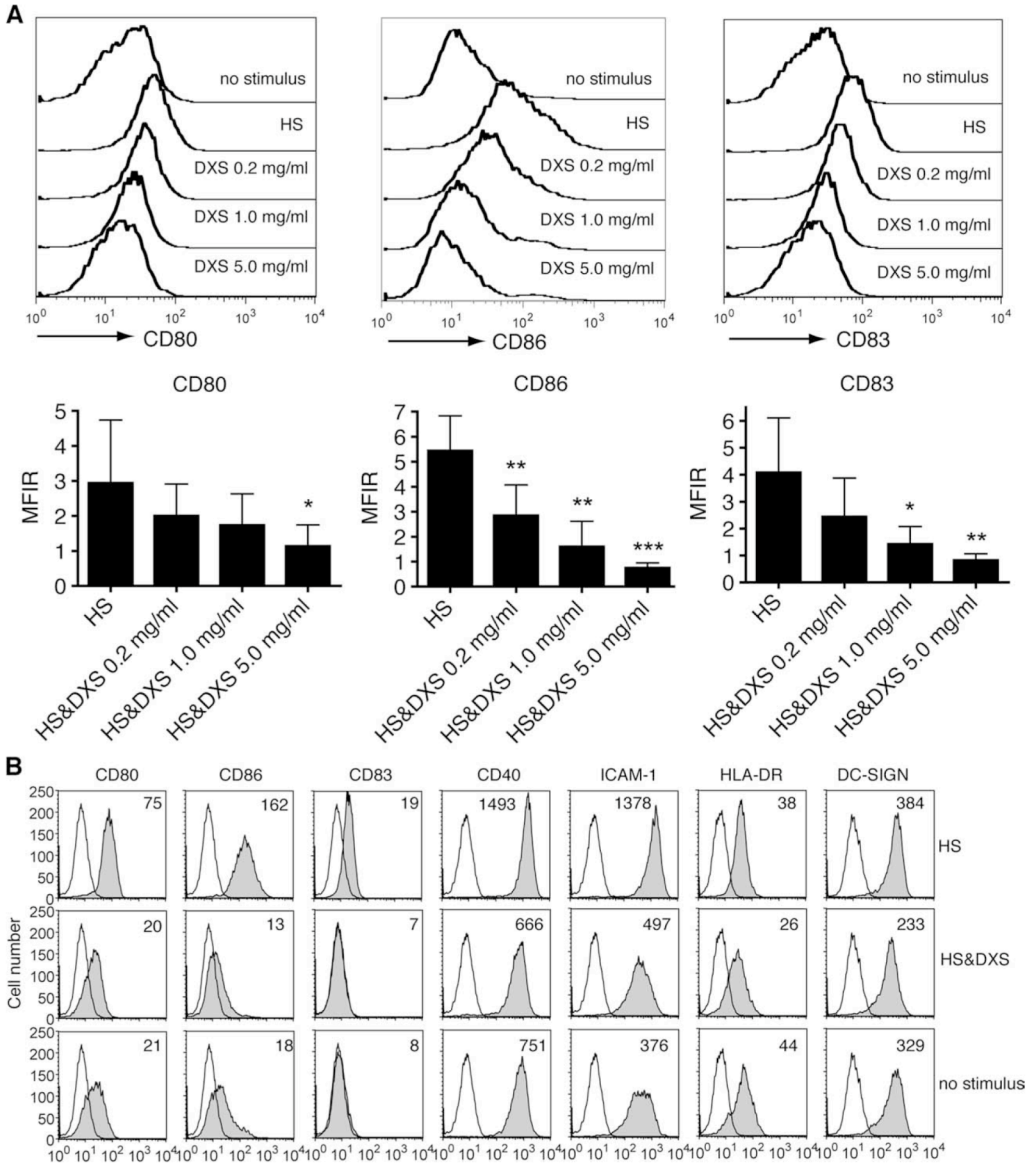

FIGURE 1. DXS prevents phenotypic maturation of human MoDC in response to HS in a dose dependent manner. *A*, Monocytes isolated from buffy coat were cultured in presence of GM-CSF and IL-4 for 6 days. DXS dose-dependently prevents HS-induced (10 μg/ml) DC maturation. Histograms show the typical expression profiles of CD80, CD86, and CD83. Data are representative of five (CD80, CD83) or eight (CD86) independent experiments with cells of different donors. To compare the levels of inhibition of up-regulation of the indicated surface molecules, the median fluorescence intensity ratios (MFIR) were calculated by dividing the median fluorescence of HS- and/or DXS-treated MoDC by the median fluorescence of immature MoDC. Mean ± SD data are shown. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ vs mature MoDC by unpaired Student's *t* test. *B*, Histograms showing the effect of 5 mg/ml DXS on HS-induced expression of the indicated cell surface molecules. DXS was added to MoDC 30 min before maturation was induced by HS (10 μg/ml) for 24 h. Afterward, cells were harvested, stained, and analyzed by FACS. The typical expression profiles of the indicated surface molecules (gray filled histogram) are shown. The isotype control or second Ab alone are indicated (open histogram). Values shown indicate the median fluorescence intensity of the expression profiles (gray filled histogram). The data shown are from one donor and representative of four to eight independent experiments.

(18, 20). It has been shown that soluble HS induces maturation of DC via TLR4 (15, 21). Mature DC are characterized by strongly reduced phagocytic activity, up-regulated Ag-presenting and T cell costimulatory molecules, as well as expression of other DC activation markers like CD83. Moreover, mature DC secrete predominantly proinflammatory cytokines such as IL-1β, IL-6, IL-12p70, and TNF-α. The

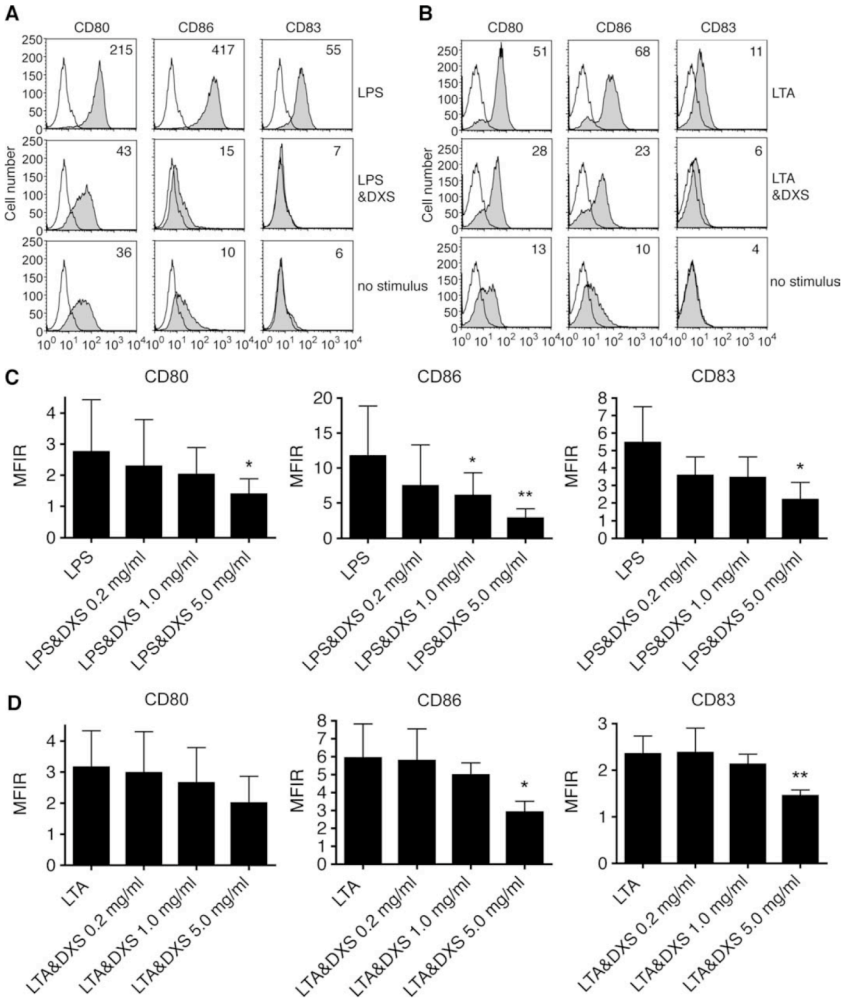

FIGURE 2. DXS prevents the up-regulation of CD80, CD83 and CD86 in response to the endogenous TLR agonists LPS and LTA in a dose dependent manner. *A*, LPS-induced up-regulation of CD80, CD86 and CD83 is inhibited by DXS in a dose-dependent manner. DXS was added to MoDC 30 min before maturation was induced by LPS (100 ng/ml) for 24 h. The typical expression profiles of the indicated surface molecule are shown (gray filled histogram). Values indicate the median fluorescence intensity of the expression profiles. Results are from one donor and representative of six (CD80, CD83) or eight (CD86) independent experiments. *B*, LTA-induced up-regulation of CD80, CD86, and CD83 is inhibited by DXS in a dose-dependent manner. DXS was added to MoDC 30 min before maturation was induced by LTA (5 μg/ml) for 24 h. The typical expression profiles of the indicated surface molecule are shown (gray filled histogram). Values indicate the median fluorescence intensity of the expression profiles. Results are from one donor and representative of four independent experiments. *C*, To compare the levels of inhibition of up-regulation of the indicated surface molecules median fluorescence intensity ratios (MFIR) were calculated by dividing the median fluorescence of LPS- or DXS-treated MoDC by the median fluorescence of immature MoDC. *, $p < 0.05$; **, $p < 0.01$ vs mature MoDC by unpaired Student's *t* test. *D*, Data show MFIR of LTA- or DXS-treated MoDC. *, $p < 0.05$; **, $p < 0.01$ vs mature MoDC by unpaired Student's *t* test.

transcription factor NF-κB is crucial for the expression of surface marker molecules and cytokine production during DC maturation.

In this study, we show that DXS interferes with activation of human DC at multiple levels by reducing immunostimulatory properties, secretion of proinflammatory cytokines, phagocytic activity, and production of the complement proteins C1q and C3. DXS prevents TLR signaling in response to endogenous and exogenous agonists by inhibiting phosphorylation of IκB-α and activation of NF-κB.

Materials and Methods

Generation and stimulation of human monocyte-derived DC (MoDC)

Human PBMC were isolated from buffy coats obtained from healthy blood donors (Regional Red Cross Blood Donation Center, Bern, Switzerland) by density gradient centrifugation over Ficoll-Paque (Amersham, Biosciences). Monocytes were isolated from PBMC as described recently (22, 23) by spontaneous aggregation and rosetting (24). In brief, purified PBMC were suspended in RPMI 1640 medium (Invitrogen) containing 2 μg/ml

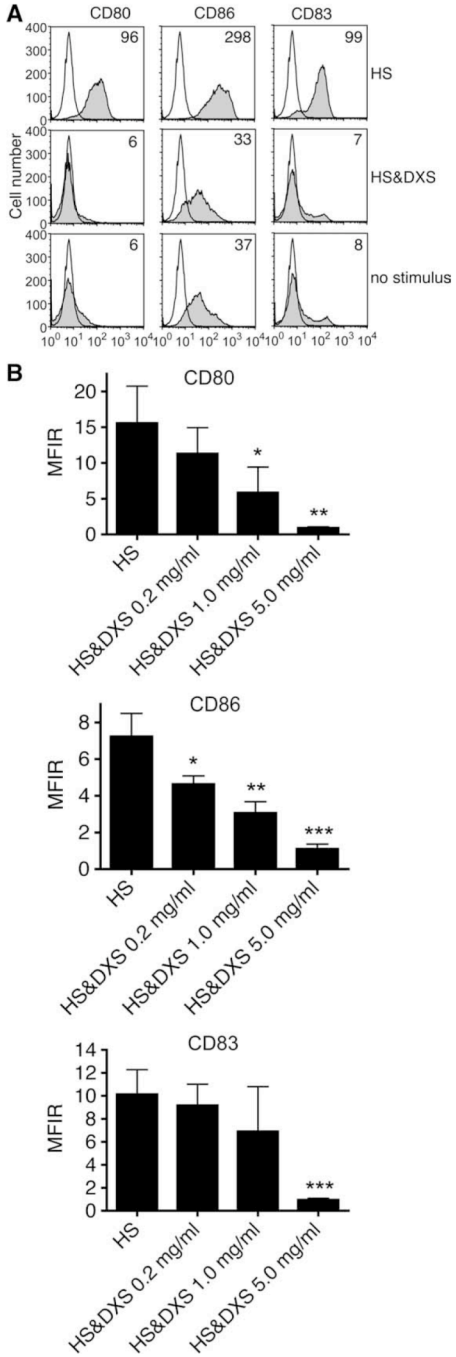

FIGURE 3. Activation of peripheral myeloid DC is prevented by DXS. *A*, Peripheral myeloid DC were isolated using a blood DC Ag-1 DC isolation kit. DXS was added to the DC 30 min before maturation was induced by HS (10 μg/ml) for 24 h. The typical expression profiles of the surface molecules CD80, CD86, and CD83 are shown (gray filled histogram). Values indicate the median fluorescence intensity of the expression profiles. Results are from one donor and representative of three independent experiments. *B*, Data show MFIR of HS- or DXS-treated peripheral myeloid DC. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ vs mature DC by unpaired Student's *t* test.

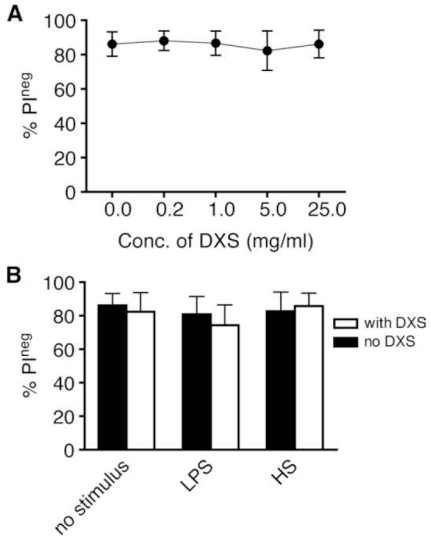

FIGURE 4. Assessing the influence of the concentration of DXS used on the viability of MoDC. *A*, MoDC were treated with indicated concentrations of DXS. Thereafter, cells were harvested and stained with propidium iodide (5 μg/ml) and analyzed by flow cytometry. Data are mean ± SD of three independent experiments with cells of different donors. *B*, MoDC were treated with LPS (100 ng/ml) or HS alone (10 μg/ml), or together with DXS (5 mg/ml). Data represent mean ± SD of three independent experiments with cells of different donors.

polymyxin B sulfate (Sigma-Aldrich). Cells were incubated for 40 min at 4°C under rotation to allow aggregation followed by 10 min of incubation on ice. Monocyte-enriched pellets were further separated from nonaggregated PBMC by a gradient of FCS (Amimed/BioConcept) and another 10 min of incubation on ice. The monocyte-enriched fractions were incubated overnight with SRBC (bioMérieux) to deplete contaminant lymphocytes by rosetting. Monocyte fractions characterized by high expression of CD14 and low expression of CD83 and CD86 were then isolated by Ficoll-Paque density gradient centrifugation. The purified mononuclear cells, mainly monocytes, were incubated for 6 days in RPMI 1640 medium (Invitrogen) containing 10% FCS (Amimed/BioConcept), 1% L-glutamine (2 mM; Invitrogen), 1% penicillin/streptomycin (100 U/ml; Invitrogen), 10 ng/ml GM-CSF (R&D Systems), and 10 ng/ml IL-4 (R&D Systems) to generate MoDC as described initially by Sallusto and Lanzavecchia (25). The cells were kept at 37°C in a 5% CO_2 humidified atmosphere. On day 3, the culture medium was replaced with fresh medium. For induction of maturation, 10 μg/ml HS (Seikagaku), 100 ng/ml LPS (Sigma-Aldrich), 5 μg/ml lipoteichoic acid (LTA; Sigma-Aldrich), 20 ng/ml TNF-α (R&D Systems), or 20 ng/ml IL-1β (R&D Systems) were added to the cultures for 24 or 48 h. Low molecular weight DXS (Sigma-Aldrich), used at the indicated concentrations, was given 30 min before HS, LPS, LTA, IL-1β, or TNF-α was added to the cells. LPS removal affinity resin (END-X B15) was purchased from Associates of Cape Cod to remove potential LPS contamination in HS.

Isolation of myeloid DC from peripheral human blood

Peripheral blood myeloid DC were isolated using a blood DC Ag-1 DC isolation kit (Miltenyi Biotec) according the manufacturer's protocol and cultured for 24 h in RPMI 1640 medium with 10% FCS, 1% L-glutamine and 1% penicillin/streptomycin. Stimulation procedures were the same as for MoDC.

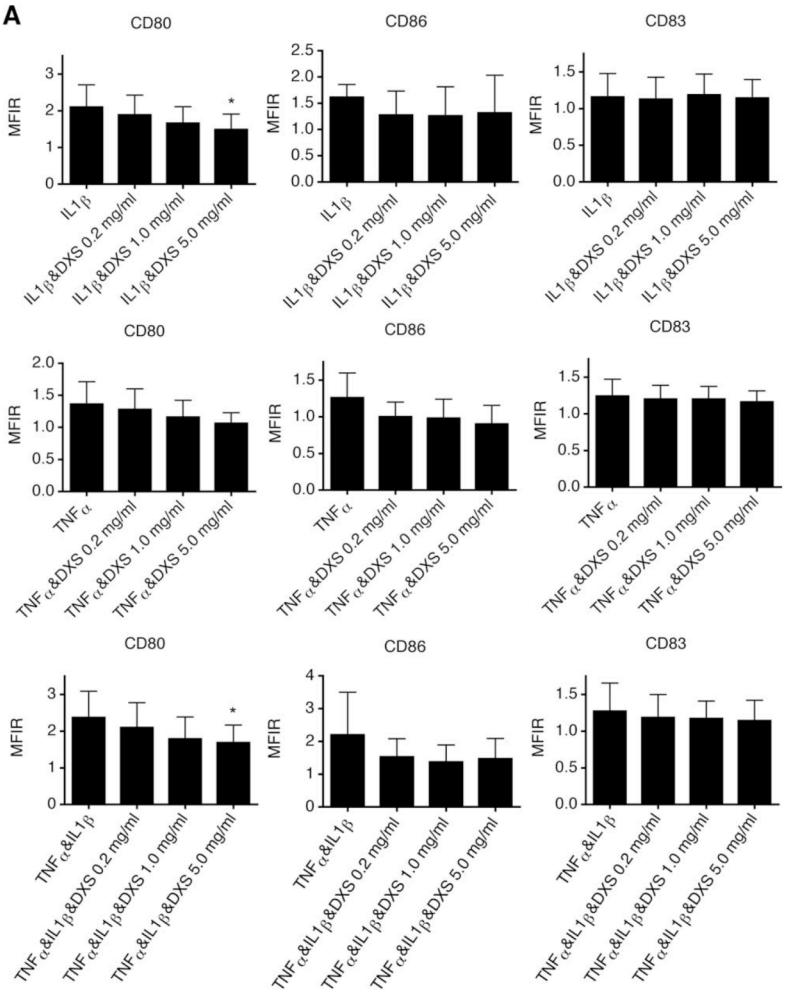

FIGURE 5. Effect of DXS on maturation of MoDC induced by proinflammatory cytokines. *A*, Influence of DXS on phenotypic maturation of human MoDC in response to TNF-α or IL-1β (each 20 ng/ml) for 24 h. Monocytes isolated from buffy coat were cultured in presence of GM-CSF and IL-4 for 6 days. To compare the levels of inhibition of up-regulation of the indicated surface molecules, the median fluorescence intensity ratios (MFIR) were calculated by dividing the median fluorescence of TNF-α-, IL-1β-, or DXS-treated MoDC by the median fluorescence of immature MoDC. Mean $\pm$ SD of five to seven independent experiment with cells of different donors is shown. *, $p < 0.05$ vs mature MoDC by unpaired Student's *t* test. *B*, Influence of DXS on phenotypic maturation of human MoDC in response to TNF-α or IL-1β (each 20 ng/ml) for 48 h. Monocytes isolated from buffy coat were cultured in presence of GM-CSF and IL-4 for 6 days. To compare the levels of inhibition of up-regulation of the indicated surface molecules, the median fluorescence intensity ratios (MFIR) were calculated by dividing the median fluorescence of TNF-α-, IL-1β-, or DXS-treated MoDC by the median fluorescence of immature MoDC. Mean $\pm$ SD of five to six independent experiment with cells of different donors are shown. **, $p < 0.01$ vs mature MoDC by unpaired Student's *t* test.

FACS analysis and cell viability

Cells were incubated with FITC-labeled mAb against CD1a, CD80, CD83, CD86 (BD Biosciences), isotype control IgG1 (BD Biosciences), PE-Cy5-labeled mAb against HLA-DR (BD Biosciences), isotype control IgG2b (BD Biosciences), unlabeled mAb against CD40, ICAM-1 (Diaclone) CD14 and DC-SIGN (BD Biosciences) followed by a PE-labeled polyclonal goat anti-mouse IgG1 (Southern Biotechnology Associates) or FITC-labeled polyclonal goat anti-mouse IgG (Sigma-Aldrich).

For determination of viability, the cells were stained with 5 μg/ml propidium iodide (Invitrogen) and analyzed by flow cytometry. As positive control, cells were treated with PBS containing 0.1% BSA (Sigma-Aldrich) and 0.1% saponin (Sigma-Aldrich) and then stained with propidium iodide. Measurements were performed with a BD FACScan flow cytometer and the obtained data were analyzed using FlowJo (Tree Star).

Binding of DXS and blocking of DC-SIGN and CR3

To examine binding of DXS to MoDC, cells were incubated with fluorescein-labeled DXS (3) for 30 min, washed three times, and analyzed immediately by flow cytometry. For receptor-blocking studies, 2×10^5 cells were incubated with 100 μg/ml mannan (Sigma-Aldrich), 20 μg/ml anti-DC-SIGN (BD Biosciences), or 50 μg/ml anti-CR3 (c7E3; Eli Lilly) in a 96-well plate (BD Biosciences). Blocking with mannan or anti-DC-SIGN was performed at 4°C for 30 min, then treatment with DXS (5 mg/ml) for 30 min followed by stimulation with HS (10 μg/ml) for 24 h. Blocking of CR3 was performed for 2 h with anti-CR3, followed by 30 min incubation with DXS and then stimulation with HS.

Measurement of immunostimulatory activity

T cells purified from human PBMC using nylon wool columns were used as responder cells for MoDC, which had been stimulated with HS or LPS, with or without DXS. Tetanus toxoid (Calbiochem) was used as Ag. A total of 1.5×10^5 autologous T cells were added to different numbers of MoDC (10,000, 5000, 1000) in 96-well tissue culture plates containing 0.2 ml of medium per well. T cells and MoDC were coincubated for 5 days and pulsed with 0.5 μCi of [^{3}H]thymidine (Amersham Biosciences) for the last 18 h. The incorporated radioactivity was then measured using a liquid scintillation counter.

Cytokine assays

MoDC (10^6 cells/ml) were treated with HS in the presence or absence of different concentrations of DXS for 24 h. The cell culture supernatants were analyzed using a Luminex multiplex array system (Bio-Rad) for IL-1β, IL-6, IL-12p70, IL-10 and TNF-α, according the manufacturer's instructions.

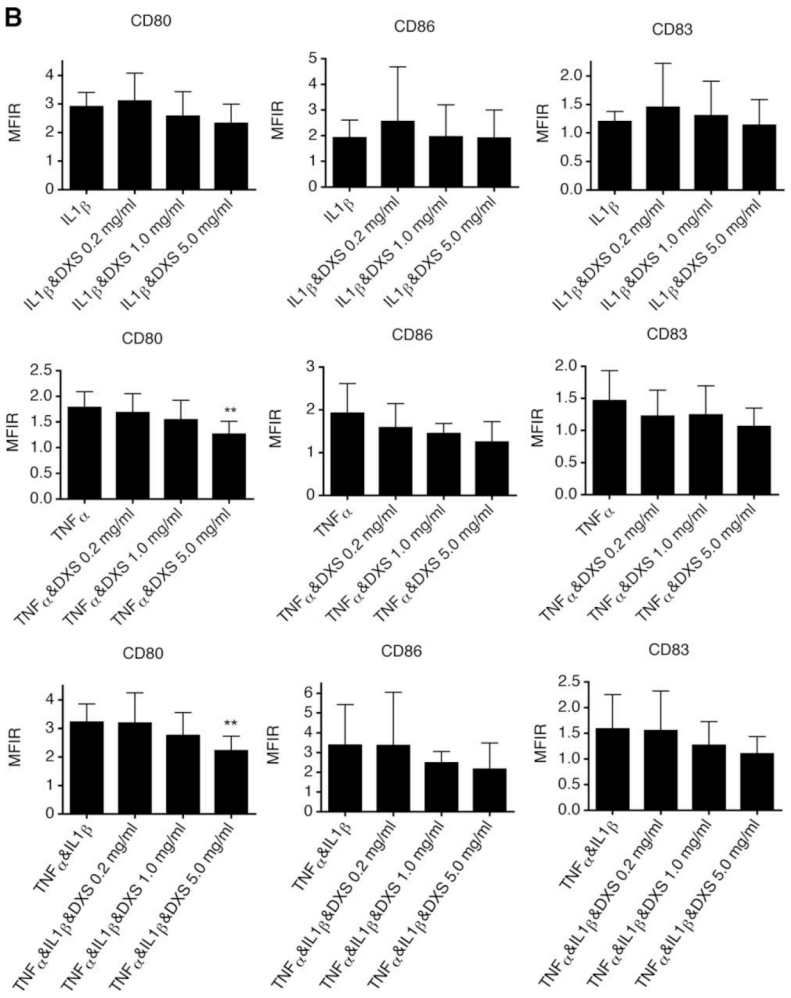

FIGURE 5. (*continued*)

C1q and C3 measurements by ELISA

Measurement was completed of C1q and C3 in supernatants of MoDC that were treated with different stimuli for 24 h, washed, and reincubated with fresh cell culture medium for 48 h. ELISA was used for measurement according to previous publications (10, 26, 27). In brief, the C1q-specific mAb 2204 was used as capture Ab and purified rabbit anti-human C1q, followed by HRP-conjugated goat anti-rabbit IgG (Jackson Immuno-Research Laboratory) was used for detection of bound C1q. Highly purified human serum C1q was used as a standard. For the detection of C3, wells were coated with a polyclonal anti-human C3 capture Ab followed by a digoxigenin-conjugated rabbit anti-human C3 Ab and HRP-conjugated sheep Fab anti-digoxigenin polyclonal Ab (Boehringer Mannheim Biochemica). The sensitivity of the ELISA for C1q was 0.125 ng/ml (10) and for C3 it was 0.03 ng/ml.

Measurement of phagocytic activity

For the analysis of phagocytic activity, 2×10^5 DC were incubated with FITC-dextran (m.w. 40,000; Invitrogen) for 1 h at 37°C. As negative control, cells were precooled before the incubation with FITC-dextran at 4°C for 1 h. The cells were washed four times and immediately analyzed by flow cytometry.

Measurement of phosphorylation of IκB-α

MoDC (2×10^6 cells/ml) were treated with HS or LPS in the presence or absence of DXS for 15 and 30 min. The cell lysate was analyzed using a Luminex multiplex array system from Bio-Rad for phosphorylation of IκB-α, according to the manufacturer's instructions.

Detection of NF-κB activation by a transcription factor ELISA

The production of NF-κB p65 was measured with a NF-κB assay kit (Active Motif) according the manufacturer's instructions. In brief, cell extract of LPS- or HS-activated DC, with or without additional pretreatment by DXS (5 mg/ml), was added to each well coated with consensus-binding site oligonucleotides of NF-κB p65. A primary Ab specific for an epitope on the bound and active form of the transcription factor was then added, followed by subsequent incubation with secondary Ab.

Statistical analysis

Data are presented as mean ± SD representing experiments with up to eight different donors. Unpaired Student's *t* tests were performed for evaluation of significance. Differences were considered as statistically significant at a value for $p < 0.05$. Data were analyzed using GraphPad Prism software.

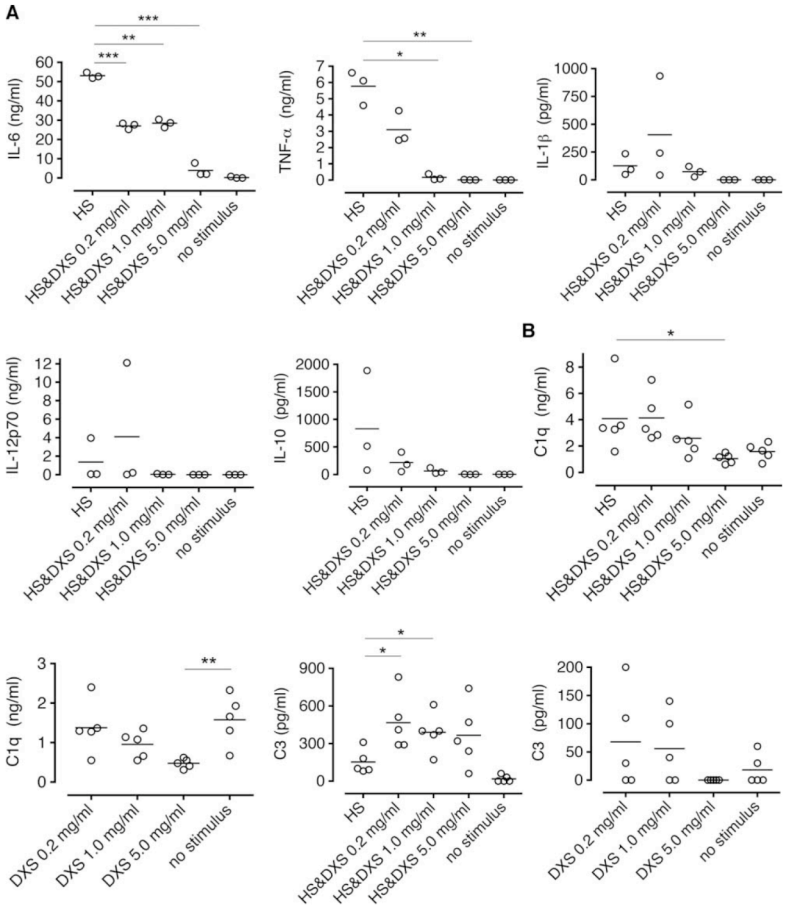

FIGURE 6. DXS prevents the secretion of proinflammatory cytokines, IL-10 and reduces the production of C1q, whereas C3 secretion is increased by MoDC. *A*, DXS prevents the release of the cytokines IL-1β, IL-6, IL-10, IL-12p70, and TNF-α by HS (10 μg/ml) treated MoDC. Immature MoDC were pretreated 30 min with the indicated concentrations of DXS before addition of HS. Supernatants after 24 h of incubation were analyzed by a multiplex suspension array. Cell-free medium was used to determine the background. Each point represents the value of one donor. Horizontal bar shows the mean of three different donors. Statistical significance between samples was indicated. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ vs mature MoDC by unpaired Student's *t* test. *B*, DXS treatment of MoDC leads to a decreased C1q production, whereas C3 secretion is increased. Individual values and mean are shown from five different individuals. Immature MoDC were pretreated 30 min with the indicated concentrations of DXS before addition of HS. After 24 h the cells were washed and incubated for 48 h with new cell culture medium. Supernatants were analyzed by ELISA. Cell-free medium was used to determine the background. Data points represent the value of one donor. Horizontal bar represents the mean of five different donors. Statistical significance between samples was indicated. *, $p < 0.05$; **, $p < 0.01$ vs mature MoDC by unpaired Student's *t* test.

Results

DXS inhibits phenotypic changes in MoDC as well as peripheral myeloid DC induced by HS, LPS, and LTA

Human immature DC were generated from monocytes isolated from buffy coat incubated for 6 days together with IL-4 and GM-CSF. Maturation was induced using HS, LPS, and LTA. To exclude that the activation through HS was due to endotoxin contamination, HS was absorbed on *Limulus* anti-LPS factor-coated silica beads. This treatment did not alter the ability of HS to induce maturation of DC, as already shown (21, 28). The same treatment of LPS eliminates its ability to induce maturation (data not shown).

To determine whether DXS affects the phenotypic maturation of MoDC induced by endogenous (HS) and exogenous TLR ligands (LPS, LTA), the cells were analyzed using flow cytometry and specific mAb against MHC class II, costimulatory, and adhesion molecules. Treatment with LPS (100 ng/ml), LTA (5 μg/ml), and HS (10 μg/ml) significantly increased the expression of CD80, CD86, CD83, CD40, and ICAM-1 (CD54) and the human MHC class II molecule HLA-DR. Incubation together with DXS significantly reduced the up-regulation of these mature DC surface markers in a dose-dependent manner, whereas DC-SIGN (CD209) expression was down-regulated (Fig. 1). The maximum inhibition by DXS was found at 5 mg/ml. Taken together, these findings indicate that DXS predominately prevents LPS- and HS-induced MoDC maturation, whereas there was less effect on LTA-induced maturation (Fig. 2).

We also examined the effect of DXS on HS-stimulated myeloid DC freshly isolated from peripheral blood. Preincubation or coincubation of peripheral myeloid DC with DXS inhibited up-regulation of the costimulatory molecules CD80/CD86 and the maturation marker CD83 (Fig. 3). These data suggest that DXS is able to prevent the HS induced maturation of myeloid DC ex vivo.

To investigate how the concentration of DXS used influences the viability of MoDC, we monitored MoDC survival at different DXS concentrations. MoDC were incubated for 24 h with different concentrations of DXS and stained afterward with propidium iodide and immediately analyzed by FACS. We found that DXS concentrations of up to 25 mg/ml, either alone or in combination with LPS or HS, did not affect the viability of MoDC within 24 h of exposure (Fig. 4).

Influence of DXS on maturation induced by TNF-α or IL-1β

MoDC were treated with TNF-α (20 ng/ml) or IL-1β (20 ng/ml), or a combination of both, for 24 or 48 h, and up-regulation of CD80, CD86, and CD83 was measured by flow cytometry. Only

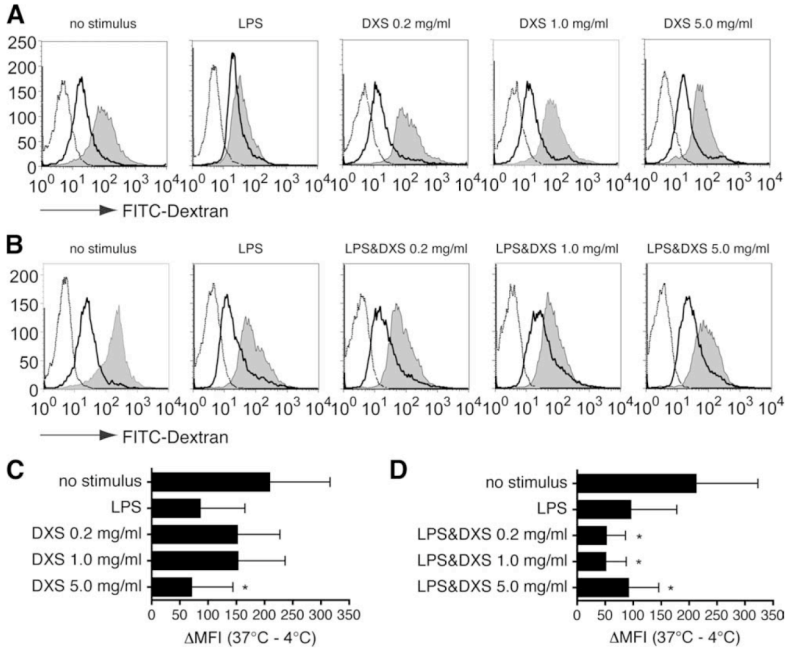

FIGURE 7. Phagocytic activity is influenced by DXS. Ag uptake capacity of MoDC treated with different concentrations of DXS with or without additional addition of LPS (100 ng/ml) was analyzed by the uptake of fluorescein-labeled DXS (m.w. 40,000, 1 mg/ml) as described in *Materials and Methods*. *A*, The profiles of the cells incubated at 37°C (gray filled histogram) and at the 4°C control (open histogram) are shown. Scattered line histogram indicates the unstained negative control. *B*, The profiles of the cells incubated at 37°C (gray filled histograms) and the 4°C control (open histogram) are shown. Scattered line histogram indicates the unstained negative control. Results shown from one donor are representative of four independent experiments. *C* and *D*, To compare the levels of phagocytic activity the mean of fluorescence intensity of cells incubated at 37°C were subtracted with the mean of fluorescence intensity of cells incubated at 4°C. Data re mean ± SD of four different donors. Statistical significance between samples was indicated. *, $p < 0.05$ vs immature MoDC by unpaired Student's *t* test.

the highest concentration of DXS did significantly inhibit the up-regulation of CD80 at 24 or 48 h of stimulation (Fig. 5), whereas no inhibitory effect was observed for CD86 or CD83. The inhibitory effect of DXS seems to be more restricted to a TLR-mediated activation of MoDC.

DXS reduces HS-induced secretion of proinflammatory cytokines and IL-10 by MoDC

HS is known to induce the secretion of proinflammatory cytokines like IL-1β, IL-6, and TNF-α (28). As assessed by multiplex suspension array, DXS significantly inhibited the HS-induced production of IL-1β, IL-6, IL-12p70, and TNF-α by MoDC in a dose-dependent manner with a maximum effect at 5 mg/ml (Fig. 6*A*). In addition, DXS also reduced secretion of the immunosuppressive cytokine IL-10 (Fig. 6*A*). Incubation of MoDC with different concentrations of DXS without a TLR agonist had no effect on secretion of these cytokines.

Reduction of C1q but increase in C3 secretion by MoDC under DXS treatment

It was recently shown in vitro and in vivo that immature DC are a rich source of C1q (10). Furthermore, it has been demonstrated that DC are able to synthesize C3 (12). Treatment of MoDC with the complement inhibitor DXS with or without HS or LPS stimulation led to a reduced production of C1q as detected in our cell culture supernatants. Surprisingly, we observed no decrease of C1q production in the HS-treated MoDC in contrast to LPS-induced maturation. The production of C3 by MoDC treated with DXS and either HS or LPS was increased, whereas the incubation with DXS alone led to a reduced C3 secretion. The amount of C3 detected in the supernatants of DXS and TLR ligand treated cells were higher than in the MoDC treated only with a TLR agonist (data for LPS not shown) (Fig. 6*B*).

DXS does not prevent maturation-induced reduction of phagocytic activity of MoDC

Immature DC capture and process Ags as a consequence of their activity; during maturation this feature disappears (7). When immature MoDC were stimulated with LPS, a significant decrease of their phagocytic activities was observed. Treatment of DC with DXS alone or together with LPS reduced the phagocytic activity of MoDC as measured by uptake of FITC-Dextran (Fig. 7, *B* and *D*). As a next step, we evaluated the effect of DXS without additional stimulation with LPS on the phagocytic activity of MoDC. As shown in Fig. 7, *A* and *C*, already DXS alone led to a functionally impaired phagocytosis with significant effect at the highest used concentration.

DXS reduces MoDC-mediated proliferation of autologous T cells

To determine whether DXS influences Ag presentation by MoDC we performed a proliferation assay. Immature MoDC were pulsed with tetanus toxoid for 3 h. Thereafter they were incubated with HS (10 μg/ml) plus DXS (5 mg/ml) for up to 24 h. These MoDC

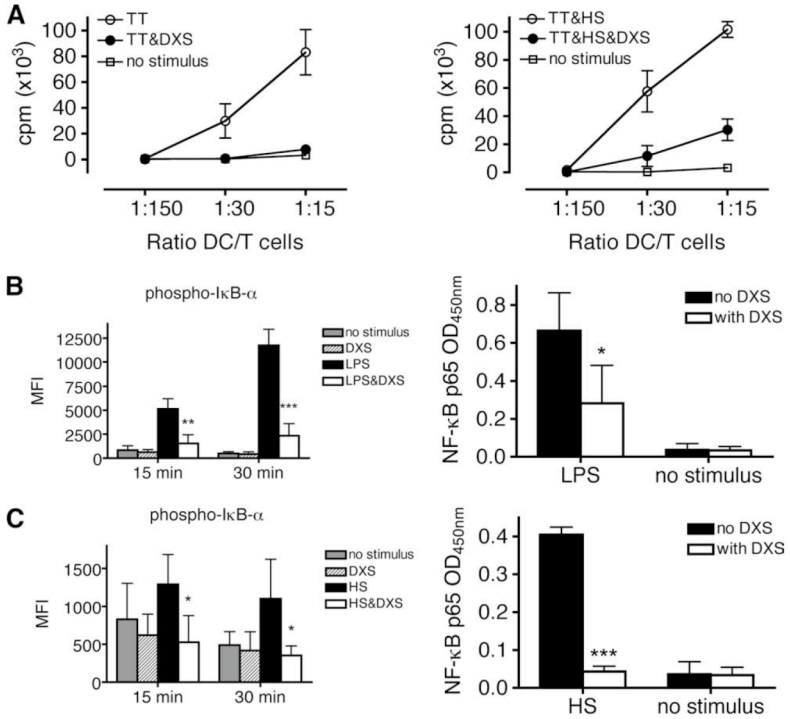

FIGURE 8. Immunostimulatory capacity of MoDC is reduced and activation of NF-κB is inhibited by DXS. *A*, T cell proliferation mediated by human MoDC is inhibited by DXS. MoDC were incubated for 24 h in the absence or presence of DXS (5 mg/ml). Cells were then harvested and incubated with autologous T cells in a 96-well plate. Stimulation experiments were conducted at several MoDC stimulator:responder T cell ratios (*x*-axis). Cells were pulsed with [^{3}H]thymidine on day 5, and [^{3}H]thymidine incorporation was assessed after 18 h. TT, Tetanus toxoid. Data represent mean ± SD of an experiment performed in triplicate and is representative of four independent experiments with cells of different donors. *B* and *C*, IκB-α phosporylation was measured in cell lysate 15 and 30 min after stimulation with LPS (100 ng/ml) (*B*) and HS (10 μg/ml) (*C*) by a Luminex multiplex array system. NF-κB activation was measured in cell extracts at 1 h after LPS (*B*) or HS (*C*) induced activation by a transcription factor ELISA. Cells were preincubated 30 min before TLR stimulation with DXS (5 mg/ml). Data represent mean ± SD of three independent experiments with cells of different donors. Statistical significance between samples was indicated. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ vs mature MoDC by unpaired Student's *t* test.

were then tested for their ability to present the Ag to T cells assessed by a standard [^{3}H]thymidine incorporation assay. MoDC incubated together with DXS showed a markedly reduced capacity to induce proliferation of autologous T cells (Fig. 8*A*).

DXS inhibits phosphorylation of IκB-α and activation of the transcription factor NF-κB

To assess the effects of DXS-mediated inhibition of maturation of MoDC on intracellular signaling, we determined the phosphorylation of IκB-α and activation status of NF-κB, the essential transcription factor for DC maturation and function. The phosphorylation of IκB-α leads to its ubiquitylation and subsequent degradation, which results in a release of NF-κB (29). Treatment with HS or LPS led to phosphorylation IκB-α measured after 15 and 30 min and after 1 h to activation and translocation of NF-κB p65 into the nucleus. Pretreatment of MoDC with DXS caused a strong abrogation of HS- and LPS-induced phosphorylation of IκB-α and activation of NF-κB as shown in Fig. 8, *B* and *C*.

DXS partially affects the differentiation of monocytes into immature DC

To investigate the effect of DXS on the differentiation of monocytes into immature DC we added different concentrations of DXS during incubation of the cells with GM-CSF and IL-4 for MoDC generation. DXS did not influence the up-regulation of the DC marker CD1a, nor the down-regulation of the monocyte marker CD14. In addition, the up-regulation of CD40 and ICAM-1 was not influenced by DXS. These data strongly support our viability test that the cells under the treatment of DXS are still alive and metabolic active. However, as compared with control DC, we observed a significant difference in expression of HLA-DR and DC-SIGN (Fig. 9*A*). In addition, the capability was tested of MoDC generated in the presence of DXS to mature upon stimulation by LPS. The stimulation pattern observed with these cells, in particular the up-regulation of the maturation surface markers CD80 and CD86 was reduced, for CD86 significantly, compared with that of MoDC generated without DXS (Fig. 9*B*).

DXS binds specifically to immature MoDC but not via DC-SIGN or CR3

To further assess the mechanism of DXS on MoDC, we have incubated the cells with fluorescein-labeled DXS (DXS-fluo). As shown in Fig. 10*A*, DXS-fluo specifically binds to immature DC, whereas significantly less binding was observed to mature MoDC. The binding of DXS-fluo was inhibited by pretreatment of the cells

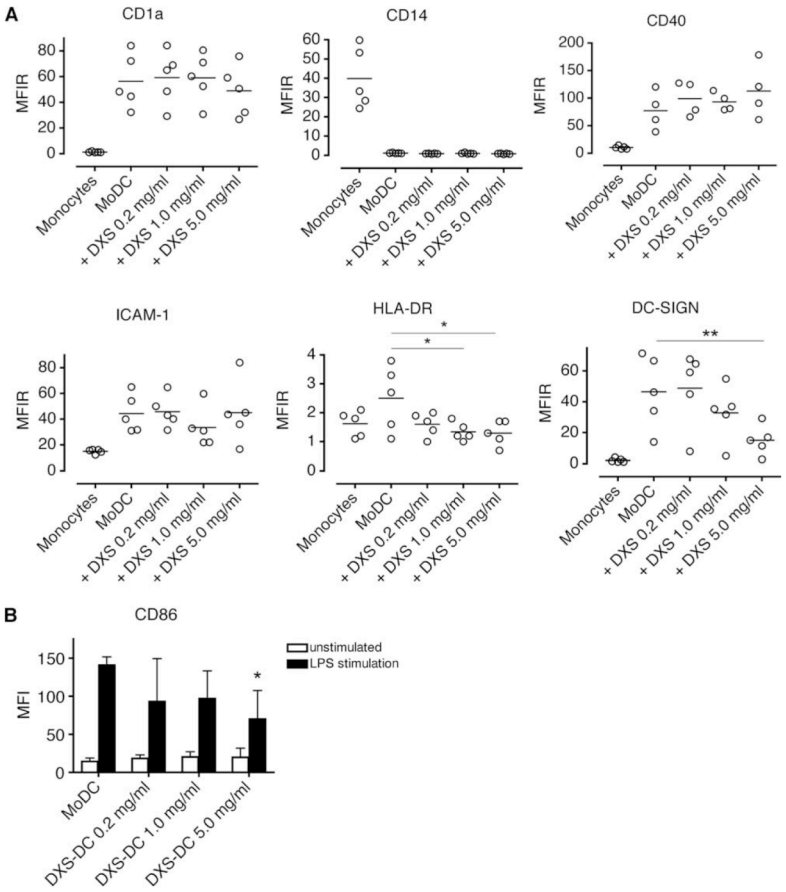

FIGURE 9. DXS partially affects the differentiation of monocytes into immature MoDC. *A*, Monocytes isolated from buffy coat were cultured in presence of GM-CSF and IL-4 for 6 days. In addition, cells were incubated with the indicated concentrations of DXS during 6 days. Monocytes were analyzed at the day of isolation and MoDC were analyzed by FACS at day 6. To compare the levels of up- or down-regulation of the indicated surface molecules, the median fluorescence intensity ratios (MFIR) were calculated by dividing the median fluorescence of monocytes, MoDC or DXS-treated MoDC by the median fluorescence of the negative control. Each point represents the value of one donor. Bars show the mean. Statistical significance between samples was indicated. *, $p < 0.05$; **, $p < 0.01$ by unpaired Student's t test. *B*, MoDC and DXS-DC (generated in presence of the indicated concentrations of DXS) were stimulated with LPS (100 ng/ml) for 24 h. Afterward, cells were analyzed for the expression of CD86 by flow cytometry. Data represent mean ± SD of three independent experiments with cells of different donors. *, $p < 0.05$ vs mature MoDC by unpaired Student's t test.

with unlabeled DXS. It has been shown that heparin, another sulfated glycosaminoglycan, binds to CR3 (30). To examine whether DXS binds to CR3, we have incubated the cells with an anti-CR3 blocking Ab. The binding of DXS-fluo was not abolished by the pretreatment of the MoDC with the blocking, neither was the inhibitory effect of DXS reversed by the blocking Ab for CR3 measured by the up-regulation of CD86 by flow cytometry (Fig. 10*B*). To assess whether DC-SIGN is involved, we have blocked DC-SIGN either with mannan (Fig. 10*C*) or with an anti-DC-SIGN blocking Ab described in the literature (31) (Fig. 10*D*). Blocking of DC-SIGN did not abolish the inhibitory effect of DXS either.

To assess whether DXS-mediated inhibition of MoDC maturation persists when the substance is removed from the cell culture medium, we thoroughly washed the cells after different preincubation periods and then stimulated them with HS or LPS. As shown in Fig. 10*E*, resistance against TLR induced activation increases depending on preincubation time with DXS, and after 24 h of preincubation the observed inhibition of CD86 expression is similar to the one observed with DXS coincubation (Figs. 1 and 2).

Discussion

In the present study we have investigated the effect of the complement inhibitor low molecular weight DXS on the maturation process of human DC. Several reports suggest that maintaining DC in an immature or semimature state (32), or activating them in an alternative manner (33), can be effective in preventing allograft rejection and favor the induction of tolerance.

Activation of vascular endothelial cell, as occurring in organ transplantation for example due to ischemia/reperfusion injury of the graft, leads to shedding of HS (18, 20). It has been shown that soluble HS serve as a danger signal, which is able to induce maturation of DC (28) via TLR4 (15). TLR4 therefore plays an important role in ischemia/reperfusion injury (34, 35) and in many stages of graft rejection (36).

The inhibitory effect of DXS is mainly observed for a TLR4 stimulation (HS, LPS), whereas the maturation induced via TLR2 (LTA) or proinflammatory cytokines like TNF-α or IL-1β seems to be less affected. Treatment of DC with DXS prevents HS- or LPS-induced up-regulation of the costimulatory molecules CD80/CD86/CD83 and CD40. Moreover, we observed a reduced expression of the human MHC class II molecule HLA-DR. Interestingly, we could also observe an inhibition of the up-regulation of the integrin ICAM-1 and a down-regulation of DC-SIGN, which is a member of the C-type lectin receptor family. The expression of the adhesion molecule ICAM-1 on DC has been reported not to influence the migration to the regional lymph nodes (37), whereas it seems to have an important role in DC-T cell interactions and induction of proliferation (38, 39). The inhibited up-regulation of

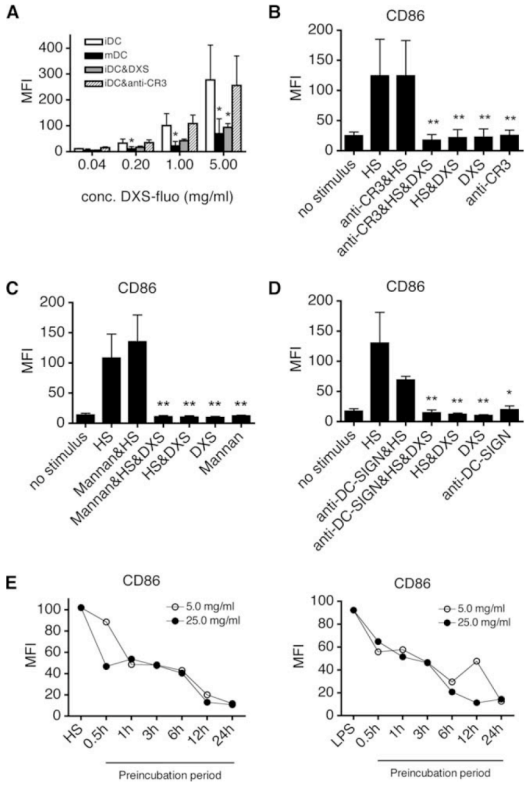

FIGURE 10. DXS specifically binds to immature MoDC. *A*, Nonstimulated immature MoDC and LPS-stimulated MoDC (48 h stimulation) were incubated for 30 min with DXS-fluo. In addition, mature MoDC were preincubated with unlabeled DXS (5 mg/ml) or with a blocking anti-CR3 Ab (50 μg/ml) for 30 min and then incubated with DXS-fluo for additional 30 min. Cells were washed three times and immediately analyzed by flow cytometry. Dead cells were excluded by propidium iodide staining. Autofluoresence of the cells was subtracted from median fluorescence intensity of DXS-fluo treated cells. Data represent mean ± SD of three independent experiments with cells of different donors. Statistical significance is indicated. *, $p < 0.05$ vs immature MoDC by unpaired Student's *t* test. *B*, Immature MoDC were incubated for 2 h with a blocking anti-CR3 (50 μg/ml), then treated for 30 min with DXS (5 mg/ml), and then stimulated with HS (10 μg/ml) for 24 h. Afterward, cells were evaluated for the expression of CD86 by flow cytometry. *C* and *D*, Immature MoDC were incubated with mannan (100 μg/ml) at 4°C or blocking anti-DC-SIGN (20 μg/ml) for 30 min before the exposure of DXS (5 mg/ml) for 30 min. The cells were then stimulated with HS (10 μg/ml) for 24 h and then analyzed for the expression of CD86 by flow cytometry. Data represent mean ± SD of three to four independent experiments with cells of different donors. Statistical significance is indicated. *, $p < 0.05$ and **, $p < 0.01$ vs mature MoDC by unpaired Student's *t* test. *E*, immature MoDC were incubated for the indicated periods with DXS (5 or 25 mg/ml), washed two times and then stimulated with HS (10 μg/ml) or LPS (100 ng/ml) for 24 h. Afterward, cells were evaluated for the expression of CD86 by flow cytometry. The results shown are from one donor and representative of two independent experiments with cells from different donors.

ICAM-1 by DXS-treated MoDC might contribute to the observed effect of a reduced capacity of these cells to induce a T cell proliferation. DC-SIGN mediates adhesion with T cells by stabilizing the DC/T cell contact and plays a crucial role in Ag capturing (40). Furthermore, there exist several reports suggesting a cross-talk between TLR and C-type lectin receptor to fine-tune the balance between immune activation and tolerance (41, 42). C-type lectin receptors are highly expressed on immature DC and our results indicate that DXS binds specifically to nonstimulated immature MoDC. Involvement of DC-SIGN was examined by receptor-blocking experiments. Our results indicate that DXS seems not to develop its inhibitory effect via DC-SIGN.

Analysis by multiplex suspension array revealed that secretion of the proinflammatory cytokines IL-1β, IL-6, and TNF-α as well as IL-12p70 in response to HS is inhibited in the presence of DXS. A reduction of the production of TNF-α and IL-1β, which are known to enhance the effect of maturation of DC induced by TLR ligands, could contribute to the inhibitory effect of DXS on DC maturation. However, we were not able to observe an up-regulation of the secretion of the immunoregulatory cytokine IL-10 after 24 h. Other known immunomodulating agents like corticosteroids (43) or vitamin D_3 (44) are known to increase the IL-10 production by DC, whereas e.g., rapamycin or FK506 (tacrolimus), similar to what we observed with DXS, did not increase IL-10 production (43).

The biological importance of these findings was confirmed by T cell stimulation assays in which DXS-treated MoDC showed a reduced capacity to induce T cell proliferation. In contrast, we found that DXS does not prevent the LPS-induced reduction of the phagocytic activity of MoDC measured by the uptake of FITC-dextran.

Immature DC were recently shown to be a rich source of functional complement components like C1q (10) and C3 (12) in vitro and in vivo. We found that DXS alone as well as in combination with HS reduced the amount of secreted C1q by DC. In contrast, the secretion of C3 was enhanced by DXS in combination with HS or LPS. The activation products of C3, such as iC3b, have been implicated in the induction of tolerance (14). In contrast, Peng et al. (12) recently suggested, that C3 synthesis by DC is crucial for full T cell activation. Furthermore, recent studies showed that signaling through CR3 in DC provides a "non-dangerous" signal (45) and renders them tolerogenic (46). We found that DXS does not bind to CR3 and blocking of CR3 could not reverse the effect of DXS.

The presence of DXS during differentiation of monocytes into immature MoDC did not affect the expression of CD1a, CD14, CD40, and ICAM-1. In contrast, we observed a significantly impaired up-regulation of HLA-DR and DC-SIGN. Therefore, DXS seems to partially influence the differentiation of monocytes into immature MoDC.

Following the observation that DXS affects DC maturation, we evaluated potential effects on signaling pathways that regulate DC immune properties. Due to the highly negative charges of HS and LPS, a direct interaction of DXS with these activating ligands is unlikely. This hypothesis was confirmed by the observation that LPS-induced down-regulation of phagocytosis was not inhibited and C3 production was increased together with DXS. It might be possible that DXS binds the positively charged acute phase plasma protein lipid binding protein, which is known to enhance the activation of immune cells by mediating the transfer of LPS to the cell surface. Whether DXS directly binds lipid binding protein or other soluble members of the TLR signaling cluster, like soluble CD14 or MD-2, and thereby inhibits TLR complex formation needs to be evaluated.

Signaling pathways that trigger TLR are dependent on Toll-IL-1R domain-containing adaptor protein and MyD88. MyD88 recruits members of the IL-1R-associated kinase family and the TNFR-associated factor 6 to finally activate NF-κB and the MAPK

pathways (29). Moreover, selective inhibition of NF-κB has been shown to be associated with tolerogenic properties of treated DC (47–49). In this study, we provide evidence that DXS significantly inhibited TLR-induced IκB-α phosphorylation and NF-κB activation, which could account for its major impact on MoDC maturation.

It should be noted that the used concentrations of DXS did not affect viability and metabolic activity of the cells, which is consistent with previously published in vitro and in vivo observations in which concentrations up to 25 mg/ml were used (5, 6). Recently, the maximum tolerated doses of DXS were determined in vivo in cynomolgus monkeys and i.v. or intraportal bolus injections of 3–6 mg/kg, followed by continuous infusion of 0.3–1.2 mg/kg/h for 6 h, and appeared to be safe (50). It should also be considered, however, that DXS binds to the surface of activated endothelial cells (3), which may limit the plasma concentration available for its inhibitory effect on DC. The in vivo concentrations of DXS required for complete or partial inhibition of DC maturation need to be elucidated in an appropriate animal model.

Our data suggest a new immunomodulatory function of the complement inhibitor and endothelial cell protectant DXS. This compound might therefore be useful as a therapeutic reagent to impede the link between innate and adaptive immunity.

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Disclosures

The authors have no financial conflict of interest.

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3-2 Paper II: Influence of DXS on the activation of human NK cells

Title: Inhibition of TLR2 induced activation of human natural killer cells by the complement inhibitor low molecular weight dextran sulfate

Authors: Rolf Spirig, Anne-Laure Millard, Nicolas J. Müller, Jörg D. Seebach and Robert Rieben

Manuscript in preparation

NK cells are a crucial part of the innate immune system and are capable to kill cells without prior immunization. It has been demonstrated, that NK cells play an important role in graft rejection and interact with DC. The previous finding that DXS is able to prevent TLR4 driven maturation of DC, led us to investigate the effect of DXS on TLR induced activation of human NK cells.

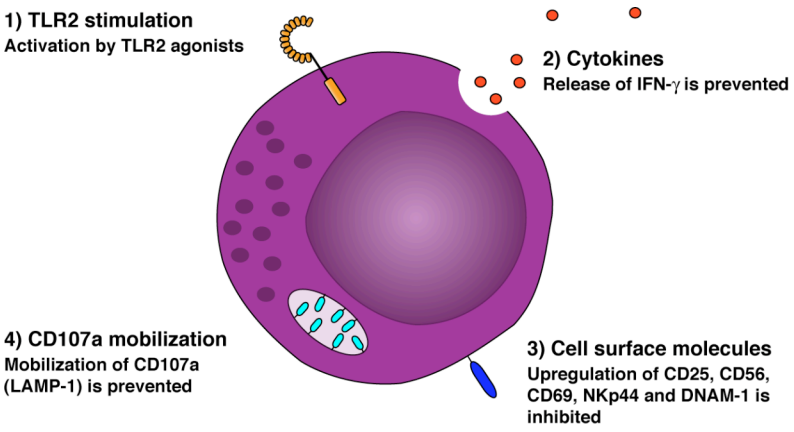

Figure 8: Influence of DXS on TLR induced activation of human NK cells.

In this study we could demonstrate, that TLR2 agonists directly induce activation of human NK cells. No activation was observed by stimulation with HA (TLR4). Pretreatment with DXS inhibited phenotypic as well as functional activation of human NK cells, which has been proven by reduced upregulation of activation markers as well as reduced frequency of IFN-γ secreting cells. These novel data indicate a novel effect of the complement inhibitor DXS on NK cells and may therefore be a useful reagent to impede activation of innate immunity.

Inhibition of TLR2 induced activation of human natural killer cells by the complement inhibitor low molecular weight dextran sulfate[1]

Running title: Inhibition of NK cell activation

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Keywords: human, natural killer cells, cell activation, transplantation

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Abstract

Natural Killer (NK) cells, complement and toll-like receptors (TLR) are important components of the innate immune system. Here, we show that the TLR2-agonists lipoteichoic acid (LTA) from gram-positive cocci *S. aureus* as well as the synthetic triacylated lipopeptide Pam3CSK4, induce direct activation of human NK cells. Treatment of NK cells with TLR2 agonists induces a significant increase of CD56, CD25, CD69, NKp44 and DNAM-1 expression. No increase of NKG2D and NKp46 was observed. Stimulation results in an increase of IFN-γ secreting NK cells. In addition, stimulation with TLR2 ligands increases the surface expression of the granulation marker CD107a (LAMP-1). Low molecular weight dextran sulfate (DXS) has been reported to inhibit the classical, alternative as well as the lectin pathway of the complement system. DXS dose-dependently prevented IFN-γ secretion of TLR2 activated human NK cells. In addition, DXS prevents up-regulation of CD56, CD25, CD69, NKp44 and DNAM-1. Moreover, recruitment of CD107a to the cell surface is prevented by DXS. These findings suggest that Pam3CSK4 and LTA are stimulators of NK cells and that DXS prevents TLR2-induced activation. DXS may therefore be a useful reagent to impede the activation of innate immunity.

Introduction

Low molecular weight dextran sulfate (DXS, MW 5000), a highly sulfated polyglucose, is known to inhibit the classical, alternative and mannan-binding lectin (MBL) complement pathways by enhancing the activity of C1-inhibitor (C1-INH) (1) and binding to factor H (2). In addition, DXS has been shown to act as an endothelial cell (EC) protectant preventing complement- and Natural Killer (NK) cell mediated EC damage (3). A combination of an anti-CD4 antibody and DXS is able to induce long-term graft survival in a rat cardiac allotransplantation model *in vivo* in a setting of prolonged cold ischemia (4). In addition, we have recently shown, that DXS inhibits toll-like receptor (TLR) 4 induced maturation of human monocyte-derived dendritic cells (MoDC) (5). Taken together, DXS can be described as a substance, which attenuates the pro-inflammatory effects of many mediators of innate immunity (6). We hypothesized, therefore, that DXS might also influence the function of NK cells.

NK cells are an important cellular component of the innate immune system and are capable to kill cells without prior immunization. Therefore, NK cells play a critical role in xeno- (7-10) as well as allotransplantation (11). Activating receptors on NK cells include NKp30, NKp44, and NKp46 (12), collectively named natural cytotoxicity receptors (NCR), the C-type lectin receptor (CLR) NKG2D, and DNAM-1 (CD226) implicated in the recognition of target cells by cytotoxic cells (13). Although NKp30 and NKp46 are detected on all NK cells regardless of their activation status, NKp44 is selectively expressed by activated NK cells (14). NK cells express germline encoded pattern-recognition receptors (PRR) as e.g. members of the TLR family. Recent studies have demonstrated the expression of TLR1 (15), TLR2 (16), TLR3 (17, 18), TLR4 (19), TLR5 (15), TLR6 (20), TLR7 (21), TLR8 (21) and TLR9 (17) on human NK cells. Although NK cells express almost all TLR, not all are functional and indirect activation via accessory cells plays an important role in NK cell activation. Several studies have demonstrated a reciprocal activation between NK cells and DC (22, 23). Activation of NK cells by DC seems to be mediated via cell-contact interactions through e.g. NKp30 or IL-15Rα as well as cytokines like IL-12, IL-15 and IL-18 (24-26).

Early inflammatory processes in organ transplantation have been associated with ischemia-reperfusion (I/R) injury. Healthy EC are covered by a layer of

glycosaminoglycans as e.g. heparan sulfate (HS) or hyaluronic acid (HA), which is crucial for the anticoagulant and anti-inflammatory properties of the endothelium. HS and HA are rapidly released under conditions of inflammation and tissue damage (27-30). It has been shown that soluble HS or HA induce maturation of DC via TLR4 (31, 32). Furthermore, TLR2 and TLR4 have been demonstrated to be involved in the early inflammatory process of I/R injury *in vivo* (33, 34). Interestingly, a recent study demonstrated the importance of TLR3 as a sensor of endogenous tissue necrosis (35).
In this study, we show a direct activation of human NK cells by TLR2 agonists. Furthermore, we demonstrate that the complement inhibitor DXS interferes with TLR2 induced activation of human NK cells at multiple levels by inhibiting up-regulation of activation receptors as well as the secretion of IFN-γ.

Material and Methods

Isolation and stimulation of human NK cells

Human peripheral blood mononuclear cells (PBMC) were isolated from healthy donors by density gradient centrifugation over Ficoll-Paque (Amersham, Uppsala, Sweden). Purification of NK cells by negative magnetic selection using the MACS system (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) has been described previously (10, 36). Isolated NK cells with a purity >95% were used directly. Pam3CSK4 (1 μg/ml; Invivogen, San Diego, California, USA), LTA (5 μg/ml; Sigma, St. Louis, MO, USA) or HA (20 μg/ml; Sigma) was added to the cells for 24 hours. Low molecular weight dextran sulfate (Sigma, MW 5000), used at the indicated concentrations, was added to the cells 60 minutes prior to Pam3CSK4 or LTA treatment. The complement inhibitory capacity of DXS was always tested prior to use for experiments with a CH50 hemolysis-assay as described previously (3).

Generation and stimulation of human monocyte-derived DC (MoDC)

PBMC were isolated from buffy coats obtained from healthy blood donors (Regional Red Cross Blood Donation Center, Bern, Switzerland) by density gradient centrifugation over Ficoll-Paque. Monocytes were isolated from PBMC as described previously (5, 37, 38). The purified monocytes were incubated for 6 days in RPMI 1640 medium (Invitrogen Life Technologies) containing 10% FCS (Amimed / BioConcept), 1% [2mM] L-Glutamine (Invitrogen), 1% [100 U/ml]

Penicillin/Streptomycin (Invitrogen), 10 ng/ml GM-CSF (R&D Systems Europe Ltd, Abingdon, Oxon, UK), and 10 ng/ml IL-4 (R&D) to generate MoDC as described initially by Sallusto and Lanzavecchia (39). The cells were kept at 37°C in a 5% CO_2 humidified atmosphere. On day 3, the culture medium was replaced with fresh medium. For induction of maturation 20 μg/ml hyaluronan (HA, Sigma) or 100 ng/ml lipopolysaccharide (LPS, Sigma) were added to the cultures for 24 or 48 hours.

Flow Cytometry

Surface expression of CD25, CD45, CD56, CD69, NKp30, NKp44, NKp46, DNAM-1 and NKG2D was analyzed on a FACSCanto (BD Biosciences). After resuspension of 1 x 10^5 cells/tube in staining buffer (PBS, 0.1% BSA), cells were incubated for 30 min at 4°C with saturating amounts of antibody (Ab). The following direct mouse IgG1 anti-human Ab were used: anti-CD45-PE, anti-CD56-AF® 488, anti-CD56-PE, anti-CD69-PE, and anti-NKG2D-PE (BD Biosciences), anti-NKp30, anti-NKp44, anti-NKp46-PE and anti-DNAM-1-PE (Miltenyi Biotec). Irrelevant mouse antibodies MOPC21, MOPC21-FITC and MOPC21-PE (mouse IgG1) from BD Biosciences were used as isotype-matched control. As secondary reagents, APC-conjugated goat anti-biotin (Miltenyi Biotec) was used. To exclude dead cells, propidium iodide (PI) gating was included in some experiments. Analysis was performed using FlowJo software (Tree Star). To compare the levels of surface expression, the mean fluorescence intensity ratios (MFIR) were calculated by dividing the geometric mean fluorescence intensity of each sample by the geometric mean fluorescence intensity of the control Ab.

Single cell analysis of IFN-γ production

A MACS® Cytokine Secretion Assay (Miltenyi Biotec, Germany) was used to identify NK cells secreting IFN-γ. Briefly, human NK cells (1 x 10^6) were stimulated in the presence or absence of DXS. After 24 hours, cytokine catch reagent (anti- IFN-γ Ab conjugated to a cell surface-specific Ab) was added and cells were incubated at 37°C for 45 min. After washing, cells were stained with anti-IFN-γ PE, and anti-CD56- or anti-CD3-FITC for 20 min at 4°C. Isotype-matched Ab were used as controls. After washing, the samples were analyzed by flow cytometry.

IFN-γ ELISA

Freshly isolated human NK cells ($5x10^5$, $2.5x10^5$ and $1.25x10^5$) were stimulated in the presence or absence of DXS. After 48 hours, supernatants were harvested and assayed for IFN-γ using an ELISA kit according to manufacturer's instructions (Mabtech AB, Germany).

CD107a degranulation assay

The frequency of NK cell degranulation was analyzed through the expression of CD107a lysosome-associated membrane protein-1 (LAMP-1) as recently described (40). The K562 cell line, which does not express MHC class I molecules, was provided from the ATCC. NK cells were incubated with K562 at an effector-target (E/T) ratio of 5:1 for 6 hours. Anti-CD107a-FITC or MOPC21-FITC (an isotype-matched control) was added directly to the co-cultures. After 1h incubation, GolgiStop reagent diluted at 1:10 (BD Pharmingen) was added to the cultures and incubated for another 5 hours. Cells were then stained with CD56–PE and analyzed on a FACSCanto (Becton Dickinson, Switzerland).

Detection of apoptosis

Cell death was evaluated by using PI and FITC-annexin V kit (BD Biosciences). PI was used in conjunction with annexin V–FITC to distinguish cells in the earlier stages of apoptosis (annexin V-FITC positive, PI negative) from those in later stages of apoptosis or those that were already dead (annexin V–FITC positive, PI positive). Briefly, 10^5 cells were washed in cold PBS and resuspended in 0.1 ml of annexin V binding buffer (10 mM HEPES, 140 mM NaCl, 5 mM KCl, 1 mM MgCl2, 1.8 mM $CaCl_2$, pH 7.4). Cells were incubated for 20 min at room temperature following addition of FITC-Annexin V and of PI solution.

Statistical analysis

Data are presented as mean ± standard deviation (SD) representing experiments with up to 4 different donors. Paired Students *t-tests* were performed for evaluation of significance. Differences were considered as statistically significant at p-values less than 0.05. Data were analyzed using GraphPad Prism software 4.0 (GraphPad, San Diego, CA).

Results

Incubation with TLR2 agonists induces phenotypic changes on human NK cells

LTA from *S. aureus* and the synthetic triacylated lipopeptide Pam3CSK4 have been described in the literature as TLR2 agonists (41, 42). To examine the effect of these two ligands on NK cell activation, we incubated purified human NK cells with LTA (5 μg/ml) or Pam3CSK4 (1 μg/ml) for 24 hours. Stimulation of human NK cells with LTA or Pam3CSK4 induced a massive up-regulation of CD56, CD25, CD69, NKp44 and DNAM-1 expression analyzed by flow cytometry. No increase was observed for NKp30 and NKp46. Pretreatment with DXS before stimulation with TLR2 agonists, significantly prevented the up-regulation of the activation markers CD56, CD25, CD69, NKp44 and DNAM-1 (Fig. 1*A* and *B*).

The TLR2 agonists Pam3CSK4 and LTA increase the frequency of IFN-γ secreting NK cells

NK cells are the main source of IFN-γ besides T cells upon activation. To examine if stimulation of NK cells with Pam3CSK4 or LTA induces secretion of IFN-γ, we measured the frequency of IFN-γ secreting NK cells after 24 hours of incubation. As shown in Fig. 2*A*, both TLR2 ligands significantly increase the frequency of IFN-γ secreting NK cells.

The endogenous TLR4 agonist HA does not increase the frequency of IFN-γ secreting NK cells

HA has been described as endogenous danger signal promoting a TLR4 driven inflammatory immune response (32, 43). Stimulation of antigen presenting cells with HA does lead to the formation of a unique TLR4 signaling-complex consisting of MD-2, CD44 and TLR4 (44). Incubation of human NK cells with HA (20 μg/ml) for 24 hours did not lead to an increased frequency of IFN-γ producing NK cells (Fig. 2*A*).

DXS inhibits TLR2 and IL-2 induced frequency of IFN-γ producing NK cells

To evaluate if DXS alone has an effect on IFN-γ secretion, we incubated NK cells with DXS alone. We could not observe any changes in the frequency of IFN-γ secreting cells induced by DXS alone (Fig. 2*B*). According to our findings that TLR2

agonists lead to a direct activation of human NK cells, we investigated the effect of DXS on TLR2 induced activation. Freshly isolated human NK cells were incubated with different concentrations of DXS prior TLR stimulation with LTA, Pam3CSK4 or IL-2. As shown in Fig. 2*B* and *C*, DXS is able to prevent the induction of IFN-γ producing cells in a dose-dependent manner.

Inhibition of TLR2 induced frequency of degranulating NK cells by DXS

Cell surface expression of CD107a correlates directly with degranulation and release of cytolytic molecules, which indicates potential killing capacity of NK cells (40). Following stimulation, CD107a is recruited from the lysosomal vesicles to the cell surface. Treatment of NK cells with TLR2 ligands significantly up-regulates both cell surface expression of CD107a and the frequency of CD107a positive NK cells which are both prevented by previous exposure to DXS (Fig. 3).

Phenotypic and functional activation of NK cells by supernatant of activated MoDC

Recent studies have revealed an intensive cross-talk between NK cells and DC (22, 23, 45). We have incubated MoDC with or without LPS (100 ng/ml) for 24 and 48 hours. Afterwards, cells were harvested and centrifuged. Supernatant was collected and incubated with freshly isolated human NK cells overnight. Subsequent activation of human NK cells was evaluated by staining of CD25, CD69, NKp30, NKp44, NKp46, NKG2D and DNAM-1. As shown in Fig. 4*A*, supernatant of LPS-activated MoDC induced up-regulation of CD25 and CD69, whereas for NKp30, NKp44, NKp46 and DNAM-1 no significant increase was observed. In contrast, NKG2D expression was reduced (Summarized in Table I). As a next step, we evaluated the capacity of the supernatants to induce IFN-γ-secretion by NK cells. We could observe a significant increase of IFN-γ-secreting NK cells after exposure to supernatant of LPS-activated MoDC (Fig. 4*B*). Furthermore, we examined cellular mobilization of CD107a. Supernatant of activated MoDC induce significant recruitment of CD107a to the cell surface, indicating activation of human NK cells (Fig. 4*C*).

Effect of DXS on NK cell viability

To investigate how the used concentrations of DXS influence the viability of NK cells, we monitored NK survival at different DXS concentrations. NK cells were

incubated for 24 hours with different concentrations of DXS and stained with PI as a marker of necrotic cells and Annexin-V-FITC for apoptotic cells and then analyzed by flow cytometry. We found that DXS concentrations of up to 5 mg/ml, either alone or in combination with LTA or Pam3CSK4, did not affect the viability of NK cells within 24 hours of exposure (Fig. 5). In contrast, less PI^+ or Annexin-V^+ cells were detected after incubation with DXS.

Discussion

Several studies in the recent years emphasize the significance of TLR expression and function on NK cell immunobiology. TLR are able to recognize pathogen associated molecules as well as endogenous danger signals released due to inflammation or tissue injury (46). NK cells have been shown to be involved in the clearance of pathogens as well as in graft rejection. Inhibition of NK cell activation could therefore be effective in attenuating xeno- (8, 47) as well as allograft rejection (11).

In the present study we investigated TLR2- and TLR4-specific stimulation on activation of human NK cells. Furthermore, we analyzed the effect of the complement inhibitor DXS on TLR-mediated NK cell activation. Lipophosphoglycan (LPG) from the protozoa *Leishmania major* has been shown to directly activate human NK cells via TLR2 to produce TNF-α and IFN-γ (16). Here we show, that LTA from gram-positive cocci *S. aureus* and the synthetic triacylated lipopeptide Pam3CSK4, both described in the literature as TLR2 agonists (41, 42), induce phenotypic and functional activation of human NK cells. Furthermore, we could demonstrate that DXS is able to prevent TLR2-mediated stimulation of NK cells. In an earlier study we could show, that DXS prevents NK cell mediated EC damage (3).

Ischemia/reperfusion injury, as occurring in organ transplantation, leads to a shedding of HS and HA, which act as dangers signals sensed by TLR4 (28, 31, 32, 48). Similarly, necrotic cell death leads to the release of intracellular dangers signals as e.g. the peptide high-mobility group box 1 (HMGB-1) or uric acid, which were shown to signal through TLR2 and TLR4 (46). Both receptors were described to be crucially involved in the early inflammatory process of I/R injury (33, 34).

Our results demonstrate that only stimulation with TLR2 agonists induces a direct activation of human NK cells. In particular, we could observe a strong stimulation with Pam3CSK4, which is described to directly bind to the heterodimer TLR2-TLR1

(49), whereas less activation was observed for treatment with LTA. This difference could be explained by the fact that accessory molecules, such as CD14, facilitate stimulation by LTA, which are not expressed by NK cells (50).

Given the importance of TLR4 in sterile inflammation, we have investigated the effect of HA on activation of NK cells. TLR4 is expressed on human NK cells, but stimulation by LPS does not directly induce activation (19). As recently demonstrated, HA does lead to a formation of a unique TLR4 complex (44). However, we could not observe an induction of IFN-γ secretion by HA treated NK cells, confirming the earlier conclusion that TLR4 may not be functional on these cells.

As a next step, we have evaluated the effect of DXS on TLR2 induced activation of NK cells. Pam3CSK4 does lead to a massive up-regulation of CD25, CD69, CD107a and secretion of IFN-γ by human NK cells. Pretreatment of NK cells with DXS significantly prevented the up-regulation of all analyzed activation markers. In addition, the number of IFN-γ secreting cells was dose-dependently inhibited. It has been demonstrated that IFN-γ deficiency prolong xenograft survival (51). Until now, only few substances have been described with an inhibitory activity on human NK cell activation. The glucocorticoid dexamethasone has been shown to suppress NK cell activity (52). Interestingly, two studies performed in the early 80s demonstrated an inhibitory effect of sugars on NK cell mediated cytotoxicity. In particular, mannose 6-phosphate, fructose 1-phosphate and fructose 6-phosphate were shown to suppress NK cell cytotoxicity at concentrations between 5-10 mg/ml (53). Stutman *et al.* showed that the monosaccharide D-mannose affected NK cell activity whereas T cell mediated cytotoxicity was not affected (54). No similar effect was observed for glucose, mannose, fucose, galactose, glucose 6-phosphate, mannose 1-phosphate, galactose 1-phosphate or galactose 6-phosphate.

DXS did not affect viability of the cells, which is consistent with previously published *in vitro* and *in vivo* observations where concentrations up to 25 mg/ml were used (4, 55, 56). Maximum tolerated doses of DXS were determined *in vivo* in cynomolgus monkeys and i.v. / intraportal bolus injections of 3-6 mg/kg, followed by continuous infusion of 0.3-1.2 mg/kg/h for 6 hours, appeared to be safe (57). Furthermore, a human clinical phase I trial with DXS has shown that systemic concentration up to 50 μg/ml could be achieved without any negative side effects (58). The *in vivo*

concentrations of DXS required for complete or partial inhibition of NK cell activation need to be elucidated in an appropriate animal model.

Taken together, we could show that Pam3CSK4 and LTA induce direct activation of human NK cells, which could be of importance in the context of TLR2-induced inflammatory immune responses. In addition, our data suggest a new immunomodulatory function of the complement inhibitor DXS. This compound might therefore be useful as a therapeutic reagent to attenuate activation of innate immunity.

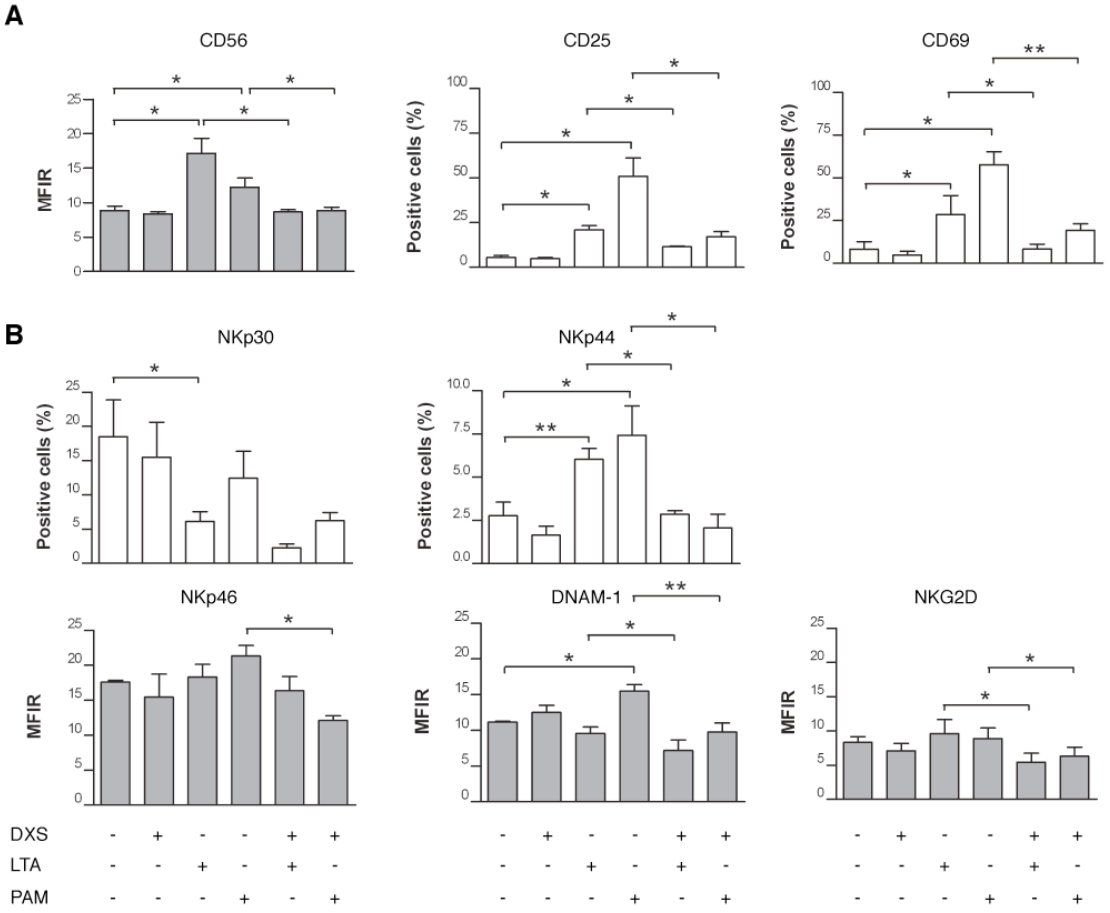

Figure 1: Characterization of the surface phenotype of NK cells stimulated with Pam3CSK4 or LTA in the presence or absence of DXS (5 mg/ml). Freshly isolated NK cells were cultured for 24h and stained for the cell surface markers as indicated. *A,* NK cells were stimulated with Pam3CSK4 (1 μg/ml) or LTA (5 μg/ml). To compare the levels of up-regulation of CD56 the mean fluorescence intensity ratios (MFIR), are shown on the y-axis (grey bars). Bars show means ± SD from four different NK donors. To evaluate the changes in CD25 and CD69 expression, the percentage of marker-positive cells are shown on the y-axis. Bars shown are the mean expression levels ± SD for CD25 and CD69 from four different NK donors. Significant differences are indicated in the graph as follows: *p<0.05, **p<0.01 (Student's *t*-test). *B,* To examine the modulation of NK cell specific activation markers NKp30, NKp40, NKp46, DNAM-1 and NKG2D, the percentage of marker-positive cells (white bars) or the MFIR (grey bars) are shown on the y-axis. Data represent mean ± SD of four independent experiments with cells from different donors. Significant differences are indicated in the graph as follows: *p<0.05, **p<0.01 (Student's *t*-test).

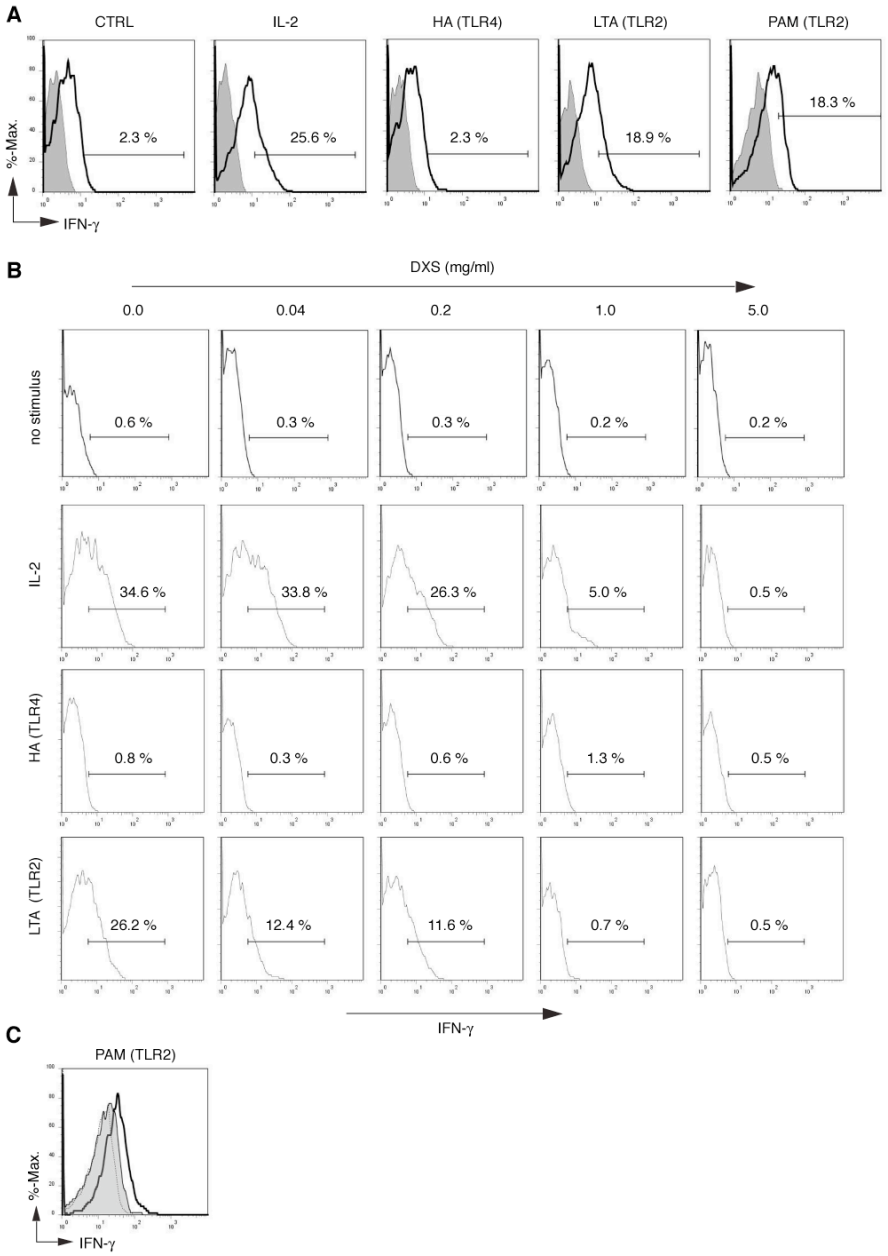

Figure 2: Stimulation of NK cells with LTA, not HA, increases the frequency of IFN-γ secreting cells. *A*, Freshly isolated NK cells were stimulated with IL-2 (400 UI/ml), LTA (5 μg/ml) or HA (20 μg/ml) for 24 hours. Afterwards, cells were harvested and analyzed by flow cytometry for the percentage of IFN-γ secreting NK cells. A representative histogram is shown out of at least 3 independent experiments with cells from different donors. *B*, DXS inhibits TLR2 induced frequency of IFN-γ producing NK cells. NK cells were incubated with indicated concentrations of DXS prior to IL-2, LTA or HA stimulation. A representative histogram is shown out of at least 2 independent experiments with cells from different donors. *C*, DXS inhibits Pam3CSK4 induced frequency of IFN-γ secreting NK. Cells were incubated with 2.5 mg/ml DXS prior to Pam3CSK4 stimulation and examined for IFN-γ secretion after 24h (grey histogram). White histogram represents stimulation with Pam3CSK4 and the dotted line is the isotype control. Displayed is a representative flow cytometry overlay of one representative donor out of 3.

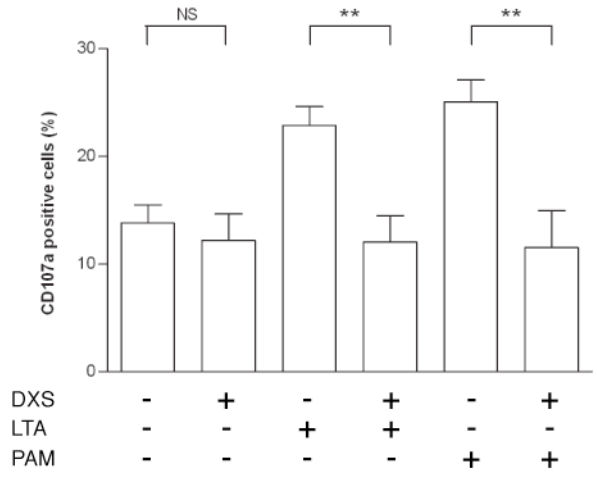

Figure 3: Stimulation of NK cells with Pam3CSK4 or LTA increases the frequency of degranulating NK cells. After stimulation, NK cells were incubated for 6 h with K562 cells. Cells were stained with isotype control or anti-CD107a mouse Ab. Plots were gated on a forward scatter/side scatter lymphocyte gate. Data represent mean ± SD of four independent experiments with cells from different donors. Significant differences are indicated in the graph as follows: **p<0.01 (Student's *t*-test).

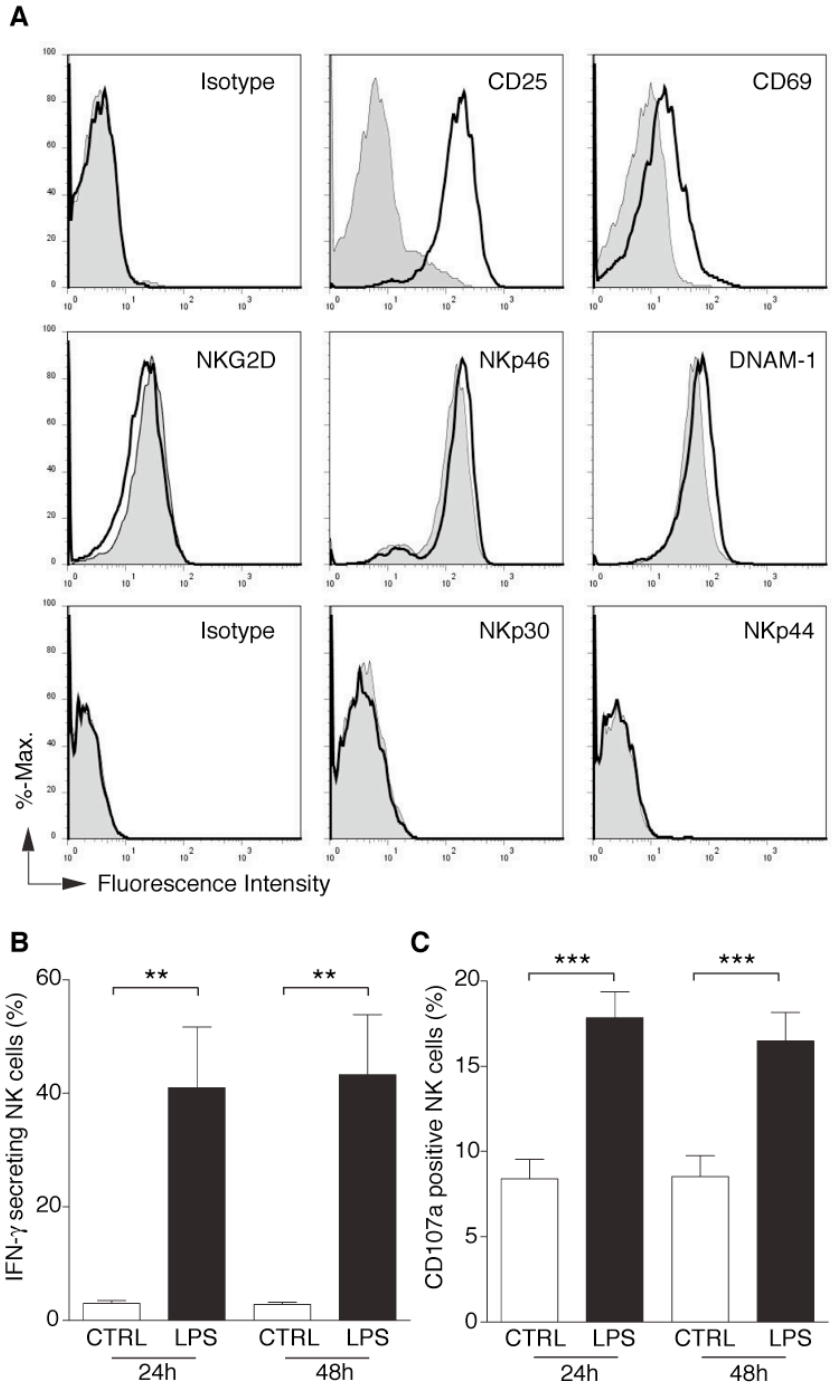

Figure 4: Characterization of phenotype and function of NK cells cultured in supernatant of LPS-activated human MoDC. *A*, Freshly isolated NK cells were incubated for 24h with supernatant of activated MoDC and then stained for the NK cell activation markers, as indicated. The histograms shown are expression levels for the indicated cell surface markers on NK. One out of four representative donors is shown. *B*, After 24h stimulation, cells were harvested and analyzed by flow cytometry for the percentage of IFN-γ secreting NK cells. Bars represent the mean frequency of IFN-γ secreting NK cells (n=4). *C*, After stimulation, NK cells were extensively incubated for 6 h with K562 cells. Cells were stained with isotype control or anti-CD107a Ab. Bars represent mean ± SD of four independent experiments with cells from different donors. Significant differences are indicated in the graph as follows: ***p<0.001 (Student's *t*-test).

Table I: Effect of MoDC-supernatant on NK cell phenotype

	Positive cells (%)		MFIR	
	DC CTRL	DC LPS	DC CTRL	DC LPS
NKG2D	85.0 ± 2.0	76.0 ± 4.0*	10.0 ± 1.0	6.7 ± 1.0**
NKp30	21.0 ± 3.0	24.0 ± 7.0	2.0 ± 0.1	2.1 ± 0.3
NKp44	2.4 ± 0.6	4.0 ± 1.0	1.1 ± 0.1	1.2 ± 0.1
NKp46	93.0 ± 1.0	93.0 ± 1.0	49.0 ± 4.0	56.0 ± 6.0
DNAM-1	98.0 ± 1.0	96.0 ± 1.0	23.0 ± 1.0	29.0 ± 2.0*
CD25	25.0 ± 3.0	96.0 ± 1.0***	2.3 ± 0.2	69.0 ± 9.0**
CD69	30.0 ± 1.0	71.0 ± 4.0**	2.1 ± 0.1	6.6 ± 1.0*

*$p<0.05$, **$p<0.01$ and ***$p<0.001$ vs. CTRL (Student's *t*-test)

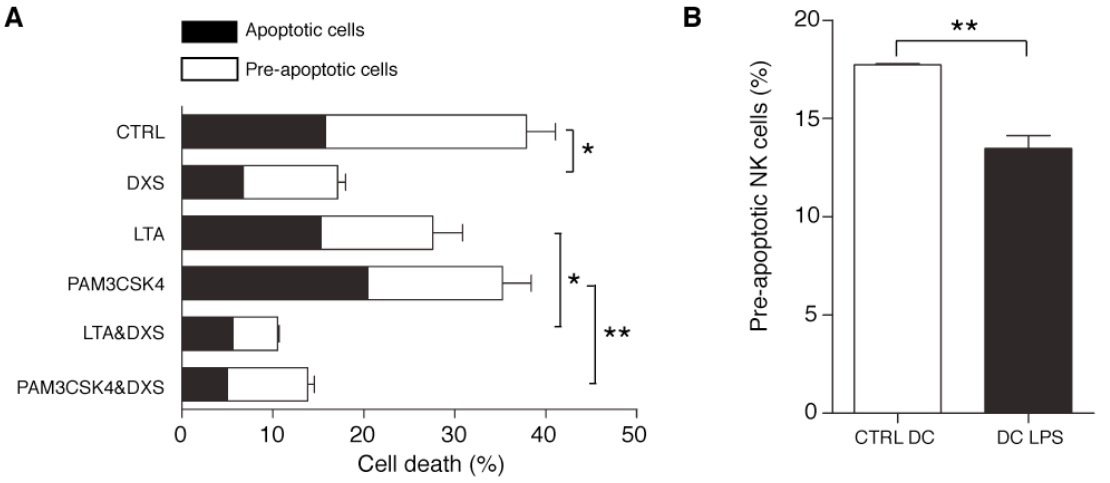

Figure 5: DXS protects NK cells from apoptosis. *A* NK cells were stimulated with Pam3CSK4 (1 μg/ml) or LTA (5 μg/ml) in the presence or absence of DXS (5 mg/ml). Bars represent mean ± SD of four independent experiments with cells from different donors. Significant differences are indicated in the graph as follows: *p<0.05, **p<0.01 (Student's *t*-test). *B*, NK cells were incubated with supernatant of activated MoDC. After 24h cells were harvested and annexin-V/PI staining was performed. The percentages of annexin-V^+, PI^- (early apoptotic) and annexin-V^+, PI^+ cells (late apoptotic) are shown. Bars represent mean ± SD of four independent experiments with cells from different donors. Significant differences are indicated in the graph as follows: **p<0.01 (Student's *t*-test).

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3-3 Paper III: Attenuation of myeloid cell inflammation by rHDL

Title: Inflammatory reactions of human myeloid innate immune cells are prevented by reconstituted high-density lipoprotein

Authors: Rolf Spirig, Alexander Schaub, Sylvia Miescher, Peter Lerch, Martin Spycher and Robert Rieben

Manuscript in preparation

Besides the cholesterol transport function, HDL is thought to have anti-inflammatory, anti-oxidative and vasoprotective activities. In this context, we hypothesized that rHDL has the capacity to attenuate activation of innate immunity by inhibiting an inflammatory response of monocytes and neutrophils in a whole blood assay and maturation of human dendritic cell *in vitro*.

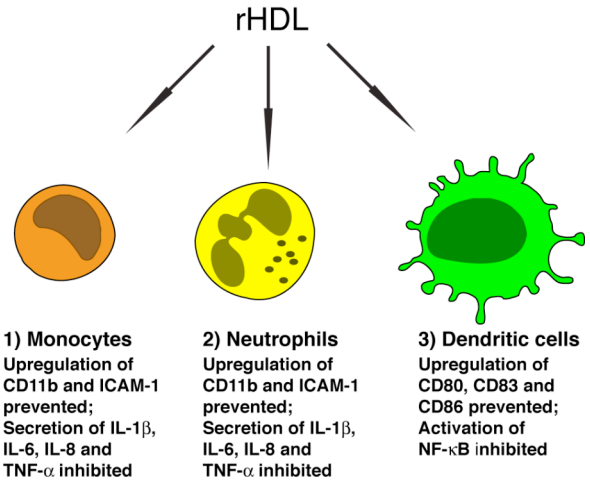

Figure 9: Influence of rHDL on activation of myeloid innate immune cells.

In this study we could show that rHDL inhibits activation of human monocytes as well as neutrophils measured by inhibition of the upregulation of CD11b and CD54. Secretion of IL-1β, IL-6, IL-8 and TNF-α was inhibited by rHDL in a dose-dependent manner. Furthermore, TLR-mediated activation of the transcription factor NF-κB was prevented. These findings suggest that rHDL prevents activation of human myeloid innate immune cells and may therefore be a useful reagent to impede the link between innate and adaptive immunity.

Inflammatory responses by human myeloid innate immune cells are inhibited by reconstituted high-density lipoprotein[1]

Running title: Inhibition of myeloid innate immune cell inflammation

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Abstract

An anti-inflammatory effects of reconstituted High Density Lipoprotein (rHDL) have been demonstrated in atherosclerosis and sepsis. An increase of adhesion molecules as well as tissue factor expression on endothelial cells in response to inflammatory or danger signals is attenuated by the treatment with rHDL. Danger signals are rapidly released under conditions of inflammation and tissue injury and induce activation of neutrophils, monocytes as well as dendritic cells (DC) through pattern recognition receptors as the toll-like receptors (TLR). Here we show the inhibitory effect of rHDL on the activation of human myeloid innate immune cells in a whole blood assay as well as on monocyte-derived human DC. Phenotypic activation of neutrophils and monocytes is prevented by rHDL by inhibiting the up-regulation of CD11b and ICAM-1. Secretion of IL-1β, IL-6, IL-8 and TNF-α was inhibited and we could observe a decrease of phagocytic activity of rHDL treated monocytes. In addition, we found a strong inhibitory effect on TLR2- and TLR4-mediated maturation of DC. Treatment of DC with rHDL prevented the up-regulation of CD80, CD83 and CD86 and it inhibited the TLR-driven activation of NF-κB. These findings suggest that rHDL prevents activation of crucial cellular players of innate immunity and could therefore be a useful reagent to impede cellular inflammation as well as the link between innate and adaptive immunity.

Introduction

A beneficial effect of treatment with reconstituted High Density Lipoprotein (rHDL), containing apolipoprotein A-I and phosphatidylcholine (PS), was described in arteriosclerosis, myocardial infarction, stroke and endotoxemia. Protective properties of rHDL on the endothelium have been described to be mediated by inhibition of up-regulation of inflammatory adhesion molecules like ICAM-1 (CD54), VCAM-1 (CD106) and E-selectin (CD62E) on endothelial cells (EC) (1) as well as reduced thrombin induced tissue-factor (TF) expression (2). A recently performed study in humans showed that rHDL reduces plasma levels of TNF-α and expression of CD11b on monocytes (3). Protection against cardiac ischemia/reperfusion (I/R) injury was demonstrated by a reduced cardiac content of TNF-α and enhanced secretion of prostaglandin in a Langendorff perfusion model (4). In myocardial infarction in rats, infusion of rHDL showed an increased phosphorylation of the MAP kinase family member extracellular-signal-related kinase (ERK) (5). The effect of rHDL in sepsis was demonstrated via binding and neutralizing lipopolysaccharide (LPS) and reduction of CD14 expression on monocytes (6). Taken together, rHDL can be described as a substance which attenuates the pro-inflammatory effects of many mediators of innate immunity. We hypothesized, therefore, that rHDL might influence inflammatory responses of important cellular players of innate immunity, namely neutrophils and monocytes, as well as professional antigen presenting cells (APC), which are crucially involved in linking the innate with the adaptive immune system.

Neutrophils are the most abundant leukocytes in the body and provide a crucial first line of defense against infections (7). On the other hand, activated neutrophils and their products like proteolytic enzymes as well as oxygen free radicals are implicated in increasing myocardial damage after infarction (8). A recent study also demonstrated that neutrophils are involved in graft rejection (9). Interestingly, neutrophils interact with dendritic cells (DC) via CEACAM-1 (CD66a), CD11b and DC-SIGN (CD209) inducing the maturation of DC (10).

Monocytes are a major myeloid cell type in the bloodstream. During conditions of inflammation, monocytes can leave the bloodstream and migrate into the inflamed tissue and differentiate either into macrophages or DC (11). Monocytes have been described to have antigen-presenting activity (12), as well as being major contributors

to cellular inflammation in arteriosclerosis (13). Early infiltration and activation of monocytes has been suggested to crucially contribute to I/R injury (14, 15).

DC are the very potent APC and pivotal for the initiation of T-cell mediated immune responses, as seen for example in allograft rejection as well as in tolerance induction (16). DC express pattern recognition receptors as e.g. members of the Toll-like receptor (TLR) family, which distinguish between conditions of tissue well-being and disease (17). Activating signals such as pathogenic molecules derived from microorganisms, e.g. lipopolysaccharide (LPS), as well as endogenous ones like hyaluronic acid (HA) have considerable effects on DC phenotype and function.

Healthy EC are covered by a layer of glycosaminoglycans like heparan sulfate (HS) or hyaluronic acid (HA), which are crucial for the anticoagulant and anti-inflammatory properties of the endothelium. HS and HA are rapidly released under conditions of inflammation and tissue damage (18-21). It has been shown that soluble HS or HA induce maturation of DC via TLR4 (17, 22). The transcription factor NF-κB is crucial for the upregulation of surface marker molecules and cytokine production during DC maturation. Recent studies have highlighted the involvement of TLR2 and TLR4 in the early inflammatory process of I/R injury *in vivo* (23, 24).

In this study, we show that rHDL interferes with the activation of human neutrophils, monocytes and MoDC at multiple levels by reducing immunostimulatory properties, secretion of proinflammatory cytokines and receptor mediated endocytosis. Furthermore, rHDL inhibits TLR induced activation of the transcription factor NF-κB.

Material and Methods

Stimulation of leukocytes in whole blood

Heparinized whole blood from healthy volunteers was collected into pyrogen-free tubes, to which 10 μg/ml phytohemagglutinin-M (PHA) was added for leukocyte stimulation. Subsequently, rHDL, apo-AI or phosphatidylcholine (PC) was added to the whole blood at concentrations ranging from 4 to 1000 μg/ml and incubated overnight at 37°C, 5% CO_2 in a humidified atmosphere. The following day all manipulations were performed at 4°C or on ice. The cells were directly stained with antibodies specific for CD14, CD54 and CD45 (all Becton Dickinson AG, Allschwil, Switzerland) 30 min on ice. Red blood cells (RBC) were lysed by a 30 min incubation

with EC Lysis Buffer (Qiagen AG, Basel, Switzerland) and gentle mixing. With the majority of RBC lysed, the tubes were centrifuged (1500 rpm, 10 min, 4°C) and resuspended in 300 µl PBS. Data acquisition and analysis was performed on a FACSCalibur flow cytometer employing the CellQuest Pro software (both BD Biosciences AG).

For routine analysis the monocyte, neutrophil and lymphocyte signals were separated from the RBC by gating on the pan-leukocyte surface marker CD45. Further subdivisions were made by granularity and CD14 expression, allowing for the distinction between monocytes, neutrophils and lymphocytes. This permitted the differential assessment of CD54 and CD11b expression on these cell populations (all antibodies by BD Biosciences).

Measurement of IL-1β, IL-6, IL-8 and TNF-α

For the analysis of cytokine production, supernatants consisting of human serum were harvested after overnight stimulation as described above. The cytokine levels in these supernatants were measured on the FACSCalibur flow cytometer by using the Cytometric Bead Array for human inflammatory cytokines (Cat. 551811, BD Biosciences AG, Allschwil, Switzerland) according to manufacturers instructions. Results are expressed as the mean of two measurements ± standard deviation of a representative experiment.

Measurement of phagocytic activity by the uptake of FITC-Dextran

For the analysis of phagocytic activity, whole blood samples were incubated with FITC-Dextran (MW 40'000, Invitrogen AG, Basel Switzerland) for 1 hour at 37°C. Monocytes were gated by staining with antibodies against CD45 and CD14 (both BD Biosciences AG). As negative control, whole blood was pre-cooled prior to the incubation with FITC-Dextran at 4°C for 1 hour. The samples were analyzed by flow cytometry (FACSCalibur, BD Biosciences AG).

Generation and stimulation of human monocyte-derived DC (MoDC)

Human peripheral blood mononuclear cells (PBMC) were isolated from buffy coats obtained from healthy blood donors (Regional Red Cross Blood Donation Center, Bern, Switzerland) by density gradient centrifugation over Ficoll-Paque (Amersham,

Uppsala, Sweden). Monocytes were isolated from PBMC as described recently (25-27) by spontaneous aggregation and rosetting (28). The purified monocytes were incubated for 6 days in RPMI 1640 medium (Invitrogen Life Technologies) containing 10% fetal calf serum (FCS; Amimed / BioConcept), 1% [2mM] L-Glutamine (Invitrogen), 1% [100 U/ml] Penicillin/Streptomycin (Invitrogen), 10 ng/ml GM-CSF (R&D Systems Europe Ltd, Abingdon, Oxon, UK), and 10 ng/ml IL-4 (R&D) to generate MoDC as described initially by Sallusto and Lanzavecchia (29). The cells were kept at 37°C in a 5% CO_2 humidified atmosphere. On day 3, the culture medium was replaced with fresh medium. For induction of maturation 100 ng/ml LPS (Sigma), 5 μg/ml lipoteichoic acid (LTA, Sigma) or 20 μg/ml HA (Sigma) were added to the cultures for the indicated time periods.

FACS analysis and cell viability

Cells were incubated with FITC-labeled monoclonal antibody (mAb) against CD80, CD83 and CD86 (BD, Franklin Lakes, NJ, USA) or Isotype Control IgG1 (BD).

For determination of viability, the cells were stained with 5 μg/ml of propidium iodide (PI; Invitrogen) and analyzed by flow cytometry. As positive control, cells were treated with PBS containing 0.1 % BSA (Sigma) and 0.1 % saponin (Sigma) and then stained with PI. Measurements were performed with a BD FACScan flow cytometer and the obtained data were analyzed using FlowJo (Tree Star Inc., Ashland, OR, USA).

Detection of NF-κB activation by a transcription factor ELISA

The production of NF-κB p65 was measured with an NF-κB assay kit (Active Motif, Rixensart, Belgium) according the manufacturer's instructions. In brief, cell extract (10 μg of total protein) of LTA activated DC, with or without additional pretreatment by rHDL (40 μg/ml), was added to each well coated with consensus-binding site oligonucleotides of NF-κB p65. A primary antibody specific for an epitope on the bound and active form of the transcription factor was then added, followed by subsequent incubation with secondary HRP-conjugated antibody.

Statistical analysis

Data are presented as mean ± standard deviation representing experiments with up to 5 different donors. Unpaired Students *t-tests* were performed for evaluation of significance. Differences were considered statistically significant at p-values less than 0.05. Data were analyzed using GraphPad Prism software 4.0 (GraphPad, San Diego, CA).

Results

Upregulation of CD11b and ICAM-1 on monocytes and neutrophils in whole blood is inhibited in a dose- and time-dependent manner by rHDL

Heparinized whole blood was stimulated with PHA and rHDL was co-incubated with the stimulated cells at concentrations ranging from 4 to 1000 µg/ml. Monocytes and neutrophils were analyzed for the expression of CD11b (CR3, Mac-1 or α_M) and ICAM-1 (CD54) as markers of cellular activation. As shown in Fig. 1, incubation with rHDL dose-dependently inhibited the up-regulation of CD11b and ICAM-1 on human monocytes (Fig. 1*A*) and neutrophils (Fig 1*B*). Near-total inhibition was achieved by adding rHDL at a concentration of 1 mg/ml for both cell types and activation markers.

PHA stimulation increases CD11b in a time-dependent manner, with a peak at around 8 hours, followed by a decrease in expression. For ICAM-1, maximum up-regulation was observed 24 hours after stimulation for monocytes and neutrophils. In both cases, treatment with rHDL reduced the up-regulation of both activation markers (data not shown).

Apo-AI or phosphatidylcholine alone are less potent inhibitors of primary human neutrophil stimulation by PHA

As rHDL consists of Apo-AI and phosphatidylcholine (PC), we have investigated, if either Apo-AI or PC alone were able to exert the same anti-inflammatory effect as the whole rHDL particle. Apo-AI and PC were added to the cells at the molarity present in the intact rHDL particle. Whereas PC alone had only minor inhibitory effects at the highest concentration, Apo-AI did not inhibit the activation of the primary human neutrophils at all, as measured by the upregulation of CD11b and CD54 (Fig. 2). This

would suggest that the presence of intact rHDL particles is required for mediating efficient anti-inflammatory activity in this experimental setting.

Secretion of proinflammatory cytokines is prevented by rHDL in whole blood

Over-night stimulation of whole blood with PHA (10μg/ml) led to a considerable secretion of IL-1β, IL-6, IL-8 and TNF-α (Fig. 3). However, co-incubation with rHDL drastically reduced PHA-induced production of these proinflammatory and chemoattractant mediators. Already at an rHDL concentration of 0.2 mg/ml we observed a considerably reduced secretion of IL-1β, IL-6 and TNF-α. Cytokine secretions were not altered after treatment with rHDL alone.

rHDL influences the phagocytic activity of monocytes in whole blood

PHA-mediated activation increased the phagocytic activity of monocytes as measured by FITC-dextran uptake. As shown in Fig. 4, a decrease of FITC-dextran uptake was observed with increasing concentrations of rHDL.

Phenotypic maturation induced by endogenous as well as exogenous TLR4 agonists of human MoDC is prevented by rHDL

It has been described, that LPS derived from gram-negative rod *E. coli* induce maturation of human MoDC via TLR4, inducing the formation of a TLR4-signalingcomplex containing MD-2, CD14 and TLR4. HA has been described as endogenous TLR4 agonist, inducing the formation of a unique TLR4 complex consisting of MD-2, CD44 and TLR4 (30). MoDC were pre-incubated with different concentrations of rHDL for 30 minutes, followed by induction of maturation by LPS or HA. As shown in Fig. 5, rHDL dose-dependently inhibited TLR4-induced phenotypic MoDC maturation measured by the upregulation of CD80, CD83 and CD86. A significant inhibitory effect of rHDL on HA-induced maturation was already observed at an rHDL concentration of 40 μg/ml.

rHDL has an inhibitory effect on TLR2 induced maturation of human MoDC

LTA from gram-positive cocci *S. aureus* has been demonstrated to induce signaling through TLR2 (31). LTA-activated MoDC were incubated with the same concentrations of rHDL (0.04, 0.2 and 1.0 mg/ml) as for TLR4 stimulation. Even at

40 μg/ml we could observe an almost complete inhibition of maturation (data not shown). As a consequence of this observation, we used lower concentrations of rHDL (1.6, 8.0 and 40 μg/ml) for similar experiments. As shown in Fig. 6*A* and *B*, at concentration of 8 μg/ml we could observe a significant inhibitory effect of rHDL on LTA induced up-regulation of CD80, CD83 and CD86 on human MoDC.

Activation of NFκB in response to LTA is prevented by rHDL

To assess the effects of rHDL-mediated inhibition of maturation of MoDC on intracellular signaling, we determined the activation status of NF-κB, the essential transcription factor for DC maturation and function. The phosphorylation of IκB-α leads to its ubiquitylation and subsequent degradation, which results in a release of NF-κB (32). As shown by transcription factor ELISA, treatment of MoDC with LTA for one hour led to activation and translocation of NF-κB p65 into the nucleus, whereas pretreatment of the cells with rHDL caused a strong abrogation of this LTA-induced activation of NF-κB (Fig. 7).

rHDL does not affect viability of human MoDC

To investigate how the used concentrations of rHDL influence the viability of MoDC, we monitored MoDC survival at different rHDL concentrations. MoDC were incubated for 24 hours with different concentrations of rHDL, stained with PI and immediately analyzed by FACS. We found that rHDL concentrations of up to 1 mg/ml, either alone or in combination with LPS, LTA or HA, did not affect viability of MoDC within 24 hours of exposure (Fig. 8).

Kinetic analysis of the effect of rHDL on the up-regulation of CD86

To assess the kinetics of rHDL-mediated inhibition of MoDC maturation, cells were incubated with rHDL at different periods prior to or after stimulation with LTA. As shown in Fig. 9, only pretreatment of the cells or co-stimulation with rHDL was able to prevent up-regulation of CD86.

Discussion

The present study is the first to demonstrate inhibition of human MoDC maturation by TLR agonists as well as inhibition of neutrophil and monocyte activation in a human

whole blood assay. Pretreatment by rHDL, followed by co-incubation with the TLR agonists, significantly inhibited up-regulation of the essential co-stimulatory molecules CD80, CD86 and CD83 on human MoDC. Furthermore, rHDL attenuated secretion of proinflammatory cytokines IL-1β, IL-6, IL-8 and TNF-α in the human whole blood assay. In parallel, up-regulation of CD11b and ICAM-1 on monocytes as well as on neutrophils was inhibited by rHDL.

Several reports suggest that maintaining DC in an immature or semi-mature state (33), or activating them in an alternative manner (34), can be effective in preventing allograft rejection and favor the induction of tolerance. Activation of vascular EC, as occurring in organ transplantation for example due to I/R injury of the graft, leads to shedding of glycosaminoglycans like HA or HS (19, 35). It has been shown that soluble HA serves as a danger signal, which is able to induce maturation of DC via TLR4 (22). TLR4 therefore plays an important role in I/R injury (24, 36, 37) and in many stages of graft rejection (38). Other reports also demonstrate an involvement of TLR2 in I/R injury (23) and TLR3 as sensor of necrotic cells (39).

The inhibitory effect of rHDL was observed for TLR4- (HA, LPS) as well as TLR2-mediated stimulation (LTA). Treatment of DC with rHDL prevents HA-, LPS- and LTA induced up-regulation of the costimulatory molecules CD80, CD86 and CD83. As rHDL has been reported to bind and neutralize LPS (6), the effect on LPS induced maturation of MoDC was expected. Interestingly, we observed a much more potent inhibitory effect of rHDL on HA-induced maturation. Already a concentration of 40 μg/ml was sufficient to significantly prevent phenotypic maturation of MoDC. It is as yet unclear whether rHDL binds and neutralizes the endogenous danger signal HA and further studies are warranted to answer this question.

Given the importance of TLR2 in I/R injury, we have evaluated the effect of rHDL on TLR2 induced activation. LTA from *S. aureus* has been published to signal via TLR2 (31) and accessory molecules as e.g. CD14 facilitate stimulation (40). Signaling pathways that trigger TLR2 are dependent on Toll-IL-1 receptor (TIR) domain-containing adaptor protein (TIRAP) and MyD88 to finally activate NF-κB and the MAP kinase pathways (32). Moreover, selective inhibition of NF-κB has been shown to be associated with tolerogenic properties of treated DC (41-43) Here we provide evidence that rHDL significantly inhibited TLR2-induced NF-κB activation, which could account for its major impact on MoDC maturation. Given the lipid-binding

capacity of rHDL, it is possible, that rHDL binds and neutralizes LTA, which could be favorable in a setting of gram-positive mediated sepsis.

We could demonstrate, that rHDL inhibited up-regulation of the integrin CD11b, which is a crucial adhesion molecule as well as receptor for complement proteins C3b and iC3b, expressed on human monocytes and neutrophils. Endothelial ligands for CD11b are ICAM-1, fibrinogen, CD154 and glycosaminoglycans. CD11b deficiency has been shown to reduce intragraft neutrophil accumulation and to prolong cardiac graft survival (44). Expression of ICAM-1 is necessary for monocyte and neutrophil transmigration (45). Furthermore, secretion of predominant proinflammatory cytokines IL-1β, IL-6 and TNF-α are dose-dependently inhibited by rHDL. Both, IL-1β and TNF-α, are potent activators of EC, macrophages and DC. IL-6 is a crucial cytokine inducing production of acute phase proteins in the liver. Interestingly, a recent study by Pasare *et al.* showed that IL-6 suppresses the inhibitory activity of regulatory T cells (Tregs) (46). Moreover, the production of the chemokine IL-8 (CXCL-8) was significantly inhibited by rHDL. IL-8 acts as a very potent chemoattractant for neutrophils (47).

In addition, it should be noted that the used concentrations of rHDL did not affect viability of the cells, which is consistent with previously published *in vitro* and *in vivo* observations. The used concentrations (up to 1 mg/ml, with already significant effects with 8 μg/ml for LTA and 40 μg/ml for HA) for inhibition of maturation of human MoDC are even much below the used concentrations of 80 mg/kg in human patients (3). The *in vivo* concentrations of rHDL required for complete or partial inhibition of DC maturation need to be elucidated in appropriate animal models.

In conclusion, our data suggest a new immunomodulatory function and anti-inflammatory effect of rHDL. This compound might therefore be useful as a therapeutic reagent to impede early cellular inflammation as well as the link between innate and adaptive immunity.

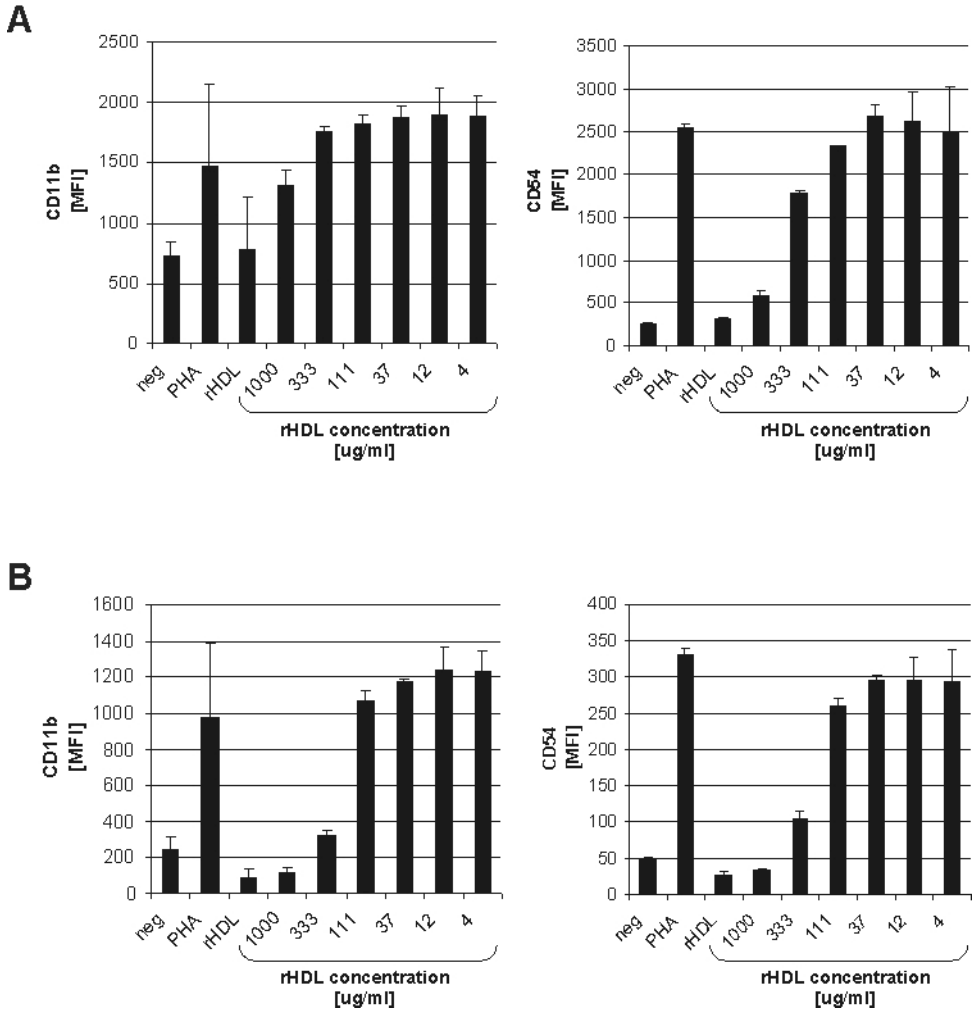

Figure 1: Stimulation by PHA is dose-dependently inhibited by coincubation with rHDL. Surface expression of CR3 (CD11b) and ICAM-1 (CD54) was measured on primary human monocytes *A* and neutrophils *B* after over night culture. The panels show cells without stimulation (neg), with PHA-stimulation (PHA), rHDL alone (rHDL) and PHA/rHDL coincubation, where the rHDL was added at different concentrations. The shown mean fluorescent intensity values ± SD are derived from duplicate overnight cell cultures of a representative experiment performed multiple times.

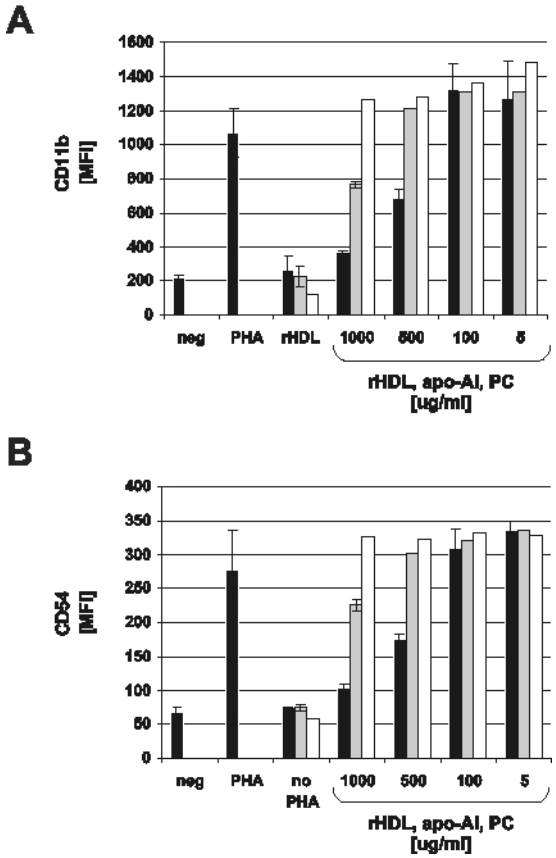

Figure 2: PHA-induced stimulation of neutrophils is only inhibited by the intact rHDL particle, but not Apo-AI or PC alone. The panel shows surface expression of CD11b *A* and CD54 *B* after overnight incubation without stimulation (neg), with PHA-stimulation (PHA), with rHDL alone (rHDL) and co-incubations of PHA with rHDL (black), Apo-AI (white) and PC (grey) at different concentrations. Apo-AI and PC were used at equimolar quantities as compared to the amount contained in rHDL at the concentrations given in the results. The mean fluorescent intensity values ± SD shown here are derived from duplicate overnight cell cultures.

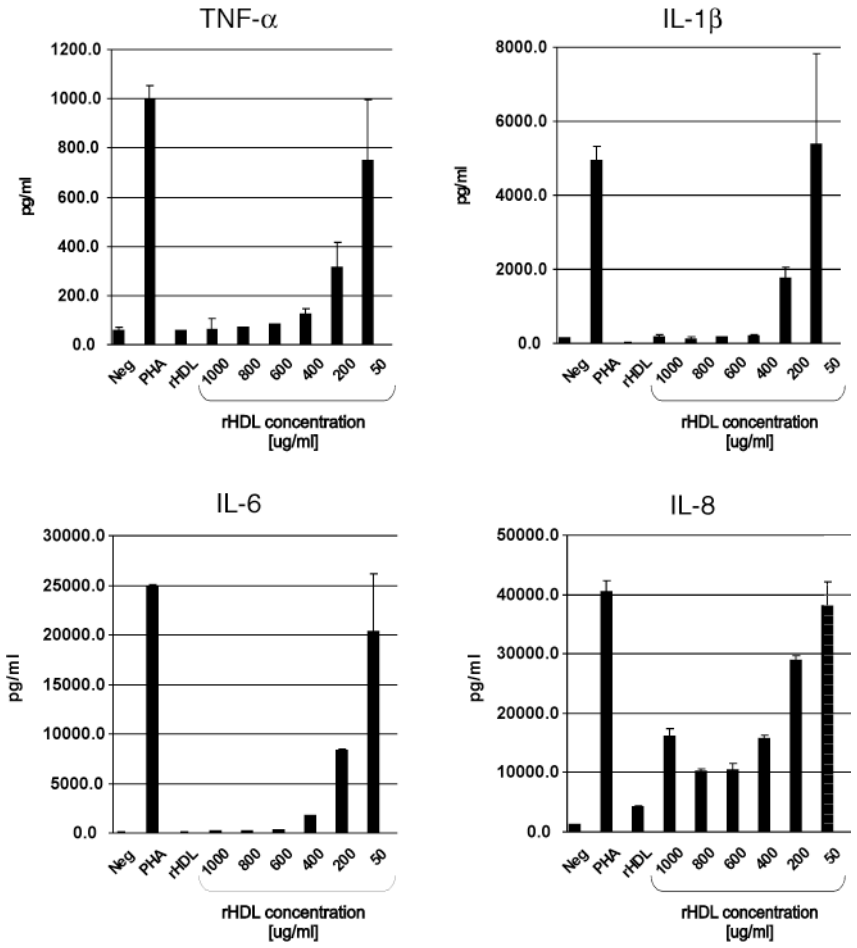

Figure 3: rHDL can dose-dependently suppress the production of proinflammatory (TNFα, IL-1β, IL-6) and chemoattractant (IL-8) cytokines in a whole blood assay setting. Cytokine levels were measured in the supernatant (serum) of overnight stimulation experiments by a bead-based flow-cytometry method. The results reflect the mean ± SD of two overnight cultures derived from a representative experiment.

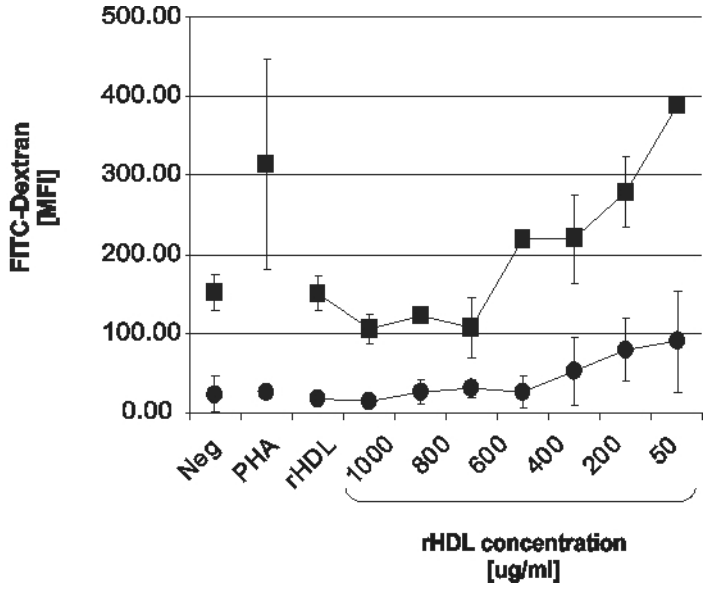

Figure 4: Phagocytic activity of primary human monocytes after stimulation is reduced in response to rHDL co-incubation. The panel shows FITC-uptake after overnight incubation without stimulation (neg), with PHA-stimulation (PHA), with rHDL alone (rHDL) and co-incubations of PHA with rHDL. Following o/n culture the whole blood samples were exposed to FITC-Dextran either at 37°C (squares) or 4°C (circles) for 1h prior to assessment of phagocytic capacity by flow cytometry. The results reflect the mean ± SD of two o/n cultures derived from a representative experiment.

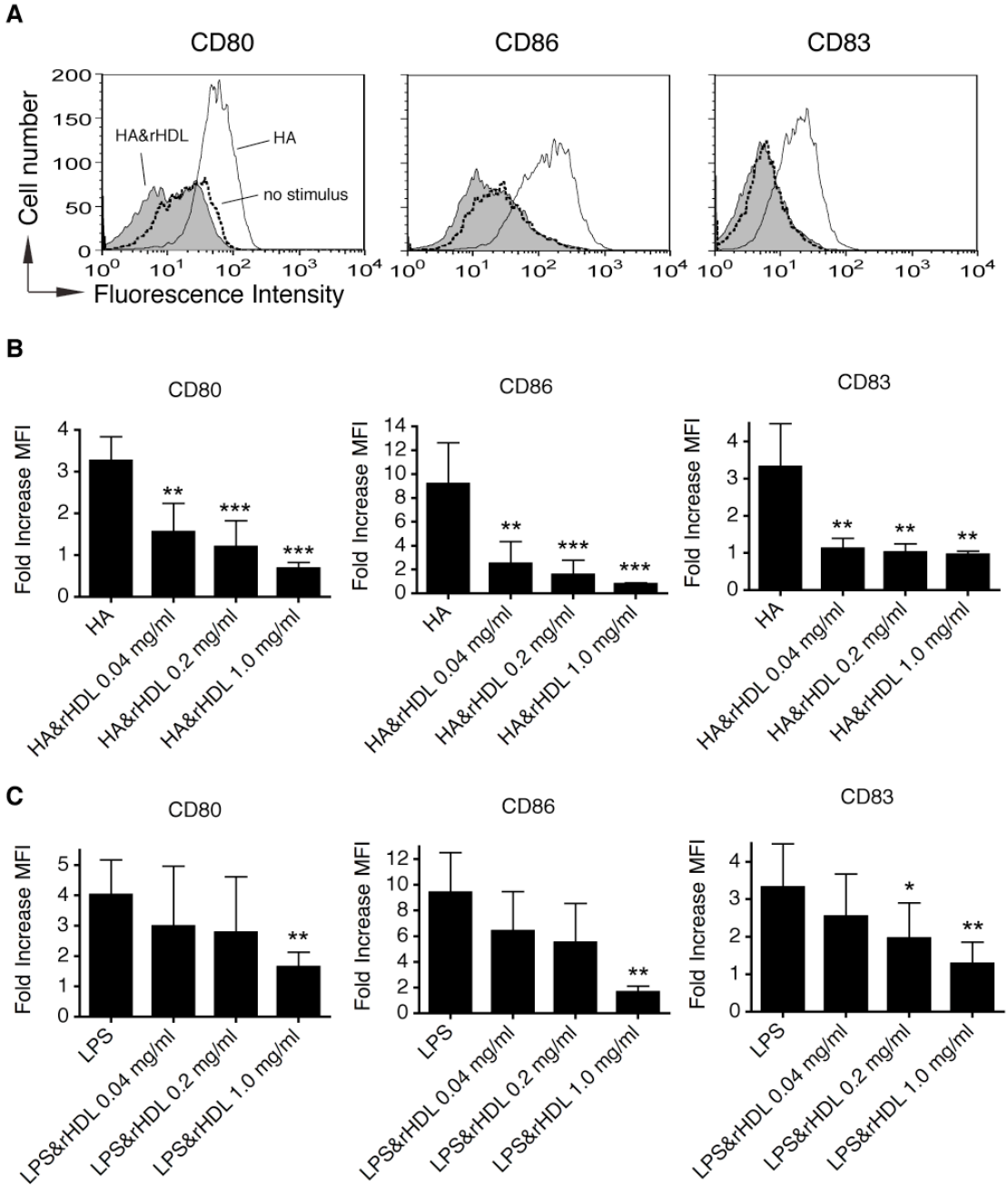

Figure 5: rHDL prevents phenotypic maturation of human MoDC in response to HA and LPS in a dose dependent manner. *A*, Monocytes isolated from buffy coat were cultured in presence of GM-CSF and IL-4 for 6 days. rHDL dose-dependently prevents HA-induced (20 μg/ml) MoDC maturation. Histograms show the typical expression profiles of CD80, CD86 and CD83. Data are representative of 5 independent experiments with cells of different donors. Grey histograms show the typical expression profiles of the indicated surface molecules for rHDL plus HA treated MoDC. White: HA only treated cells; Dotted line: no stimulus. *B*, To compare the levels of inhibition of up-regulation of the indicated surface molecules, the median fluorescence intensity (MFI) ratios were calculated by dividing the median fluorescence of HS- and/or rHDL-treated MoDC by the median fluorescence of immature MoDC and indicated as fold increase in the MFI. Mean values ± SD are shown as column graphs (n=5). *p<0.05; **p<0.01; ***p<0.001 vs. mature MoDC (unpaired Student's *t*-test).

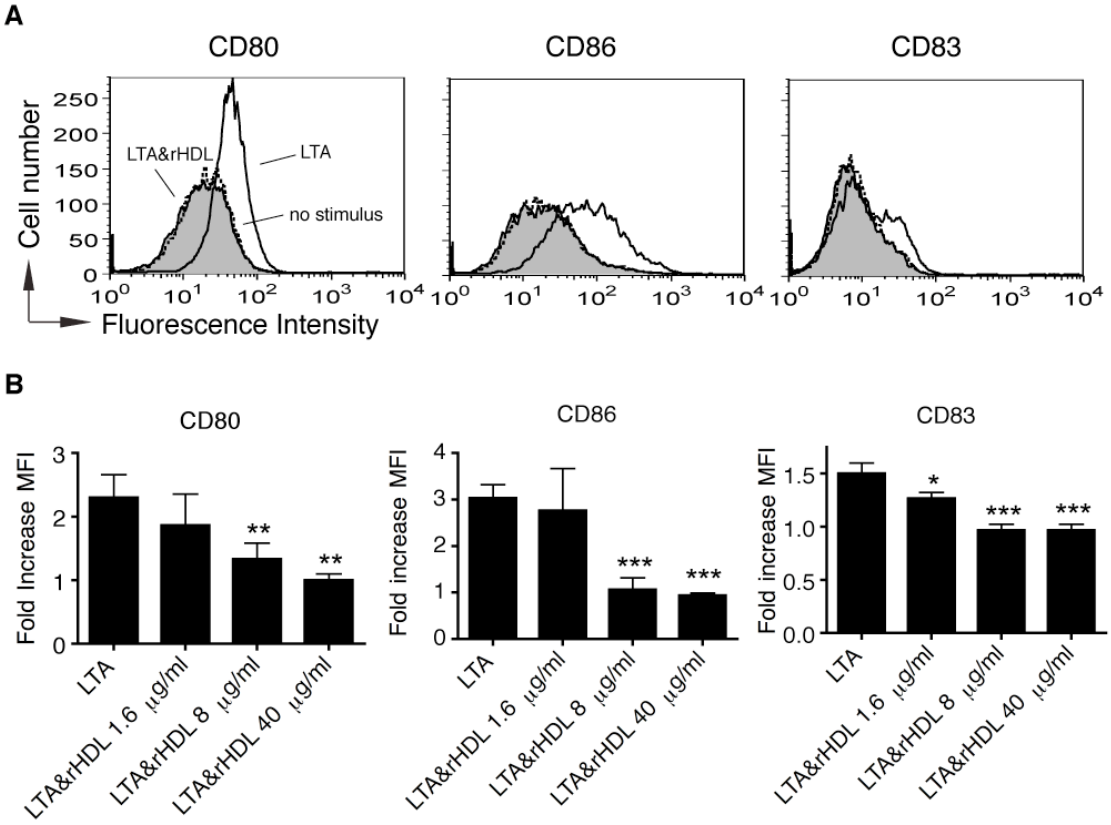

Figure 6: rHDL prevents phenotypic maturation of human MoDC in response to LTA in a dose dependent manner. *A*, rHDL dose-dependently prevents LTA-induced (5 μg/ml) MoDC maturation. Histograms show the typical expression profiles of CD80, CD86 and CD83. Data are representative of 3 independent experiments with cells of different donors. Grey histograms show the typical expression profiles of the indicated surface molecules for rHDL plus LTA treated MoDC. White: LTA only treated cells; Dotted line: no stimulus. *B*, To compare the levels of inhibition of up-regulation of the indicated surface molecules, the median fluorescence intensity (MFI) ratios were calculated by dividing the median fluorescence of HS- and/or rHDL-treated MoDC by the median fluorescence of immature MoDC and indicated as fold increase in the MFI. Mean values ± SD are shown as column graphs (n=3). *p<0.05; **p<0.01; ***p<0.001 vs. mature MoDC (unpaired Student's *t*-test).

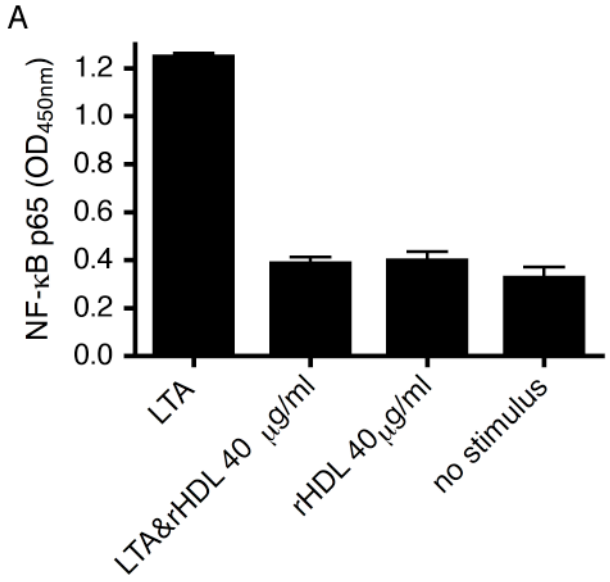

Figure 7: LTA induced activation of NF-κB is inhibited by rHDL. NF-κB activation was measured in cell extracts at 1 hour after LTA induced activation by a transcription factor ELISA. Cells were preincubated 30 min before LTA stimulation with rHDL (40 μg/ml). A representative experiment performed in duplicates from one donor out of three is shown. The bars represent mean ± SD.

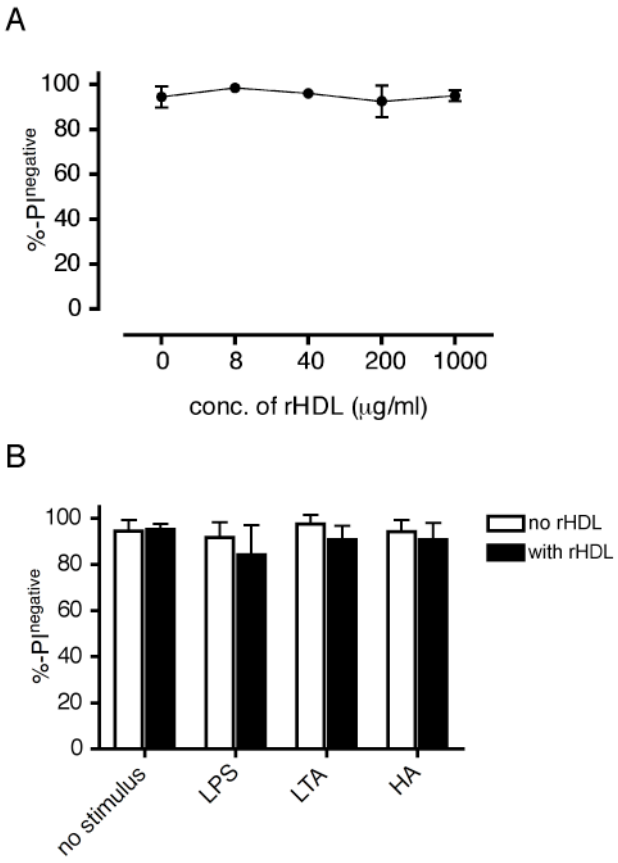

Figure 8: *A*, MoDC were treated with indicated concentrations of rHDL. Thereafter, cells were harvested and stained with PI (5 μg/ml) and analyzed by flow cytometry. Shown are mean ± SD of three independent experiments with cells of different donors. *B*, MoDC were treated with LPS (100 ng/ml), HA (20 μg/ml) or LTA alone (5 μg/ml), or together with rHDL (1 mg/ml). The bars represent mean ± SD of three independent experiments with cells of different donors.

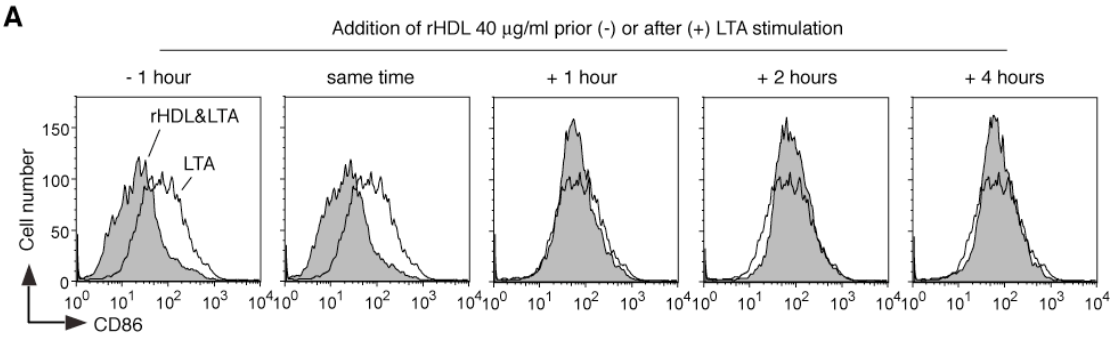

Figure 9: Effect of rHDL on MoDC maturation given prior to or after TLR stimulation. rHDL (40 µg/ml) was given to the cells at the indicated time point prior to or after stimulation with LTA (5 µg/ml) for 24 hours. The cells were then evaluated for the expression of CD86 by flow cytometry. The shown results are from one donor and representative of two independent experiments with cells from different donors.

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3-4 Paper IV: A role of hypoxia-inducible factor 1α in maturation of human DC

Title: Selective TLR agonists induce activation of HIF-1α in human dendritic cells in the absence of hypoxia

Authors: Rolf Spirig, Siamak Djafarzadeh, Tomas Regueira, Sidney Shaw, Jukka Takala, Stephan M. Jakob, Robert Rieben and Philipp M. Lepper

Manuscript submitted for publication

The transcription factor hypoxia-inducible factor 1α (HIF-1α) has recently been shown to be induced by LPS even under normoxic conditions in murine and human macrophages and to enhance the inflammatory reaction. We hypothesize, that TLR stimulation of human DC leads to the functional expression of HIF-1α.

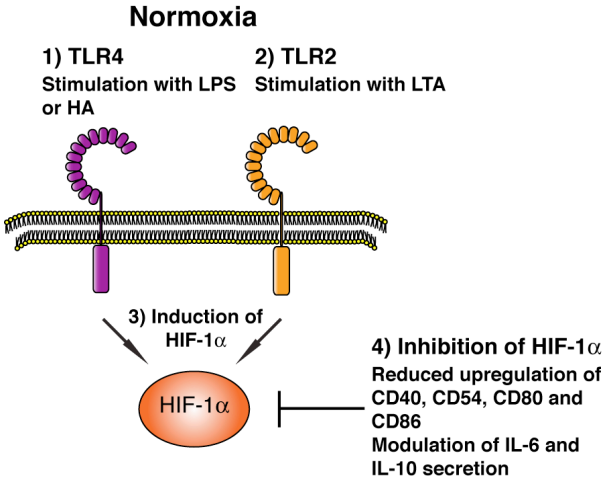

Figure 10: Stimulation of human DC with TLR agonists induce the expression of HIF-1α. Inhibition of this transcription factor inhibited maturation of DC.

In this study we could show, that TLR4 and TLR2 agonists induce the expression of the transcription factor HIF-1α under normoxic conditions in human DC. Phenotypic maturation of DC in response to LPS was amplified under hypoxic conditions. Inhibition of HIF-1α by chemical agents resulted in reduced phenotypic maturation. Secretion of VEGF is reduced as well as IL-6 and IL-10 production. No significant effect on TNF-α and IL-1β secretion was observed. Therefore, HIF-1α might represent a novel target for inhibition of DC maturation.

Selective TLR agonists induce activation of HIF-1α in human dendritic cells in the absence of hypoxia

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Keywords: human, dendritic cells, transcription factors, cell activation, inflammation

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Abbreviations: MoDC: Monocyte-derived DC
HIF-1α: hypoxia-inducible factor 1α
CTM: chetomin

[*]These authors contributed equally to this work

Abstract

Dendritic cells (DC) are professional antigen presenting cells that represent an important link between innate and adaptive immunity. Danger signals such as toll-like receptor (TLR) agonists induce maturation of DC leading to a T-cell mediated adaptive immune response.

In this study, we show that exogenous as well as endogenous inflammatory stimuli for TLR4 and TLR2 induce the expression of HIF-1α in human monocyte-derived DC, suggesting a functional TLR-HIF pathway under normoxic conditions. Inhibition of HIF-1α by the antagonist chetomin prevented phenotypic maturation of human DC mediated by pro-inflammatory stimuli as shown by a reduced up-regulation of CD40, CD80, CD86 and ICAM-1. On the functional level, inhibition of HIF-1α was associated with a reduced secretion of IL-6 and IL-10, whereas TNF-α and IL-1β were not significantly affected. Induction of HIF-1α by hypoxia or $CoCl_2$ did not result in maturation of human DC. However, phenotypic maturation of DC induced by LPS was amplified under hypoxic conditions. In addition, we could show that TLR stimulation resulted in an increase of VEGF secretion. These results suggest that the transcription factor HIF-1α plays a crucial role in TLR-mediated activation of human DC. These results demonstrate for the first time that HIF-1α can be induced in human DC under normoxic conditions in a time-dependent manner by both endogenous and exogenous TLR2- and TLR4 agonists leading to DC maturation.

Introduction

DC are a unique leukocyte population of professional antigen presenting cells (APC) that play an important role in bridging innate and adaptive immunity [1]. They are crucial for inducing T-cell mediated immune responses, as seen in infection, allograft rejection, as well as in the induction of peripheral tolerance [2, 3]. DC continuously scan their environment. For this purpose, they express pattern recognition receptors (PRR) including Toll-like receptors (TLR), nucleotide-binding oligomerization domain (NOD)-like receptors, C-type lectin receptors and others. Activating signals such as pathogen-derived molecules, e.g. LPS or lipoteichoic acid (LTA), induce maturation of the cells. Recent studies have shown that some of these PRR can also sense the presence of endogenous molecules, which are released in the context of tissue injury [4]. Ischemia/reperfusion (I/R) results in shedding and degradation of the glycosaminoglycans heparan sulfate (HS) [5-8] and hyaluronic acid (HA) [9, 10] from the endothelial cell surface. It has been shown that HS and HA induce maturation of DC via TLR4 *in vitro* [11, 12]. Further, it has been demonstrated that TLR2 and TLR4 are crucially involved in I/R injury *in vivo* [13-17]. Additionally, maturation of DC is influenced by their microenvironment. Recent evidence suggests, that Toll-like receptor ligation can lead to stabilization of the transcription factor hypoxia-inducible factor 1α (HIF-1α) under normoxic conditions. HIF-1α has been described as a key regulator of a broad range of cellular and systemic responses to hypoxic conditions. HIF-1α is also involved in myeloid cell-mediated inflammation [18]. Furthermore, HIF-1α is a master regulator of the bactericidal capacity of phagocytes [19]. Lipopolysaccharides (LPS) induce the expression of HIF-1α in murine macrophages [20] and dendritic cells (DC) [21]. Additionally, HIF-1α knock-out mice develop less clinical signs of sepsis [22]. These findings suggest that HIF-1α may also be an important mediator of inflammatory responses in the absence of hypoxia. HIF-1α activation by TLR might have a functional consequence on DC. Thus, we investigated the effect of TLR2 and TLR4 activation by endogenous and exogenous agonists on the induction of the transcription factor HIF-1α in human monocyte-derived DC (MoDC) under normoxic conditions. Results show that stimulation of human MoDC with exogenous as well as endogenous TLR agonists induces the expression of HIF-1α in a time-dependent manner. Inhibition studies of HIF-1α transcriptional activity

further suggests that this transcription factor is crucially involved in MoDC maturation and function

Results

Induction of HIF-1α by exogenous and endogenous TLR4 agonists on human MoDC under normoxia

To investigate if HIF-1α could be induced by TLR stimulation under normoxic conditions, MoDC were incubated with different TLR ligands for the indicated time periods in a normal humidified cell incubator. We used LPS (1 μg/ml, Fig. 1A) and HA (20 μg/ml) as exogenous and endogenous TLR4 agonists, respectively. HIF-1α expression at the protein level was determined by Western blot analyses. As shown in Fig. 1B and C, both TLR4 agonists induced the expression of HIF-1α in a time-dependent manner. In parallel, cells were stained for the expression of the co-stimulatory molecules CD80 and CD86 as markers of cell maturation to confirm activation of the cells (Fig. 1D and E). To rule out contamination of HA with LPS, we incubated the cells with polymyxin B, an inhibitor of the biological activities of endotoxin. As shown in Fig. 2, polymyxin B did not affect maturation of MoDC induced by HA whereas the effect of LPS was abolished.

HIF-1α expression is induced by TLR2

To investigate, if the induction of HIF-1α is restricted to TLR4 signaling, we tested the effect of the TLR2 agonist LTA on the expression of HIF-1α. As shown in Fig. 3A, LTA also induced the expression of HIF-1α in a time dependent manner under normoxic conditions. To confirm activation of the cells, they were stained for the expression of the co-stimulatory molecules CD80 and CD86 after 24 hours (Fig. 3B).

Hypoxia and induction of HIF-1α by $CoCl_2$ does not induce maturation of MoDC

Next we analyzed, if the induction of HIF-1α by $CoCl_2$ alone, without stimulation of TLR, leads to an activation of MoDC. As shown in Fig. 4A and B, treatment with $CoCl_2$ led to the stabilization of HIF-1α but did not lead to a significant increase of the expression of the co-stimulatory molecules CD80 and CD86. Furthermore, we investigated the effect of hypoxic conditions (1.5 % oxygen) on the maturation of MoDC. Hypoxia alone did not lead to phenotypic maturation of human MoDC (Fig.

4C and D), whereas the cells still matured if they were incubated together with LPS (Fig. 4E). In addition, MoDC stimulated with LPS under hypoxic conditions demonstrated a significant increase of double positive $CD80^+/CD86^+$ cells (Fig. 4F) compared to stimulation under normoxia.

Inhibition of HIF-1α impairs the phenotypic maturation of human MoDC

To further characterize the role of the transcription factor HIF-1α on the maturation process of human MoDC, we used CTM (Chetomin) [23] to block HIF-1α. MoDC were pretreated with CTM for 3 hours and then stimulated with LPS, LTA or HA for 24 hours. Afterwards cells were analyzed for expression of CD40, CD80, CD86 and ICAM-1 (CD54) by flow cytometry. Inhibition of HIF-1α by CTM significantly inhibited the up-regulation of these markers compared with the positive controls treated with the TLR agonist only (Figs. 5A,B and C). To examine if the inhibitors influenced the expression levels of the indicated surface molecules under steady state conditions without stimulus, we incubated MoDC for 24 hours with CTM and then analyzed the cells by flow cytometry. As shown in Fig. 5D, CTM alone led to a modest decrease in CD80 and ICAM-1 expression. Dead cells were always excluded from analysis by PI staining.

Production of VEGF is prevented by inhibition of HIF-1α

The *vegf*-gene is under the control of the transcription factor HIF-1α. To analyze if the inhibition by CTM was successful, we measured the concentrations of secreted VEGF in the supernatant of MoDC. As shown in Fig. 6, treatment with CTM significantly inhibited the TLR-induced production of VEGF.

Inhibition of HIF-1α modulates secretion of proinflammatory cytokines and IL-10

The functional consequence of HIF-1α inhibition on the secretion of cytokines by MoDC was evaluated using a Luminex multiplex array system. Supernatants of LPS, HA and LTA stimulated cells were assayed for IL-1β, IL-6, IL-10 and TNF-α. Stimulation of MoDC with LPS resulted in a massive secretion of the proinflammatory cytokine IL-6. Inhibition of HIF-1α by CTM led to a significant reduction of IL-6 secretion after 24 hours of stimulation. No significant reductions were observed for the stimulations with HA and LTA (Fig. 7A). Furthermore, no

significant changes of TNF-α and IL-1β production were observed for any of the used TLR agonists (Fig 7B and C). Inhibition of HIF-1α activity resulted in a reduced secretion of IL-10, in particular for the stimulation with the endogenous TLR4 ligand HA (Fig. 7D).

Effect of LPS stimulation on mitochondrial respiration of MoDC

As maturation is an energy dependent process, we analyzed the functional effect of HIF-induction under normoxic conditions on energy metabolism, and measured the mitochondrial respiration of MoDC, which leads to generation of ATP. DC were incubated with 1 μg/ml of LPS under normoxic conditions. After 4 hours of incubation no significant changes in maximal oxygen consumption (state 3) was observed (complex I activity in LPS-treated cells 56 ± 30 pmol/s/million cells versus 63 ± 27 pmol/s x 1/million cells in controls ($p>0.05$) and complex II activity in LPS-treated cells of 54 ± 19 pmol/s/million cells versus 53 ± 32 pmol/s/million cells in controls ($p>0.05$).

Discussion

In the present study, we investigated the effect of TLR2 and TLR4 stimulation on the induction of HIF-1α in human MoDC under normoxic conditions. It is well known that TLR play an important role in sensing pathogens and are therefore crucially involved in the defense against infections. In addition, several reports have emphasized the importance of TLR2 and TLR4 in conditions of so called "sterile inflammation" in accordance with the danger model introduced by P. Matzinger [24, 25].

Our results demonstrate that HIF-1α can be induced under normoxic conditions in a time-dependent manner by both TLR2- as well as TLR4-stimulation in human DC. Up to now, HIF-1α induction under normoxic conditions by TLR ligands has been shown only for stimulation with LPS in murine bone-marrow derived DC [21]. In our experiments, LTA from the gram-positive cocci *S. aureus*, a ligand for TLR2 [26], showed an induction of HIF-1α, comparable to the stimulation induced by LPS from the gram-negative rod *E. coli*. We also examined the potential effect of HA on HIF-1α induction. Recent evidence suggests that such endogenous TLR agonists play a crucial role in human disease states such as I/R injury, that occurs in solid organ

transplantation, myocardial infarction, autoimmunity or trauma. HA, a non-sulfated glycosaminoglycan, is a major constituent of the extracellular matrix (ECM). Under conditions of tissue injury or inflammation, HA is degraded and shed from the ECM and cell surface and provides an endogenous danger signal [9, 27]. HA induced the expression of HIF-1α, but peaked already at around 6 hours, whereas LPS and LTA demonstrated highest induction levels at the end of our measurement period which was 24 hours after stimulation. Differences in HA and LPS stimulated HIF-1α induction may relate to the recently reported distinctive pattern of gene expression in macrophages induced by these two different agonists or the composition of the respective receptor complex [28].

As a next step, we examined the influence of induction of HIF-1α by $CoCl_2$ or hypoxia alone on the maturation process of MoDC. At the dose used, $CoCl_2$ is a widely accepted inducer of HIF-1α activation [29-31]. Different ranges of oxygen levels have been considered as hypoxic conditions, starting from almost anoxic conditions of 0.1 % up to 2 % O_2. Oxygen levels in the body vary from around 16 % in the pulmonary alveoli down to approximately 6 % in other tissues [32]. Furthermore, oxygen gradients exist within tissues depending on the distance of cells from the blood vessels. Currently there is no consensus regarding a critical threshold level and in the present study we used the commonly quoted value of 1.5% O_2 [33, 34]. Neither hypoxia nor $CoCl_2$ induced phenotypic maturation of MoDC examined after 24 hours. However, we observed an amplification of LPS induced phenotypic maturation of MoDC under hypoxia. Consequently, hypoxia seems to augment an inflammatory response elicited in the presence of a danger signal associated with infection or ischemia. In contrast, hypoxia without pro-inflammatory stimuli does not induce activation, which may serve as a protection mechanism of the host preventing development of an autoimmune response. These data are consistent with the finding of Jantsch *et al.* in murine DC for hypoxia [21]. In contrast, a recent study has shown that human MoDC differentiated from monocytes under permanent hypoxic (1 % O_2) conditions exhibit a reduced up-regulation of CD40, CD80, CD83 and CD86 in response to LPS, whereas the secretion of TNF-α, CCL22 and IL-1β was increased [35]. Another study demonstrated, that MoDC generated under hypoxic conditions (1 % O2) change their chemokine releasing profile and exhibit a reduced Ag-uptake capacity [36]. Obviously, hypoxic conditions during the differentiation process of

monocytes into DC exert another effect on the cells than exposure of already fully differentiated DC. Differentiation of monocytes into DC under low hypoxic conditions (e.g. 1 % O_2) would be the conditions of novel recruited monocytes into inflamed tissue. On the other hand, tissue-resident DC are exposed to oxygen levels of around 6 % with subsequent reduction of O_2 due to inflammation. The evaluation of the effect of different O_2 levels on differentiation or maturation of human MoDC would be important but is beyond the scope of this study.

As a next step, we investigated the functional importance of HIF-1α expression. Functional gene knock-down using siRNA has been tested, but has shown to influence the activation status of MoDC in our hands. Accordingly, we used the chemical inhibitor CTM [23] to prevent HIF-1α activation. CTM has been shown to disrupt the CH1 domain of p300 and thereby prevents the binding of HIF to its target gene [23]. Pretreatment of MoDC with CTM followed by TLR stimulation with LPS, LTA or HA significantly inhibited up-regulation of the co-stimulatory molecules CD40, CD80 and CD86 which are crucially involved in providing the second signal for T cell activation. In addition, up-regulation of the adhesion molecule ICAM-1 was inhibited. Signaling pathways that trigger TLR2 and TLR4 responses are mainly dependent on Toll-IL-1 receptor (TIR) domain-containing adaptor protein (TIRAP) and MyD88. MyD88 recruits members of the IRAKs and TNF receptor-associated factor 6 (TRAF6) to the IKK complex (consisting of IKK-α, IKK-β and IKK-γ) to finally activate NF-κB. Activation of NF-κB is controlled by IKK kinases, mainly by IKK-β. A recent study demonstrated, that IKK-β is crucial for HIF-1α accumulation in macrophages thereby linking innate immunity with the hypoxic response [37].

LPS has shown to activate in an early phase the MyD88-dependent pathway of NF-κB activation leading to the production of IL-1β, IL-6 and TNF-α. Interestingly, several studies have highlighted the existence of a MyD88-independent pathway for LPS, which activates the interferon regulatory factor 3 (IRF3) pathway and involves the late phase of NF-κB activation. In contrast, stimulation with TLR2 agonists has been shown so far to be strictly MyD88-dependent [38]. HA has originally been described to trigger only TLR4 [12], whereas an involvement of TLR2 was reported later on [39]. HA signaling has been reported so far to be MyD88-dependent [39, 40]. The co-activator protein p300 is involved in the MyD88-independent and the TRIF-dependent TLR pathway. Activation of the TRIF-dependent signaling pathway is

suggested to be mainly restricted to TLR3 stimulation with e.g. poly I:C [38]. On this basis it seems likely that an effect of CTM, which specifically inhibits binding of p300, on the TLR pathway itself can be excluded.

As a consequence of TLR stimulation MoDC secrete proinflammatory cytokines like IL-1β, IL-6 and TNF-α as well as the immunoregulatory cytokine IL-10. Our data indicate that secretion of IL-6 was reduced for stimulation with HA and LPS when HIF-1α was inhibited by CTM. In contrast, TNF-α and IL-1β secretion was unaffected. As stimulation with TLR agonists in combination with CTM does not affect TNF-α and IL-1β we conclude, that the treatment with CTM does not affect cell viability and metabolic activity of the cells. Amounts of secreted IL-10 after inhibition of HIF-1α were decreased. Particularly for stimulation with HA we could observe a significant reduction. Other known immunomodulating agents like corticosteroids [41] or vitamin D_3 [42] are known to increase IL-10 production by DC whereas e.g. rapamycin or FK506 (tacrolimus) [41] or low molecular weight dextran sulfate [43] do not increase IL-10 production.

VEGF is a down-stream target of HIF-1α and has been shown to be produced by macrophages [44], neutrophils [45], fibroblasts [46], EC [47] and T cells [48]. One recent study has shown that MoDC secrete VEGF in response to LPS [49]. Our results confirm the finding that LPS can induce production of VEGF in human MoDC. Furthermore, LTA and HA were able to induce VEGF production, which was abolished with treatment of CTM confirming the functional inhibition of HIF-1α.

To determine if induction of HIF-1α by TLR-ligands under normoxia leads to a change of mitochondrial respiration, we examined the mitochondrial electron transport chain, which finally leads to generation of ATP. We could not detect a significant change of mitochondrial respiration after stimulation with LPS which is consistent with a previous published study [50].

In summary, we demonstrate that endogenous and exogenous TLR4 and TLR2 agonists induce HIF-1α stabilization under normoxic conditions and that TLR-mediated HIF-1α production plays an important role in the maturation of DC. Hypoxia alone was not able to induce maturation of DC, however, when DC were exposed to both, LPS and hypoxia, maturation was markedly enhanced. These results indicate a synergistic effect of hypoxia and inflammation that is most likely regulated by HIF-1α. Inhibition of HIF-1α by compounds like CTM, which results in

modulation of DC maturation and function, may therefore represent a novel target for future modulation of DC biology in various human disease states.

Materials and Methods

Generation and stimulation of human monocyte-derived DC (MoDC)

Human peripheral blood mononuclear cells (PBMC) were isolated from buffy coats obtained from healthy blood donors (Regional Red Cross Blood Donation Center, Bern, Switzerland) by density gradient centrifugation over Ficoll-Paque (Amersham, Uppsala, Sweden). Monocytes were isolated from PBMC as previously described [43, 51, 52]. Purity of isolated monocytes was characterized by high expression of CD14 (>96 % positive cells) and absent expression of CD1a and DC-SIGN. Monocytes were incubated for 6 days in RPMI 1640 medium (Invitrogen Life Technologies) containing 10% fetal calf serum ((FCS, Amimed / BioConcept, Allschwil, Switzerland), 1% [2mM] L-Glutamine (Invitrogen), 1% [100 U/ml] Penicillin/Streptomycin (Invitrogen), 10 ng/ml GM-CSF (R&D Systems Europe Ltd, Abingdon, Oxon, UK), and 10 ng/ml IL-4 (R&D) to generate MoDC as described initially by Sallusto and Lanzavecchia [53]. Immature MoDC were characterized by absent expression of CD14 and high expression of CD1a, HLA-DR, DC-SIGN and phagocytic activity. Maturation of these cells leads to a massive up-regulation of CD40, CD80, CD83, CD86, ICAM-1, CCR7 and HLA-DR. Mature MoDC exhibit a very potent T cell proliferation capacity and reduced Ag-uptake as previously described [43, 52, 54]. Cells were kept at 37°C in a 5% CO_2 humidified atmosphere. On day 3, the culture medium was replaced with fresh medium. For induction of maturation 1 μg/ml LPS (Sigma Aldrich, Buchs, Switzerland), 5 μg/ml LTA or 20 μg/ml HA (Sigma Aldrich, Buchs, Switzerland) were added to the MoDC cultures for the indicated periods.

FACS analysis and cell viability

Cells were incubated with FITC- or PE-labeled monoclonal antibody (mAb) against CD80, CD86 (BD, Franklin Lakes, NJ, USA), isotype control IgG1 (BD), or unlabeled mAb against CD40, ICAM-1 (Diaclone, Besançon, France) followed by a FITC-labeled polyclonal goat anti-mouse IgG (Sigma).

For determination of viability, the cells were stained with 5 μg/ml of propidium iodide (PI; Invitrogen) and analyzed by flow cytometry. As positive control for PI staining, cells were treated with PBS containing 0.1 % BSA and 0.1 % saponin (Sigma Aldrich, Buchs, Switzerland). Measurements were performed with a BD FACScan flow cytometer and analyzed using the FlowJo software (Tree Star Inc., Ashland, OR, USA).

Hypoxic conditions

Cells were incubated in a hypoxia chamber (1.5 % oxygen) for the indicated time-periods.

SDS-PAGE and Western blotting

DC were lysed in 60 mM Tris-HCl, 8.5% glycerol, and 2% SDS. Protein concentration was determined with the Quanti-IT assay kit and read with the Qubit fluorometer (Invitrogen). Equal amounts of protein (20 μg per line) were loaded and separated by 4-12% SDS-PAGE. Gels were then transferred to nitrocellulose membranes with the iBlot dry blotting system (Invitrogen). Equal loading was verified by staining the extracted gel with SimplyBlue SafeStain (Invitrogen). Afterwards, the membranes were blocked for 30 min with incubation buffer (10 mM Tris-HCl pH 7.5, 100 mM NaCl and 0.1% w/v Tween 20) supplemented with 5% non-fat dry skim milk, and incubated overnight with the primary antibodies against HIF-1α (Novus Biologicals, Littleton, CO, USA; dilution 1:2000) and actin (Sigma Aldrich, Buchs, Switzerland; dilution 1:3000). Membranes were washed with incubation buffer and incubated for 1 hour with horseradish peroxidase-coupled goat polyclonal anti-rabbit IgG (dilution 1:3000). Finally, the membranes were developed with a chemiluminescence detection kit (Pierce, Rockford, IL, USA). All Western blotting experiments were performed in triplicates.

Cytokine assays

MoDC (10^6 cells/ml) were treated with LPS, LTA or HA for 24 hours. Cell culture supernatants were analyzed using a Luminex multiplex suspension array system from Bio-Rad (Bio-Rad, Hercules, CA, USA) for IL-1β, IL-6, IL-10, TNF-α and VEGF

(all kits from BioSource, Invitrogen, Carlsbad, CA, USA) according the manufacturer's instructions.

Inhibitors and agonists of HIF-1α

Blocking of HIF-1α was achieved using the commercially available inhibitor CTM (Chetomin, HIF-1α transcriptional inhibitor, Invitrogen). Induction of HIF-1α in control experiments was achieved using $CoCl_2$ (Sigma Aldrich, Buchs, Switzerland), which is a potent inhibitor of post-translational hydroxylation of a proline residue in the oxygen-dependent degradation (ODD) domain of HIF-1α and thus inhibits HIF-1α degradation.

High-resolution respirometry

DC were centrifuged for 5 min (350 g) and resuspended in respiration buffer (110 mM sucrose, 0.5 mM EGTA, 3.0 mM $MgCl_2$, 80 mM KCl, 60 mM K-lactobionate, 10 mM KH_2PO_4, 20 mM taurine, 20 mM HEPES, 1.0 g/l BSA, pH 7.1) at a concentration of 3-5 x 10^6 cells/ml. Cells were incubated with 1 µg/mL LPS for 4 h . Respiration rates were measured at 37ºC in a high-resolution oxygraph (Oxygraph-2k, Oroboros Instruments, Innsbruck, Austria). For assessment of mitochondrial complex activity cells were first permeabilized with digitonin (8.1 µM) for 5 min. Afterwards, for complex I-dependent maximal respiration stimulation, substrates added were glutamate (10 mM) and malate (5 mM), followed by addition of adenosine diphosphate (ADP) (0.25 mM) causing a sudden burst of oxygen uptake as ADP is converted into ATP. After a stable signal was reached and marked, rotenone (0.5 µM) was added to inhibit complex I, and then complex II-dependent respiration was stimulated by adding succinate (10 mM), while complex III was inhibited by antimycin A (0.5 µM) (Sigma Aldrich, Buchs, Switzerland). Respiration rates were calculated and recorded using DatLab software for data acquisition and analysis; Oroboros Instruments, Innsbruck, Austria.

Statistical analysis

Data are presented as mean ± standard deviation (SD) representing experiments with up to 10 different donors. Unpaired Students *t-tests* were performed for evaluation of significance. Cellular respiration was compared using the paired t-test. Differences

were considered as statistically significant at p-values less than 0.05. Data were analyzed using GraphPad Prism software 4.0 (GraphPad, San Diego, CA).

Acknowledgements

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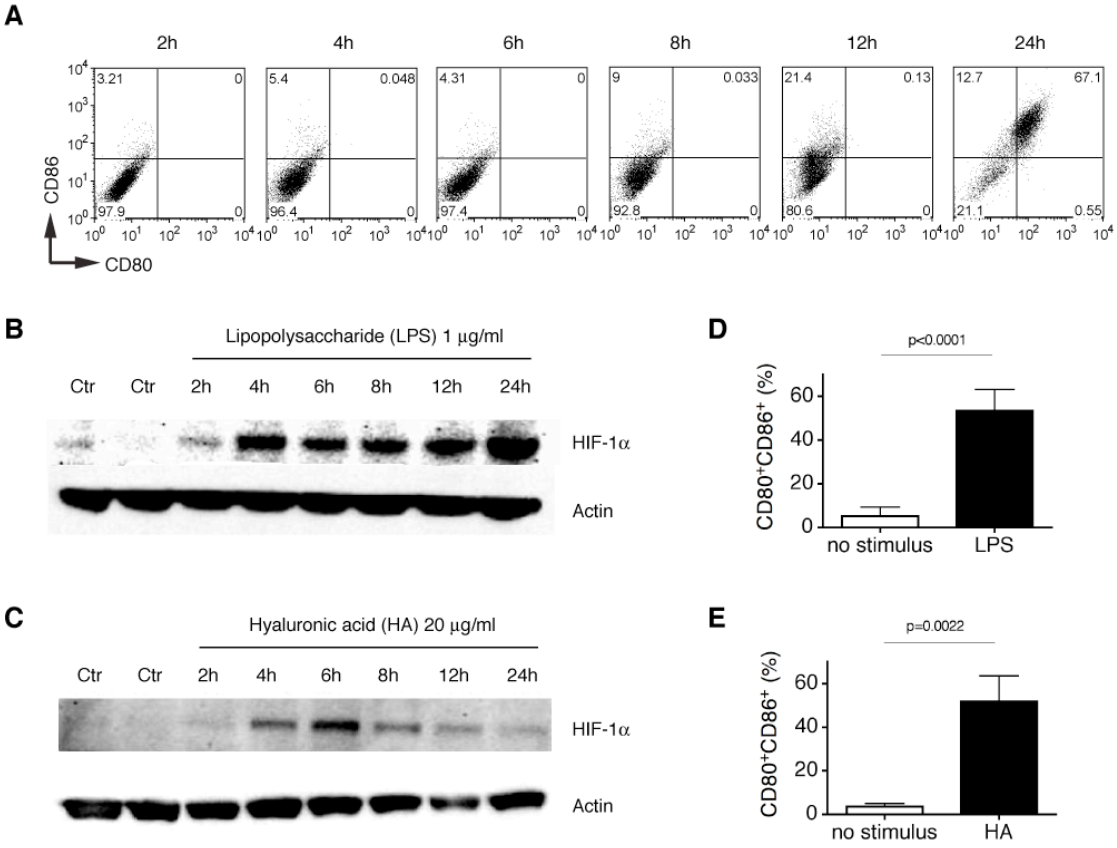

Figure 1: Time dependent induction of HIF-1α by the TLR4 agonists LPS and HA. *A*, Monocytes isolated from buffy coat were cultured in presence of GM-CSF and IL-4 for 6 days. MoDC were incubated with LPS (1 μg/ml) for the indicated periods. Afterwards cells were harvested and analyzed by flow cytometry for the expression of CD80 and CD86. *B* and *C*, Cell lysates from MoDC that had been stimulated with either LPS (1 μg/ml) or HA (20 μg/ml) for the indicated time-periods were prepared. Equal amounts of protein were loaded and separated by SDS page, transferred onto membranes and probed for HIF-1α and actin. *D* and *E,* Percentages of $CD80^+$ $CD86^+$ double-positive cells after stimulation with LPS or HA. Values of 3 to 4 experiments with cells from different donors are shown as mean values ± standard deviation.

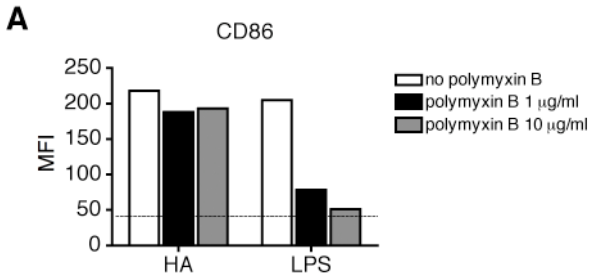

Figure 2: Polymyxin B does not affect stimulatory activity of HA. MoDC were incubated with polymyxin B (1 or 10 μg/ml) and then stimulated with HA (20 μg/ml) or LPS (1 μg/ml) for 24 hours. Afterwards, cells were washed and analyzed for the expression of CD86 as a maturation marker. Y-axis shows the median fluorescence intensity (MFI). The dotted line indicates the MFI of non-stimulated MoDC. Data are representative of three independent experiments.

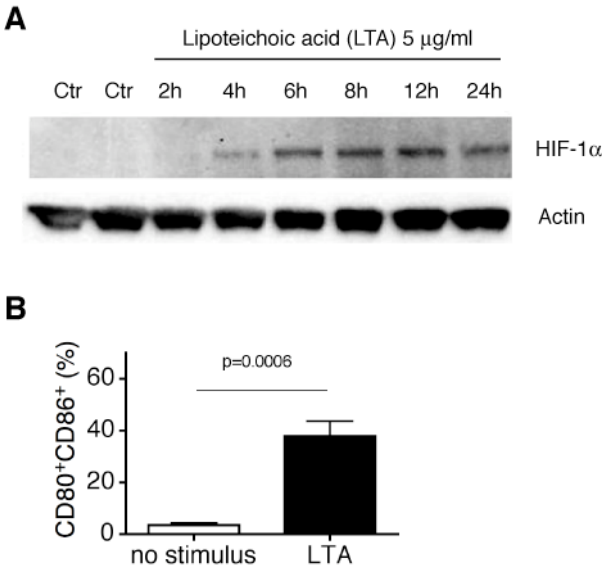

Figure 3: Time-dependent induction of HIF-1α by the TLR2 agonist LTA in MoDC. *A*, Cell lysates of MoDC stimulated with LTA (5 μg/ml) for the indicated time-periods. Equal amounts of protein were loaded and separated by SDS page, transferred onto membranes and probed for HIF-1α and actin. *B*, Percentages of $CD80^+$ $CD86^+$ double-positive cells stimulated with LTA are shown as mean values ± standard deviation of 3 experiments with cells from different donors.

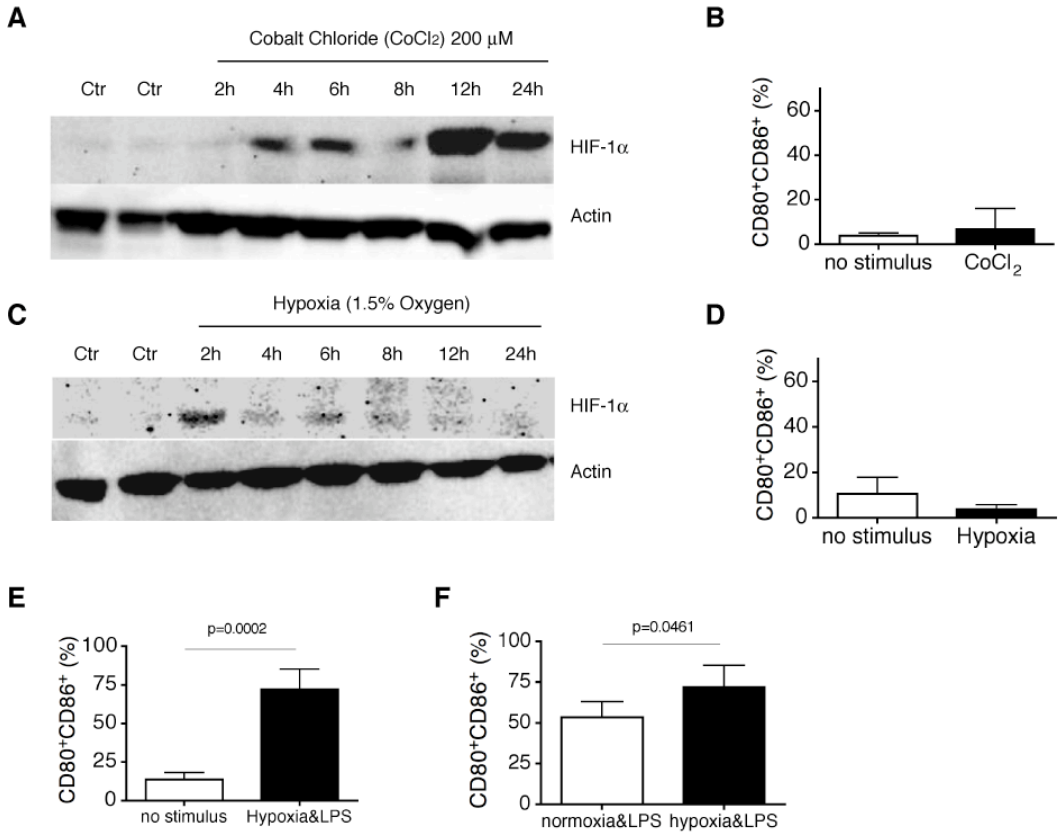

Figure 4: Induction of HIF-1α by Hypoxia or $CoCl_2$ alone does not induce MoDC maturation. *A*, Cell lysates were prepared from MoDC exposed to $CoCl_2$ under normoxic conditions for the indicated time-periods. Equal amounts of protein were loaded and separated by SDS page, transferred onto membranes and probed for HIF-1α and actin. *B*, Percentages of $CD80^+$ $CD86^+$ double-positive cells stimulated with $CoCl_2$ are shown as mean values ± standard deviation of 3 experiments with cells from different donors. *C*, Cell lysates were prepared from MoDC exposed to hypoxia (1.5 % oxygen) for the indicated time-periods. Equal amounts of protein were loaded and separated by SDS page, transferred onto membranes and probed for HIF-1α and actin. *D*, Percentages of $CD80^+$ $CD86^+$ double-positive cells under hypoxic conditions are shown as mean values ± standard deviation of 3 experiments with cells from different donors. *E*, Percentages of $CD80^+$ $CD86^+$ double-positive cells stimulated with LPS under hypoxic conditions are shown as mean values ± standard deviation of 4 experiments with cells from different donors. *F*, Percentages of $CD80^+$/$CD86^+$ double-positive cells stimulated with LPS under normoxic or hypoxic conditions are shown as mean values ± standard deviation of 4-5 experiments with cells from different donors.

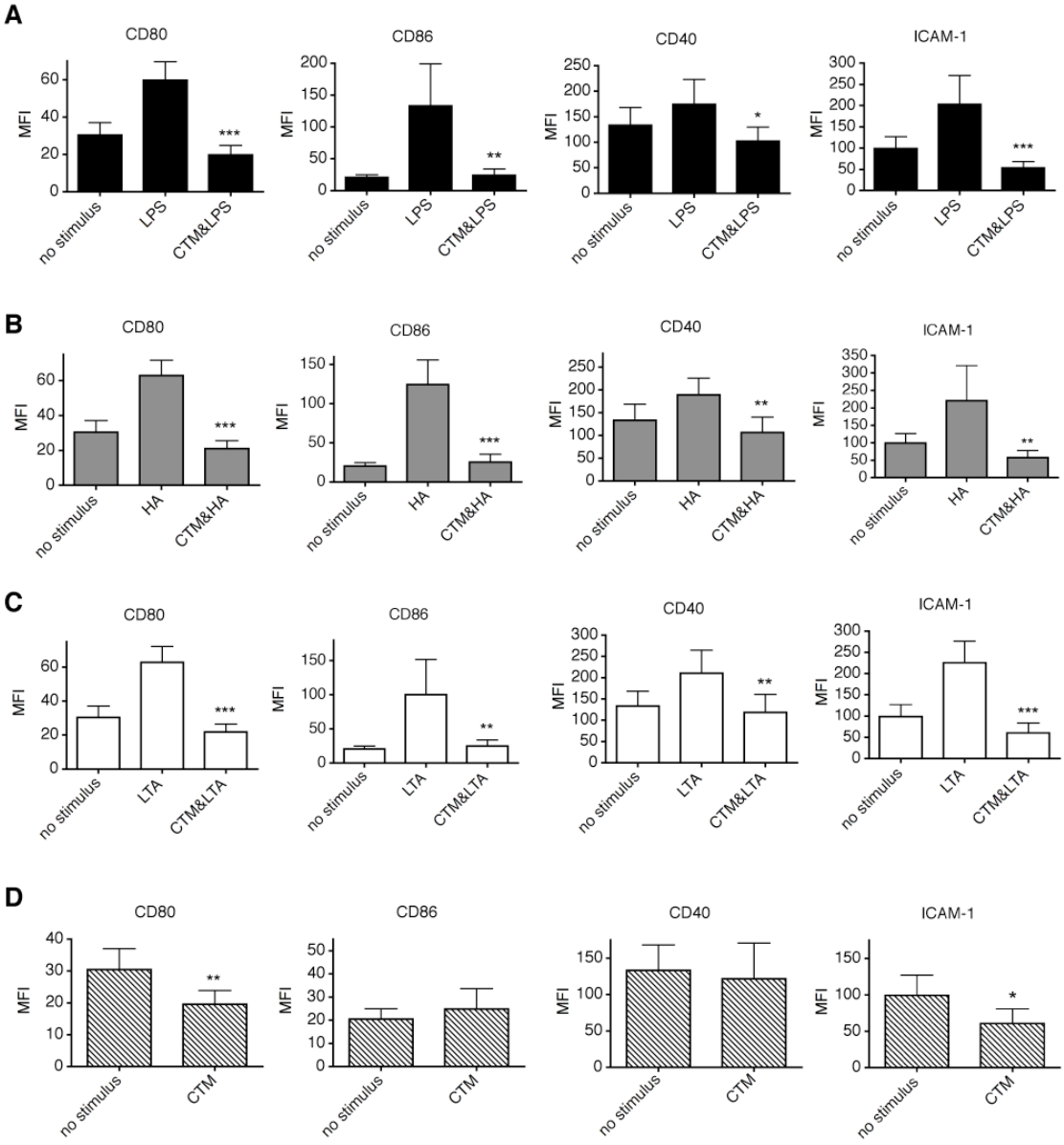

Figure 5: Inhibition of HIF-1α impairs phenotypic maturation of MoDC. *A*, Monocytes isolated from buffy coat were cultured in the presence of GM-CSF and IL-4 for 6 days. Inhibition of HIF-1α expression by CTM inhibited maturation of MoDC in response to LPS. MoDC were incubated with CTM (200 nM) for 3 hours, afterwards cells were stimulated for 24 hours with LPS (1 μg/ml) (*A*), HA (20 μg/ml) (*B*) and LTA (5 μg/ml) (*C*). Cells were washed and analyzed for the expression of CD80, CD86, CD40 and ICAM-1 . Y-axis shows the median fluorescence intensity (MFI). Mean values ± standard deviation of 6 experiments with cells from different donors are shown. *p<0.05; **p<0.01; ***p<0.001 vs. mature MoDC (unpaired Student's *t*-test) *D*, Influence of HIF-1α inhibitor CTM on the expression of CD80, CD86, CD40 and ICAM-1 expression of non TLR-stimulated MoDC. *p<0.05; **p<0.01 vs. non-treated MoDC (unpaired Student's *t*-test).

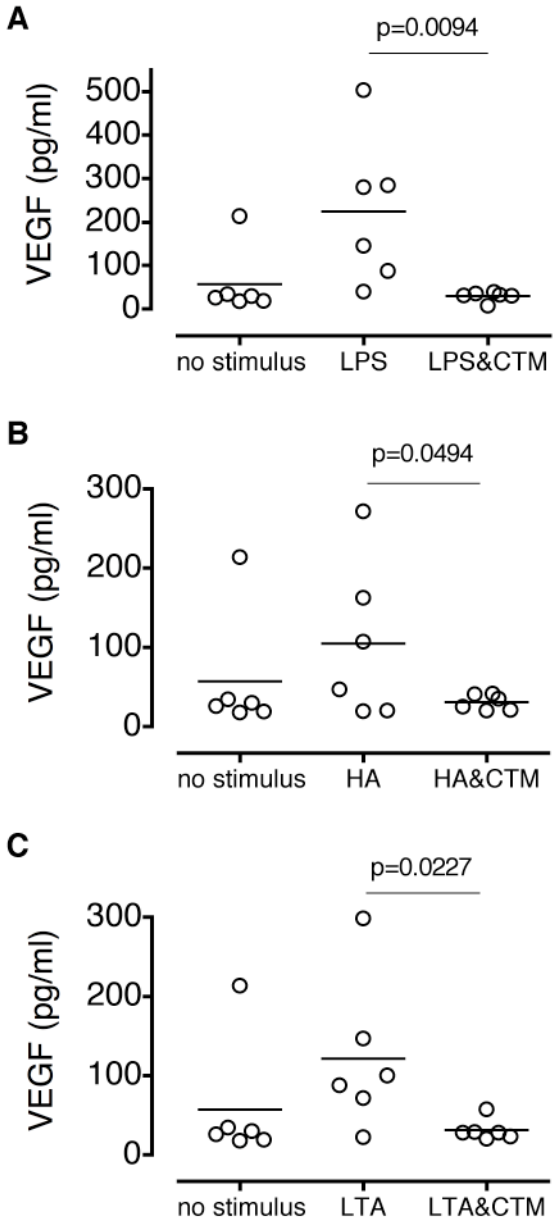

Figure 6: Inhibition of HIF-1α by CTM prevents VEGF production of MoDC. *A*, *B* and *C*, MoDC were incubated with CTM (200 nM) for 3 hours, then cells were stimulated with LPS (1 μg/ml) (*A*), HA (20 μg/ml) (*B*) and LTA (5 μg/ml) (*C*). After 24 hours cells were harvested and the supernatant assayed for VEGF with a Luminex multiplex array system. Each point represents an experiment with cells from a different donor. Significant p-values comparing stimulated vs. CTM treated MoDC are shown in the figure.

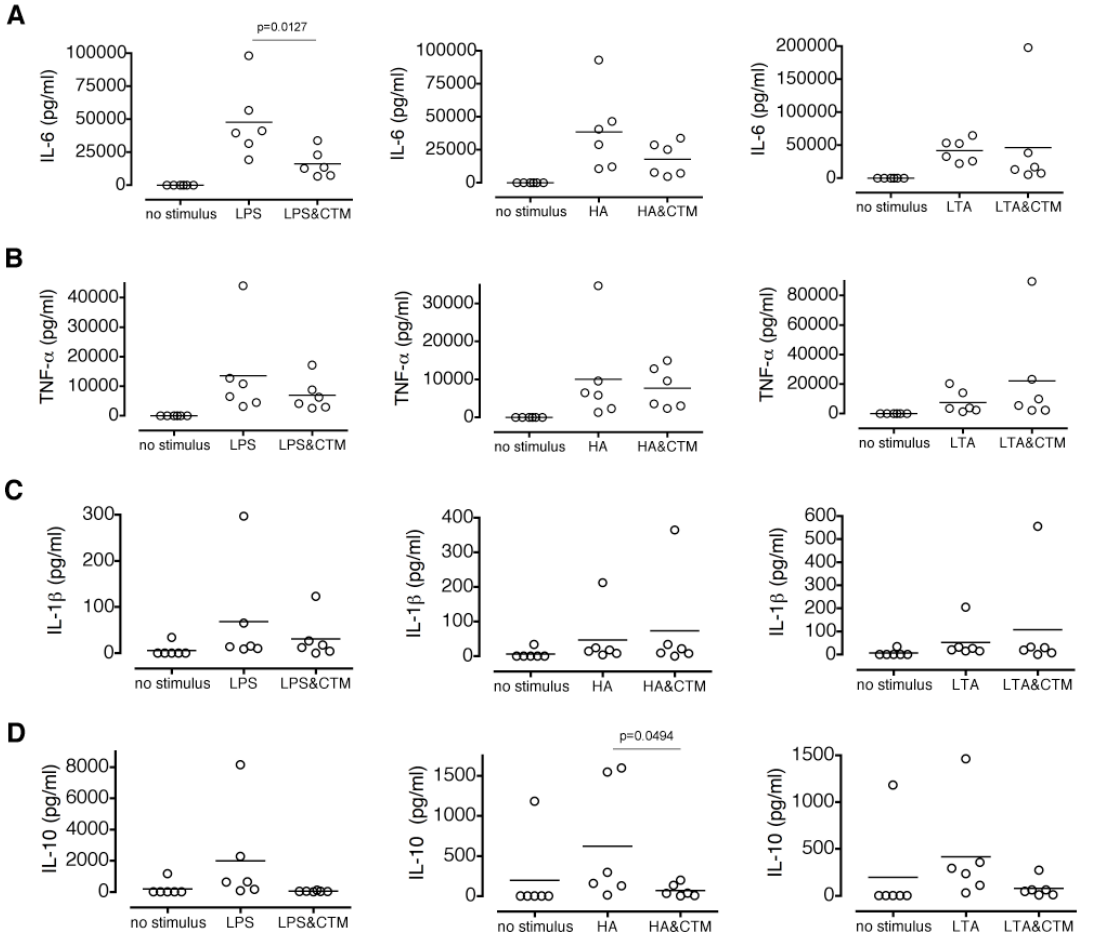

Figure 7: Modulation of the secretion of proinflammatory cytokines as well as IL-10 by inhibition of HIF-1α. *A*, *B, C* and *D*, MoDC were stimulated with LPS (1 μg/ml), HA (20 μg/ml) and LTA (5 μg/ml). After 24 hours of incubation, cells were harvested and the supernatants examined for the amounts of IL-6 (*A*), TNF-α (*B*), IL-1β (*C*) and IL-10 (*D*) with a Luminex multiplex array system. Each point represents an experiment with cells from a different donor. Significant p-values comparing stimulated vs. CTM treated MoDC are shown in the figure.

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3-5 Paper V: TLR induced production of ET-1 in human DC

Title: TLR2 and TLR4 stimulation leads to production of ET-1 in human DC

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Manuscript in preparation as short communication

Endothelin-1 (ET-1) has been described to be mainly secreted by endothelial cells and to act as a potent vasoconstrictor. Moreover, ET-1 is involved in the pathogenesis of I/R injury. LPS induce secretion of ET-1 by macrophages. Therefore we hypothesized, that stimulation of MoDC with different TLR ligands induces production ET-1.

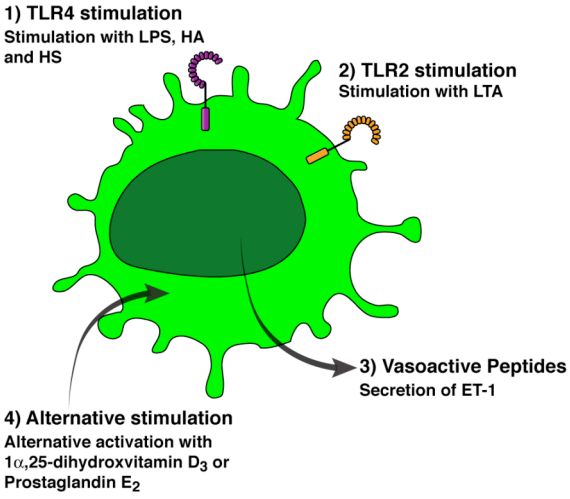

Figure 11: TLR stimulation of human DC leads to production of the vasoactive peptide ET-1.

We could demonstrate, that stimulation of human MoDC with TLR2 and TLR4 agonists induce the secretion of ET-1 in a dose- and time-dependent manner. Alternative stimulation of MoDC with the active form of vitamin D_3 (1α,25-dihyroxvitamin D_3) and LPS amplifies secretion of VEGF as well as ET-1. Interestingly, alternative-activation of MoDC with prostaglandin E_2 and LPS only amplifies secretion of VEGF whereas ET-1 production was reduced. According to our novel data, TLR-mediated induction of ET-1 production by human DC should be considered as a potential source of increased amounts of ET-1 in e.g. I/R injury.

Stimulation of human dendritic cells with TLR2 and TLR4 agonists leads to secretion of the vasoactive peptide ET-1

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Abbreviations: ET-1: Endothelin-1;
MoDC: monocyte-derived DC
VEGF: Vascular Endothelial Growth Factor

Abstract

Endothelin-1 (ET-1) is mainly secreted by endothelial cells and acts as a potent vasoconstrictor. In addition ET-1 has also been shown to have pleiotropic effects on a variety of other systems including adaptive immunity. There are two main ET-1 receptors, ET_A and ET_B, which have different tissue and functional distributions. Dendritic cells (DC) are pivotal antigen-presenting cells linking the innate with the adaptive immune system. DC are sentinels expressing pattern-recognition receptors as e.g. the toll-like receptors (TLR) for detecting danger signals released from pathogens or tissue injury. Here we show for the first time that stimulation of human monocyte-derived DC with exogenous as well as endogenous selective TLR4 and TLR2 agonists induces the production of ET-1 in a dose- and time-dependent manner. In contrast, maturation in the presence of 1α,25-dihydroxyvitamin D_3, i.e. alternatively-activated DC, results in a marked potentiation of the endothelin response. Surprisingly, stimulation of human monocytes with LPS did not lead to secretion of detectable amounts of ET-1. These results may suggest a role of ET-1 as important player in human DC biology and innate immunity in general.

Introduction

Dendritic cells (DC) are the most potent antigen-presenting cells (APC) and play a central role in bridging innate and adaptive immunity. They are pivotal for the initiation of T-cell mediated immune responses. DC residing in the peripheral tissue are normally in a phenotypically and functionally immature state [1]. DC express toll-like receptors (TLR), which distinguish conditions of well-being from conditions of disease. Activating signals such as pathogenic compounds derived from microorganisms, e.g. lipopolysaccharide (LPS), as well as endogenous molecules like hyaluronan (HA) induce maturation of DC via TLRs [2]. The equilibrium between pro- and anti-inflammatory signals in the microenvironment determines the phenotype and behavior of DC at the site of inflammation. Exposure of DC to anti-inflammatory molecules like IL-10 [3], TGF-β [3], 1α,25-dihydroxyvitamin D_3 (calcitriol) [4], prostaglandin E_2 [5] or glucocorticosteroids [6] following stimulation with LPS are generally described as so called "alternatively-activated" DC (AA-DC) [7]. AA-DC are considered to exhibit tolerogenic properties, i.e. increase of IL-10 secretion and induction of T regulatory cells, and to prolong organ allograft survival [8]. A recent study has demonstrated, that AA-DC secrete substantially higher levels of VEGF when the cells are pretreated with 1α,25-dihydroxyvitamin D_3 or prostaglandin E_2 (PGE_2) prior proinflammatory stimulation with LPS, TNF-α, *S. aureus* or CD40L [9].

Endothelins, including endothelin-1 (ET-1), ET-2 and ET-3 are widely distributed in tissues and mainly produced by endothelial and epithelial cells. Endothelin-1 (ET-1) is a potent vasoconstrictor [10] but has also been shown to exert pleiotropic effects in different biological processes such as cancer [11], ischemia/reperfusion (I/R) injury [12] diabetes and adaptive immune response [13]. Two endothelin receptors have been identified, endothelin receptor A (ET_A) and endothelin receptor B (ET_B) [10] and selective receptor agonists are currently in clinical use for the treatment of diseases including pulmonary hypertension and melanoma. [13].

Recently Ehrenreich *et al.* have demonstrated, that human macrophages produce ET-1 in response to LPS [14]. One additional study has also shown that stimulation of human monocyte-derived DC (MoDC) with TNF-α or gram-positive cocci *S. aureus* results in secretion of ET-1. These reports suggest that endothelin secretion may be regulated through specific TLR activation in dendritic cells and that the endothelin

system may be involved in linking innate and adaptive immune responses. We hypothesized therefore, that stimulation of human MoDC with selective TLR4 and TLR2 agonists would induce ET-1 and possibly also alter endothelin receptor expression. Our data show that stimulation with exogenous as well as endogenous selective TLR agonists indeed induce secretion of ET-1, which was enhanced when cells were alternatively-activated with 1,25-dihydroxyvitamin D_3. The endothelin system could therefore play an important role in DC biology and various diseases where TLR receptors and DC are critically involved.

Results and discussion

LPS does not induce ET-1 production in human monocytes

Since it has previously been shown that macrophages secrete ET-1 upon stimulation with LPS [14], we investigated the effect of LPS stimulation on human monocytes. Freshly isolated human monocytes, characterized by a high expression of CD14, low expression of MHC class II and absent expression of CD1a and DC-SIGN (CD209) [15], were stimulated with different concentrations of LPS (1, 10, 100 and 1000 ng/ml) for 24 and 48 hours. Afterwards, cells were harvested and supernatants were assayed for the amount of secreted ET-1. In our hands LPS stimulated human monocytes did not produce detectable amounts of ET-1 for (data not shown).

LPS and LTA induce ET-1 production in human MoDC

As a next step, we evaluated the potency of DC to secrete ET-1 in response to selective TLR ligands. TNF-α and *S. aureus* were previously shown to induce secretion of ET-1 by MoDC [16]. Although stimulation with *S. aureus* implicates an involvement of TLRs, responses induced by selective TLR activation have not previously been investigated. Lipopolysaccharide (LPS) and lipoteichoic acid (LTA) were characterized as selective TLR4 and TLR2 agonists. respectively [17]. MoDC, characterized by a high expression of CD1a, DC-SIGN and MHC class II, were stimulated with different concentrations of LPS from *E. coli* (1 ng/ml, 10 ng/ml, 100 ng/ml and 1000 ng/ml) for 24 and 48 hours. As shown in Fig. 1*A* and *B*, LPS stimulated secretion of ET-1 in a dose- and time-dependent manner. These results demonstrate that ET-1 is inducible in DCs by stimulation with TLR4 agonists. In addition, we evaluated the potency of LTA to induce secretion of ET-1 via activation

of TLR2. LTA also induced secretion of ET-1 in a time and dose-dependent manner , but appeared to be appeared to be less potent than LPS (Fig. 1*A*).

Endogenous TLR agonists induce secretion of ET-1 in human MoDC

Several reports in the last years have identified endogenous ligands of TLRs, which are released in response to tissue injury and sterile inflammation. We therefore evaluated whether stimulation of MoDC by the endogenous molecules hyaluronic acid (HA) or heparan sulphate (HS), both constituents of the glycocalyx of endothelial cells (EC), induces secretion of ET-1. Stimulation of MoDC with HA resulted in production of ET-1, whereas a much lower response was observed for HS. Interestingly, shedding of HA has been previously been observed in a setting of hyperglycemia induced vascular dysfunction [18] and ET-1 has been demonstrated to be an important mediator under these conditions [13, 19]. Moreover, shedding of HA and HS has been demonstrated as a result of I/R injury where elevated ET-1 levels have also been described [12, 20, 21].

Evaluation of ET-1 secretion by alternatively-activated MoDC

Different protocols for the generation of AA-DC have been described in the literature [3, 4, 6]. AA-DC were shown to secrete highly elevated amounts of VEGF, whereas FGF levels were not increased [9]. Accordingly, we evaluated whether ET-1 secretion in response to TLR agonists may be altered in AA-DC. Stimulation procedures were the same as those described by Riboldi *et al.* [9]. As anti-inflammatory stimuli we used dexamethasone, PGE_2 and 1α,25-dihydroxyvitamin D_3, followed by stimulation by LPS for 48 hours. Cell supernatants were assayed for ET-1, VEGF and IL-10. In keeping with previous reports we observed a significant increase of VEGF secretion in AA-DC treated with PGE_2 and 1α,25-dihydroxyvitamin D_3 (Fig. 2) [9]. In contrast, treatment with dexamethasone did not increase secretion of VEGF (data not shown). No increase in IL-10 secretion could be observed for PGE_2 and 1α,25-dihydroxyvitamin D_3 (Fig. 2). This discrepancy may be due to differences in experimental protocols used for the generation of AA-DC. Commonly, cells are exposed to these anti-inflammatory molecules during the differentiation process from monocytes into immature MoDC for 6 days whereas in our study protocol pre-treatment of mature DC was only for 1 hour.

Strikingly, we observed a massive enhancement of ET-1 secretion of AA-DC generated with 1α,25-dihydroxyvitamin D_3, whereas a marked decrease was observed for treatment with PGE_2 and dexamethasone (Fig. 2, data not shown for dexamethasone). PGE_2 has been previously been shown to inhibit ET-1 production by EC [22] and we now observed the same effect in human MoDC.

Influence of TLR stimulation on the expression of ET_A and ET_B

Stimulation of MoDC by LPS for 48 hours led to an increase in ET-1 gene expression as determined by TaqMan quantitative qRT-PCR. In contrast both ET_A and ET_B receptor transcription were down regulated (Fig. 3). Further studies on a functional level are necessary to determine whether the time course of ET_A and ET_B receptor protein translation and receptor binding follows a similar time course.

Concluding Remarks

These results show for the first time, that stimulation of MoDC by exogenous as well as endogenous selective TLR agonists induces secretion of ET-1. The data demonstrate, that the endogenous TLR4 agonist HA induced secretion of ET-1 by MoDC. Shedding and synthesis of HA has been demonstrated in diabetes [18] and I/R injury [23], conditions in which circulating ET-1 levels are increased. Furthermore, we show that stimulation of MoDC with the TLR2 agonist LTA also induces secretion of ET-1.

Alternatively activated DC have previously been shown to have pro-angiogenic properties and upregulate VEGF production [9]. Our results confirm these findings and show that stimulation of MoDC in an alternative manner with PGE_2 or 1α,25-dihydroxyvitamin D_3 enhanced VEGF production. In addition the present results extend these findings and demonstrate that ET-1 formation is also enhanced in these cells. Interestingly, only 1α,25-dihydroxyvitamin D_3 induced an increase of ET-1 production by MoDC. PGE_2 has previously been shown to inhibit production of ET-1 by EC [22], whereas 1,25-dihydroxyvitamin D_3 has been reported to antagonize the effect of ET-1 [24]. Hence, 1α,25-dihydroxyvitamin D_3 seems to possess the properties to on one hand induce production of ET-1 and on the other hand to antagonize its functional response. This may relate to differential regulation of ET-1 by 1α,25-dihydroxyvitamin D_3 in different cell types.

DC, TLR and ET-1 have all been shown to be crucially involved in transplantation and autoimmune disorders. Shedding of constituents of the glycocalyx, the protective layer of EC, has been demonstrated in I/R injury as well as under conditions of hyperglycemia. Our data suggest that the interplay of the endothelin system with the innate immune system could be of interest in the context of novel immunomodulatory therapies.

Material and Methods

Generation and stimulation of human monocyte-derived DC (MoDC)

PBMC were isolated from buffy coats obtained from healthy blood donors (Regional Red Cross Blood Donation Center, Bern, Switzerland) by density gradient centrifugation over Ficoll-Paque (Amersham, Uppsala, Sweden). Monocytes were isolated from PBMC as described previously [15, 25, 26]. Purified monocytes were incubated for 6 days in RPMI 1640 medium (Invitrogen Life Technologies) containing 10% FCS (Amimed / BioConcept), 1% [2mM] L-Glutamine (Invitrogen), 1% [100 U/ml] Penicillin/Streptomycin (Invitrogen), 10 ng/ml GM-CSF (R&D Systems Europe Ltd, Abingdon, Oxon, UK), and 10 ng/ml IL-4 (R&D) to generate MoDC as described initially by Sallusto and Lanzavecchia [27]. The cells were kept at 37°C in a 5% CO_2 humidified atmosphere. On day three the culture medium was replaced with fresh medium. For induction of maturation, hyaluronan (HA, Sigma), lipoteichoic acid (LTA, Sigma), lipopolysaccharide (LPS, Sigma) or heparan sulfate (HS, Seikagaku, Tokyo, Japan) were added to the cultures for 24 or 48 hours.

LPS removal affinity resin (END-X B15) was purchased from Associates of Cape Cod (Falmouth, MA, USA) to remove potential LPS contamination in HS. Treatment of HA with polymyxin B (Sigma), an inhibitor of LPS, did not affect biological activity of HA.

Maturation of cells was examined with the light microscope to confirm aggregation of the cells, which is characteristic for mature MoDC [28]. Additionally, analysis of CD86 expression was performed in some experiments by flow cytometry.

Generation of alternatively-activated DC (AA-DC)

Immature MoDC were pretreated with 1α,25-dihydroxyvitamin D_3 (Sigma; active form of Vitamin D_3), dexamethasone (Sigma) or prostaglandin E_2 (Sigma) at the

indicated concentrations for one hour [9]. Afterwards, cells were stimulated with LPS (1 μg/ml) for 48 hours.

Flow cytometric analysis

Cells were incubated with FITC-labeled monoclonal antibody (mAb) against CD86 (BD, Franklin Lakes, NJ, USA) and Isotype Control IgG1 (BD). Measurements were performed with a BD FACScan flow cytometer and the data analyzed using FlowJo (Tree Star Inc., Ashland, OR, USA).

Cytokine assays

MoDC (10^6 cells/ml) were treated with LPS and/or 1,25-dihydroxyvitamin D_3, PGE_2 or dexamethasone for 48 hours. The cell culture supernatants were analyzed using a Luminex multiplex array system from Bio-Rad (Bio-Rad, Hercules, CA, USA) for IL-10 and VEGF according the manufacturer's instructions.

Quantitative Real Time PCR

Gene expression analysis of ET-1, ET_A and ET_B receptors were evaluated using quantitative reverse transcription (RT)–PCR with real-time detection using TaqMan probes (Perkin-Elmer Applied Biosystems, Perkin–Elmer Europe B.V., Rotkreuz, Switzerland). Total RNA was isolated from cell pellets using TRIzol reagent (Invitrogen Life Technologies, Paisley, U.K.) based on the single step guanidinium acid–phenol method. cDNA samples were used for real-time quantitative PCR, performed with the ABI Prism 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as endogenous control. Quantitative values were obtained from the threshold cycle number at which the increase in signal associated with an exponential growth of PCR products was detected (C_T). Each sample was normalized to its content of the endogenous control, GAPDH, to standardize the amount of total RNA added to each reaction (ΔC_T). ΔC_T values were used for statistical analyses. *N* target = $2^{(DC_T sample-DC_T control)}$.

Measurement of Endothelin-1 (ET-1)

ET-1 levels were determined by specific radioimmunoassay after solid phase extraction on C18 reverse phase columns as previously described by Shaw *et al.* [29].

Statistical analysis

Data are presented as mean ± standard deviation representing experiments with up to 3 different donors. Unpaired Students *t-tests* were performed for evaluation of significance. Differences were considered as statistically significant at p-values less than 0.05. Data were analyzed using GraphPad Prism software 4.0 (GraphPad, San Diego, CA).

Acknowledgements

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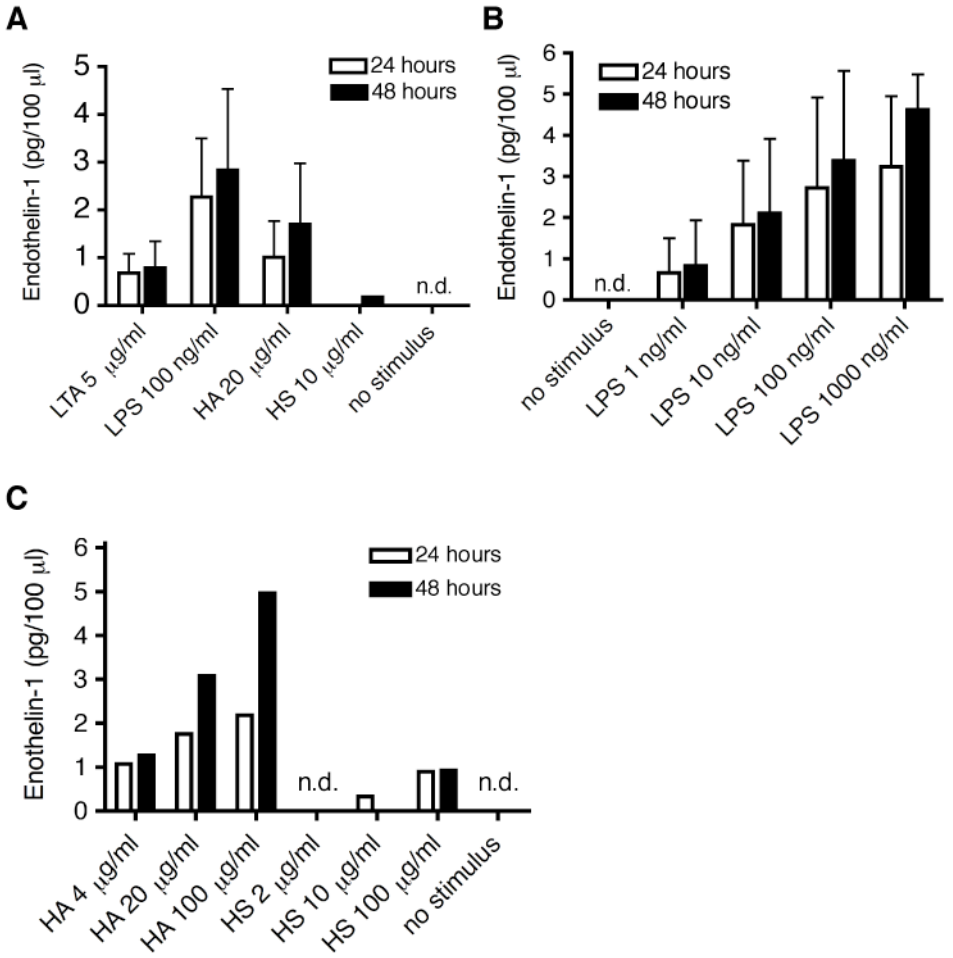

Figure 1: Activation of MoDC by selective TLR agonists induces secretion of ET-1. (A), MoDC were stimulated with LTA (5 µg/ml), LPS (100 ng/ml), HA (20 µg/ml) and HS (10 µg/ml) for 24 or 48 hours. Afterwards, supernatants were evaluated for the amount of secreted ET-1. Data represent mean ± SD of three independent experiments with cells from different donors. (B), MoDC were stimulated with LPS (1, 10, 100 and 1000 ng/ml) for 24 or 48 hours. Data represent mean ± SD of three independent experiments with cells from different donors. (C), MoDC were stimulated with HA (4, 20 and 100 µg/ml) and HS (2, 10 and 50 µg/ml) for 24 or 48 hours. One representative donor out of two is shown. n.d.: not detectable.

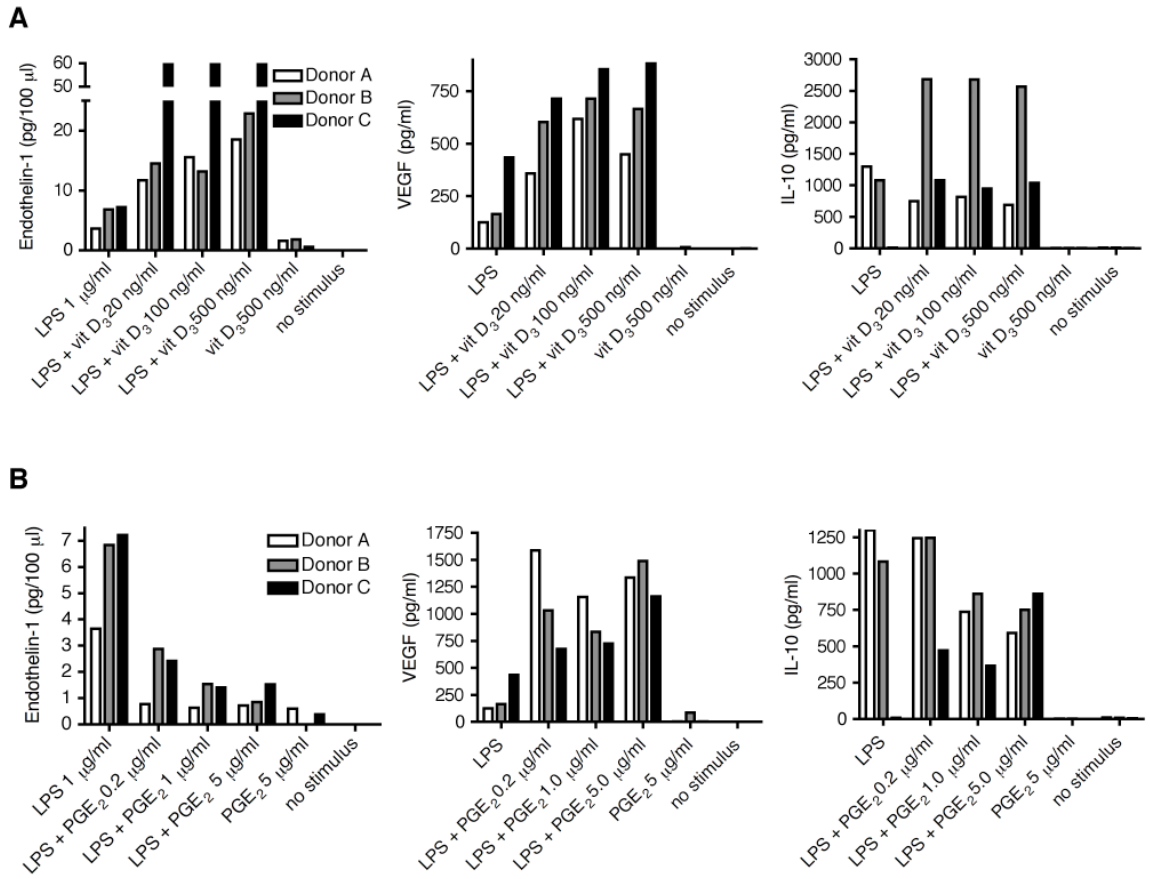

Figure 2: Secretion of ET-1 by AA-DC. (A), MoDC were pretreated with 1,25-dihyroxyvitamin D_3 at the indicated concentrations for one hour. Afterwards, cells were stimulated with LPS (1 µg/ml) for 48 hours. Supernatants were assayed for the amount of secreted ET-1, VEGF and IL-10. (B), MoDC were pretreated with PGE_2 at the indicated concentrations for one hour. Afterwards, cells were stimulated with LPS (1 µg/ml) for 48 hours and supernatants assayed for the amount of secreted ET-1, VEGF and IL-10. Values from three different individuals are shown as column-graphs.

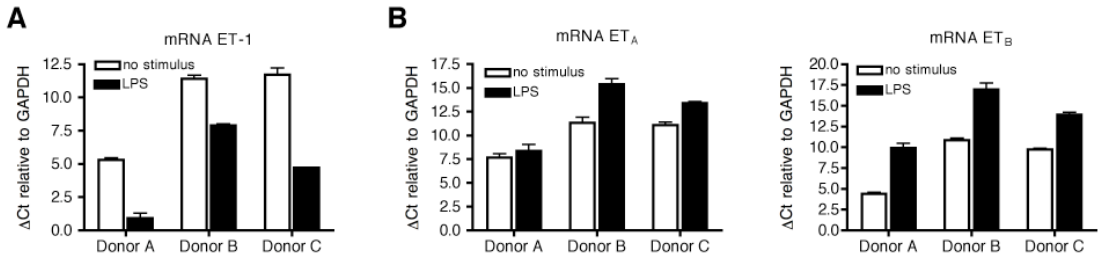

Figure 3: Influence of LPS stimulation on ET-1, ET_A and ET_B mRNA. (A), MoDC were stimulated with LPS or 48 hours. Afterwards, mRNA levels were by qRT-PCR for ET-1. ΔCt values of mRNA for ET-1 relative to GAPDH are indicated at the y-axis. (B) ΔCt values of mRNA of ET_A and ET_B relative to GAPDH are indicated at the y-axis. Data represent mean ± SD of triplicates of cells from different donors.

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4 General Discussion and Outlook

The role of the innate immune system in organ transplantation has often been neglected. It has been shown that targeting the adaptive immune system by immunosuppressive regimens could not prevent chronic allograft failure (174). In addition, long-term immunosuppression of patients has revealed severe side effects associated with infections, cancer, nephrotoxicity, liver disease and graft vasculopathy (175). The induction of donor-specific graft tolerance would represent the ideal solution to avoid these negative complications. Unfortunately, tolerance induction is experimentally achievable in small rodents, but fails up to now in a clinical setting. Prolonged ischemia time of the graft with subsequent reperfusion, as well as brain death of the donor, have been considered to play a major role in the failure of tolerance induction protocols in humans (3, 4, 174). Inhibition of the complement system has been demonstrated to prolong graft survival and to attenuate I/R injury (176). Furthermore, in accordance with the danger model introduced by Matzinger, it has been suggested that I/R injury leads to a release of danger signals, which are able to induce activation of various cells of the innate immune system through ligation to PRR (68). Recent studies revealed an important role of TLR2 and TLR4 in mediating an early proinflammatory reaction in I/R injury (75, 77, 78, 177). DC as professional APC provide a crucial link between the innate and adaptive immune system. In organ transplantation, they are involved in priming effector T cells to accelerate graft rejection. On the other hand, they can induce regulatory T cells and promote graft acceptance (129).

DXS, a highly sulfated polyglucose, has been shown to inhibit all three major activation pathways of the complement system (178). Furthermore, our group has demonstrated that DXS protect graft EC from complement-mediated damage in xeno- as well as in allotransplantation *in vivo* (167, 168). According to these previous findings in our laboratory, we hypothesized that DXS, beside its protective effect on the endothelium, affects activation of innate immune cells, in particular DC. I/R injury leads to dramatic changes on the surface of EC, which leads to a shedding of HS (85, 86). Subsequently, soluble HS could act as danger signal for DC leading to a TLR4 mediated activation (88). A main objective of this work was to evaluate the effect of DXS on HS-induced maturation of DC. We could observe an inhibitory effect of DXS on TLR4-mediated activation of human MoDC *in vitro*, as shown by a reduced upregulation of costimulatory molecules such as CD40, CD80 and CD86. In

addition, production of proinflammatory cytokines IL-1β, IL-6, IL-12 and TNF-α was significantly reduced (Paper I). The functional consequence of the observed effect has been confirmed with a low capacity of these MoDC to induce a T cell proliferation. Neutralizing of TNF-α and IL-6 has been suggested to promote a tolerance-inducing environment (179). Interestingly, TLR-induced production of IL-6 has been demonstrated to inhibit the regulatory function of Treg (73). In addition, IL-6 and TGF-ß lead to differentiation of T cells into IL-17 producing Th17 cells and further suppress Treg induction (71). Moreover, IL-12 is a crucial cytokine of polarizing a Th1 mediated graft rejection (180). In addition, blockade or absence of the costimulatory signal has been shown in several studies to induce T cell anergy and to prolong graft survival (181).

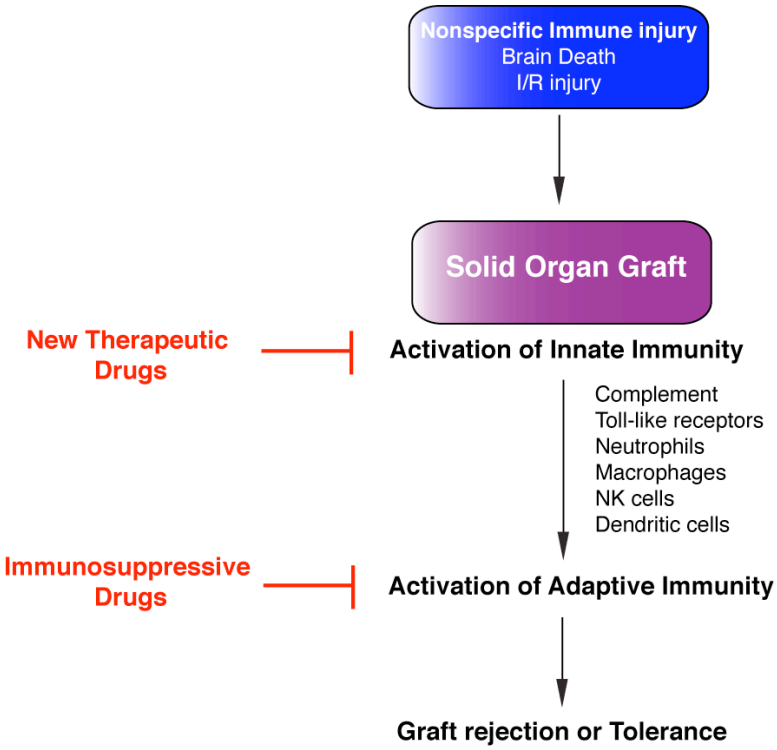

Figure 12: Graft rejection as well as tolerance induction are influenced by the innate immune system. Brain death or I/R injury activate the innate immune system, which leads to a release of danger signals. Danger signals or DAMP activate complement and TLR bearing innate immune cells.

Graft-specific production of complement by non-hepatic cells has shown to accelerate graft rejection (32). Others as well as ourselves could demonstrate that DC produce the complement proteins C1q and C3. Treatment of MoDC with DXS decreases C1q production, whereas C3 is increased. The increase of C3 might be explained by an enhanced secretion of TGF-β, which has been shown to increase C3

production in monocytes via the protein kinase-c (PKC) pathway *in vitro* (182, 183). Furthermore, the proinflammatory cytokine IL-1α has been shown to increase C3 synthesis by human hepatoma-derived HepG2 cells (184). It needs to be examined if the elevated levels of C3 in our setting are due to elevated TGF-β or IL-1α production of these DXS treated MoDC.

DC not only prime T cells, but also intensively interact with other cells of the immune system, such as neutrophils, EC and NK cells. An increasing amount of studies revealed an important cross-talk between NK cells and DC. Not many studies so far investigated the influence of TLR on the activation of NK cells, which almost express all described TLR. We could show that stimulation of NK cells with exogenous TLR2 agonists induces activation, which has been confirmed by secretion of IFN-γ and up-regulation of CD25, CD56, CD69, NKp44 and DNAM-1 expression, whereas the endogenous TLR4 agonists HA did not result in activation (Paper II). Furthermore, treatment of NK cells by DXS prevented TLR2-mediated activation. It remains to be evaluated if endogenous agonists of TLR2, such as e.g. serum amyloid A or HMGB-1, have the capacity to induce activation of NK cells. Regarding the important described cross-talk between NK cells with DC, it would be of interest to investigate, if DXS interferes with the reciprocal activation pathway between these two cell types.

Given the importance of TLR2 and TLR4 in I/R injury, the observed novel inhibitory effect of DXS on DC as well as NK cells *in vitro*, may contribute for the observed beneficial effect of DXS *in vivo*. Most of the described agents in the literature, which modulate or inhibit DC or NK cell function, have no inhibitory effect on complement activation. Therefore, DXS might be a very interesting candidate in organ transplantation to target many different mediators of an innate immune response and thereby attenuate I/R injury. But in a setting of transplantation, DXS has to be combined with another agent, like e.g. cyclosporine or an anti-CD4 antibody (as published by us), as it is on its own not sufficient to prevent graft rejection. In addition, further investigations targeting the molecular mechanisms of the inhibitory effect of DXS are of great interest. Different mechanisms could account for the observed effects. DXS might bind soluble components of the TLR signaling complex such as soluble CD14, MD-2 or lipid binding protein (LBP). It might also be possible that DXS induces the expression of molecules, which suppresses maturation.

Induction of heme-oxygenase 1 (HO-1), a protein involved in heme catabolism with cytoprotective properties, by cobalt protoporphyrin has been demonstrated to prevent DC maturation (185). Enhanced expression of HO-1 in the context of DXS-treatment has been shown in an earlier study of our laboratory in a xenotransplantation setting (167), whereas no significant changes of intragraft mRNA levels of HO-1 were detected in a previous published allotransplantation model (168). Our results indicate that the inhibitory effect of DXS is not due to receptor-mediated interactions with DC-SIGN or CR3. But there would still be the possibility that other sugar-binding receptors are involved as e.g. macrophage galactose-type lectin (MGL), which has been associated with tolerogenic properties of DC (186) and downregulates T cell effector function (187).

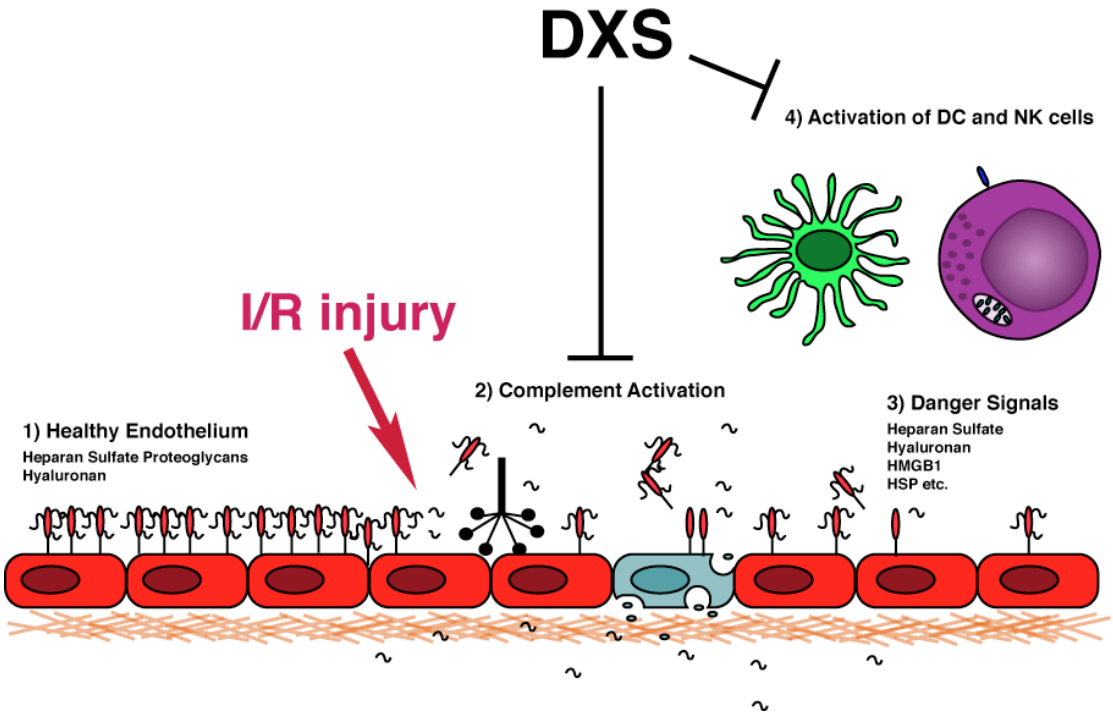

Figure 13: Effect of DXS in I/R injury. I/R injury leads to a release of danger signals, which induce activation of DC and NK cells. DXS prevents activation of DC, NK cells as well as complement.

In another study (Paper III), we could demonstrate that rHDL exerts an inhibitory effect on DC maturation. rHDL has been shown to have protective effects on blood vessels as well as to prevent activation of monocytes. Our data provided insights into a novel effect of rHDL on TLR induced activation of DC. We could demonstrate a strong effect of rHDL on HA (TLR4) and LTA (TLR2) induced activation of MoDC. Furthermore, our data show inhibition of the activation of monocytes and neutrophils

in a whole blood assay. Inhibition of neutrophil activation might further enhance the effect on DC, as it has been shown that activated neutrophils could induce maturation of DC in a cell-contact manner via interactions of CR3 with DC-SIGN. The pleiotropic inhibitory effect of rHDL on innate immune cells would be favorable in a setting of organ transplantation in order to attenuate inflammation and impede the link between the innate with the adaptive immune system.

The effect of DXS on the maturation of DC has to be further examined in an appropriate *in vivo* model. Our data provide novel evidence that these two substances might have an impact on the maturation-process of DC, which would be helpful in a setting of organ transplantation. Combination with other immunoregulatory or immunosuppressive reagents might therefore provide a novel tolerance inducing protocol, considering the more clinical relevant setting of a prolonged ischemic time of the donor organ.

Another possible application of DXS in organ transplantation might be the generation of maturation-resistant MoDC *in vitro* for the use of cell-based therapies in transplantation. Based on our data, DXS was shown, after prolonged exposure with subsequent removal of the substance, to generate maturation-resistant DC. Generation of maturation resistant donor- as well as recipient DC has been shown in animal models to prolong graft survival. In addition, DXS might be a suitable substance as constituent of organ preservation-solutions, protecting the endothelium and influencing intragraft passenger-leukocytes. DXS was shown to have an anti-inflammatory effect when added to blood cardioplegia solution in a porcine model of cardiopulmonary bypass (188). Similar applications could be considered for rHDL.

According to our new finding that DXS prevents TLR mediated immune responses, it might be considered to extend the use of this substance. The involvement of TLR has been shown in autoimmune diseases (189-191) as well as in sepsis (192). It would be of interest to examine whether treatment with DXS would reveal a beneficial effect in these diseases.

Further investigations were performed on the effect of TLR stimulation in human DC in the context of hypoxic conditions, HIF-1α expression and ET-1 secretion. Ischemia is always associated with hypoxic conditions. Our data indicate a contribution the transcription factor HIF-1α in TLR-induced maturation of human MoDC (Paper IV). The transcription factor HIF-1α enhances myeloid cell

inflammation *in vivo*, and others as well as ourselves could show that maturation of MoDC by LPS is enhanced under hypoxic conditions. It needs to be evaluated if HIF-1α exerts a similar effect in I/R injury, enhancing TLR-mediated inflammation and maturation of DC. In a model of sepsis with mice with a conditional knock-out of HIF-1α in the myeloid cell lineage, reduced levels of proinflammatory cytokines were found (99). Therefore, inhibition of this transcription factor might be a novel target to attenuate endogenous TLR-mediated maturation of DC. However, contribution of HIF-1α in DC maturation *in vivo* needs to be examined. In contrast, the systemic induction of the HIF-1α pathway by erythropoietin (EPO) was shown to attenuate renal I/R injury (193). Obviously, HIF-1α exerts distinctive effects in different cells. In addition, it would be interesting to investigate the influence of DXS on HIF-1α. Moreover, analysis of HIF-α expression and function in other DC subsets like plasmacytoid DC needs to be investigated.

In the same line, our data show that stimulation of human DC by exogenous as well as endogenous TLR agonists induce production of the vasoactive peptide ET-1 (Paper V). Expression of ET-1 is controlled by the transcription factor HIF-1α. Elevated ET-1 has been associated with I/R injury (103, 194). According to our results, TLR stimulated DC might be considered as a potential additional source of ET-1 in I/R injury and thus might represent a novel target for reduction of ET-1 production. DXS was shown to reduce tissue ET-1 levels (188). If DXS also prevents ET-1 secretion by human DC has to be evaluated. In addition, the capacity of pDC to secrete ET-1 in response to TLR stimulation needs to be determined.

In conclusion, this work provides novel results on inhibitory effects of DXS on maturation of MoDC, which might complement the protective effect on the graft endothelium observed *in vitro* and *in vivo*. In addition, as DXS seems to exert an anti-inflammatory effect on many different members of the innate immune system, it might be considered as an additional regimen in a combined tolerance inducing protocol in a more clinically relevant setting. Moreover, new data providing a link between the expression of the transcription factor HIF-1α and TLR induced maturation of human MoDC have been provided. We could also show that stimulation of MoDC with TLR agonists induces the secretion of ET-1. Both results provide new indications for modulation of DC biology and might be useful for the development of new experimental or therapeutic approaches targeting DC.

5 References Introduction and General Discussion

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6 Appendix

6-1 Acknowledgements

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6-2 Curriculum vitae

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01.2002-07.2003	Undergraduate Assistant at the Botanical Institute of the University of Basel, Section Plant Physiology
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08.2002-10.2002	Teacher (Representative) at the Bezirksschule Grenchen in Mathematics, Physics and Biology
08.1997-10.1998	Trainee program in banking for high school alumni; CREDIT SUISSE Zurich

Awards and Prizes

Poster Prize, 5th International Conference on Innate Immunity, Chania, Greece

Travel Award, 5th International Conference on Innate Immunity, Chania, Greece

Travel Award, 11th European Meeting Complement in Human Disease, Cardiff, UK

6-3 List of publications, abstracts and presentations

Publications in peer-reviewed journals

Spirig, R., C. van Kooten, C. Obregon, L. Nicod, M. Daha and R. Rieben. 2008. The complement inhibitor low molecular weight dextran sulfate prevents TLR4 induced phenotypic and functional maturation of human dendritic cells. *The Journal of Immunology 181: 878-890*

Daubenberger, C.A., ***R. Spirig***, M.E. Patarroyo and G. Pluschke. 2007. Flow cytometric analysis on cross-reactivity of human-specific CD monoclonal antibodies with splenocytes of *Aotus nancymaae*, a non-human primate model for biomedical research. *Veterinary Immunology and Immunopathology 119: 14-20*

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Reviews

Spirig, R., T. Gajanayake, O. Korsgren, B. Nilsson and R. Rieben. 2008. Low Molecular Weight dextran sulfate as complement inhibitor and cytoprotectant in solid organ and islet transplantation. *Molecular Immunology 45: 4084-4094*

Manuscripts in preparation/submitted

Spirig, R., S. Djafarzadeh, T. Regueira J. Takala, S.M. Jakob, R. Robert and P.M. Lepper. Selective TLR agonists induce activation of HIF-1α in human dendritic cells in the absence of hypoxia. *Manuscript submitted for publication*

Spirig, R., I. Potapova, J. Boden-Shaw, R. Rieben and S. Shaw. TLR2 and TLR4 stimulation leads to production of ET-1 in human DC. *Manuscript in preparation*

Spirig, R., A-L. Millard, N.J. Müller, J.D. Seebach and R. Rieben. Inhibition of TLR2 induced activation of human natural killer cells by the complement inhibitor low molecular weight dextran sulfate. *Manuscript in preparation*

Spirig, R., A. Schaub, S. Miescher, P. Lerch, M. Spycher and R. Rieben. Inflammatory reactions of human myeloid innate immune cells are prevented by reconstituted high-density lipoprotein. *Manuscript in preparation*

Published Abstracts

Spirig, R., A-L. Millard, J.D. Seebach and R. Rieben. 2008. The complement inhibitor low molecular weight dextran sulfate prevents TLR2 mediated activation of human natural killer cells. *Molecular Immunology 45: 4095-4182*

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Spirig, R., Carolina Obregon, Laurent Nicod and Robert Rieben. 2006. The cytoprotectant low molecular weight dextran sulfate inhibits toll-like receptor induced phenotypic and functional maturation of human dendritic. *American Journal of Transplantation 6: 4-1145*

Professional Memberships

Swiss Society of Allergology and Immunology
Swiss Society of Laboratory Animal Science
Swiss Cardiovascular Research and Training Network

Oral presentations at international conferences

Spirig R., S. Djafarzadeh. J. Takala, S.M. Jakob, R. Robert and P.M. Lepper. Induction of hypoxia-inducible factor 1 alpha by toll-like receptors in human dendritic cells; World Immune Regulation Meeting II, 17.-20.03.08, Davos, Switzerland

Spirig, R., C. van Kooten, C. Obregon, L. Nicod, M. Daha and R. Rieben. Modulation of toll-like receptor mediated maturation of professional human antigen-presenting cells by the complement inhibitor low molecular weight dextran sulfate; 11th European Meeting Complement in Human disease, 8.09.-11.09.07, Cardiff, UK

Poster presentations at international conferences

Spirig, R., AL. Millard, J.D. Seebach and R. Rieben. The complement inhibitor low molecular weight dextran sulfate prevents TLR2 mediated activation of human natural killer cells. XXII International Complement Workshop, 29.9.-2.10.08, Basel, Switzerland

Millard, AL., ***R. Spirig***, R. Rieben, M.K.J. Schneider, W. Bossart, N. J. Mueller and J. D. Seebach. Low molecular weight dextran sulfate protects endothelial cells from cytomegalovirus infection. XIV International Congress of Virology, 10.08.-15.08.08, Istanbul, Turkey

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Spirig, R., C. Obregon, L. Nicod and R. Rieben. Low molecular weight dextran sulfate attenuates activation of innate immunity by inhibiting dendritic cell maturation; 7th International Conference on New Trends in Immunosuppression and Immunotherapy, 16.-19.02.06, Berlin, Germany

Oral presentations at national congresses

Spirig, R., C. van Kooten, C. Obregon, L. Nicod and R. Rieben. Modulation of dendritic cell activation by the vascular cytoprotectant low molecular weight dextran sulfate; Meeting of Swiss Immunology PhD students, 26.-28.3.07, Wolfsberg, Switzerland

Spirig, R., C. van Kooten, C. Obregon, L. Nicod and R. Rieben. Inhibition of human dendritic cell activation by the vascular endothelial protectant low molecular weight dextran sulfate; 12th Cardiovascular Biology and Clinical Implications Meeting, 5./6.10.06, Muntelier, Switzerland

Spirig, R., C. van Kooten, C. Obregon, L. Nicod and R. Rieben. Inhibition of Toll-like receptor induced phenotypic and functional maturation of human dendritic cells by the complement inhibitor low molecular weight dextran sulfate; International Summer School of the University of Bern, 15.08.06, Heiligenschwendi, Switzerland

Poster presentations at national congresses

Spirig, R., S. Djafarzadeh. J. Takala, S.M. Jakob, R. Robert and P.M. Lepper. Toll-like receptor induced expression of hypoxia-inducible factor 1α in human dendritic cells; Annual Congress Swiss Society of Allergology and Immunology, 17./18.4.08, Fribourg, Switzerland

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Spirig, R., C. van Kooten, C. Obregon, L. Nicod and R. Rieben. Low molecular weight dextran sulfate modulates toll-like receptor induced human dendritic cell activation; Annual Congress Swiss Society of Allergology and Immunology, 19./20.04.07, Basel, Switzerland

Spirig, R., C. Obregon, L. Nicod and R. Rieben. The vascular endothelial cell protectant dextran sulfate inhibits phenotypic and functional maturation of human dendritic cells; Annual Congress Swiss Society of Cardiology, 7.-9.06.06, Basel, Switzerland

Spirig, R., C. Obregon, L. Nicod and R. Rieben. The endothelial cell protectant low molecular weight dextran sulfate inhibits phenotypic and functional maturation of dendritic cells; Annual Congress Swiss Society of Allergology and Immunology, 30./31.03.06, Zurich, Switzerland

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Spirig, R., C. Obregon, L. Nicod and R. Rieben. Vascular endothelial cell protection prevents maturation of human dendritic cells; Annual Meeting SCARTNet, 27.01.06, Bern, Switzerland

Spirig, R., C. Obregon, L. Nicod and R. Rieben. Consequence of vascular endothelial cell protection on antigen presentation by dendritic cells; 11th Cardiovascular Biology and Clinical Implications Meeting, 6.-8.10.05, Thun, Switzerland

6-4 Declaration of Originality

Last name, first name: **Spirig Rolf**

Matriculation number: **98-056-658**

I hereby declare that this thesis represents my original work and that I have used no other sources except as noted by citations.
All data, tables, figures and text citations which have been reproduced from any other source, including the internet, have been explicitly acknowledged as such.
I am aware that in case of non-compliance, the Senate is entitled to divest me of the doctorate degree awarded to me on the basis of the present thesis, in accordance with the "Statut der Universität Bern (Universitätsstatut; UniSt)", Art. 20, of 17 December 1997.

Place, date Signature

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