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REGULATION OF DENDRITIC CELL DIFFERENTIATION, MATURATION AND ACTIVATION

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发光的不一定是金子，但是金子总会发光的。

All that glitters is not gold, but gold will glitter forever.

To my parents and my family

ABSTRACT

The human immune system efficiently protects the host from exogenous pathogens such as bacteria, virus and parasites and endogenous threats such as damaged cells and tumors. Invasion of pathogens and other threats activate the innate immunity causing secretion of pro-inflammatory cytokines with the subsequent antigen presentation and activation of the adaptive immunity resulting in T and B cell responses. The professional antigen presenting cell (APC), the dendritic cell (DC), is crucial for connecting the innate and adaptive immune system. DCs have a critical role for activating efficient immune responses, and they are key targets for negative regulatory mechanisms that ensure adequate inflammatory responses. In addition, based on their unique capacity, DCs have been used in many immunotherapy trials since DC based vaccines have demonstrated an ability to induce anti tumoral immunity. However, many aspects of DC biology are still unexplored, especially the regulation of DC differentiation, maturation and activation.

The aim of this thesis was to investigate the regulating mechanisms of novel regulators and conventional chemotherapeutic drugs and their effect on the differentiation, maturation and activation of DCs.

Initially, we investigated the gene expression of suppressor of cytokine signaling (SOCS) members and their regulating role in LPS induced DC maturation. We showed that SOCS2, SOCS3 and SOCS6 are significantly induced after LPS treatment, and that SOCS2 influences the maturation of human monocyte-derived DC (moDC). Furthermore, we demonstrated that various toll-like receptor (TLR) ligands induce SOCS2 gene expression in human DCs, and that TLR4 signaling regulates SOCS2 transcription in an autocrine/paracrine type I IFN loop via STAT3 and STAT5. Using SOCS2 deficient mouse, we revealed that although SOCS2 does not regulate murine lymphoid DC differentiation *in vivo*, SOCS2 is necessary for the differentiation of GM-CSF and IL-4 induced DCs *in vitro*, likely by regulating GM-CSF signaling. Similar to human DCs, SOCS2 also affects LPS induced mouse DC maturation, and thereby regulates the antigen presenting ability of DCs with consequences for activation of $CD4^+$ T cell. In this thesis, we also investigated the effect of conventional chemotherapeutic drugs on human DCs. We demonstrated that Dexamethasone, Doxorubicin, Cisplatin and Irinotecan inhibit the differentiation of human moDC to various extents. However, Cisplatin treatment of human DCs leads to increased T cell activation, a potentially beneficial effect of Cisplatin mediated by the increased expression of IFN-β cytokine.

In conclusion, we have demonstrated that SOCS2 positively regulates the differentiation, maturation and antigen presenting ability of DCs. Furthermore, TLR4 signaling regulates SOCS2 transcription in an autocrine/paracrine type I IFN loop. The differentiation of human moDC may be negatively influenced by Cisplatin, but treatment of human DCs may cause increased T cell activation, a finding deserving clinical exploration.

LIST OF PUBLICATIONS

I. Jin Hu, Ola Winqvist, Amilcar Flores-Morales, Ann-Charlotte Wikström, Gunnar Norstedt.
SOCS2 Influences LPS Induced Human Monocyte-Derived Dendritic Cell Maturation.
PLoS ONE. 2009 Sep 25; 4(9): e7178.

II. Jin Hu, DaoHua Lou, Berit Carow, Malin E. Winerdal, Martin Rottenberg, Ann-Charlotte Wikström, Gunnar Norstedt, Ola Winqvist.
LPS regulates SOCS2 transcription in a Type I interferon dependent autocrine-paracrine loop.
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III. Jin Hu, Berit Carow, Ann-Charlotte Wikström, Martin Rottenberg, Gunnar Norstedt, Ola Winqvist.
SOCS2 expression in bone marrow derived dendritic cells is a positive regulator of T cell activation.
Manuscript.

IV. Jin Hu, Johan Kinn, Ali Zirakzadeh, Amir Sherif, Gunnar Norstedt, Ann-Charlotte Wikström, Ola Winqvist.
The effects of chemotherapeutic drugs to human monocyte-derived DC differentiation and antigen presentation.
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Publications not included in the thesis

Carolina Gustavsson, Paolo Parini, Jovanca Ostojic, Louisa Cheung, Jin Hu, Fahad Zadjali, Faheem Tahir, Kerstin Brismar, Gunnar Norstedt, Petra Tollet-Egnell.
Cocoa butter and safflower oil elicit different effects on hepatic gene expression and lipid metabolism in rats.
Lipids. 2009 Nov;44(11):1011-27.

CONTENTS

LIST OF ABBREVIATIONS

APC	Antigen presenting cell
BCR	B cell receptor
BM	Bone marrow
BMDC	Bone marrow derived dendritic cell
BMM	Bone marrow derived macrophage
CDP	Common-DC progenitor
CIS	Src homology 2 domain-containing protein
CpG	Cytidine-phosphate-guanosine
CTL	Cytotoxic T lymphocyte
DAMP	Danger-associated molecular pattern
DC	Dendritic cell
dsRNA	Double-stranded RNA
Flt3L	FMS-like tyrosine kinase 3 ligand
GM-CSF	Granulocyte-macrophage colony-stimulating factor
iDC	Immature dendritic cell
IFN	Interferon
IL	Interleukin
IRAK	Interleukin 1-associated kinase
IRF	Interferon regulatory factor
JAK	Janus kinase
LPS	lipopolysaccharide
MAL	MyD88-adaptor-like protein
MAPK	Mitogen-activated protein kinase
MDA5	Melanoma differentiation-associated gene 5
MDP	Macrophage-DC progenitors
MHC	Major histocompatibility complex
MUC-1	Mucin-1
MyD88	Myeloid differentiation factor 88
mDC	Myeloid dendritic cell
moDC	Monocyte-derived dendritic cell
NF-κB	Nuclear factor kappa B
NLR	Leucine-rich repeat-containing receptor
NOD	Nucleotide-binding oligomerization
OVA	Ovalbumin
PAMP	Pathogen-associated molecular pattern
pDC	Plasmacytoid dendritic cell
pre-DC	Precursor dendritic cell
PRR	Pattern recognition receptor
RIG	Retinoic acid-inducible gene
RLR	Retinoic acid-inducible gene (RIG)-I-like receptor
SH2	Src homology 2
SIGIRR	Single immunoglobulin IL-1related protein
siRNA	Small interfering RNA
SLE	Systemic lupus erythematosus

SOCS	Suppressor of cytokine signaling
ssRNA	Single-stranded RNA
ST	Suppressor of tumorigenicity
STAT	Signal transducer and activator of transcription
TAK1	TGF beta activated kinase 1
TCR	T cells receptor
TGF-β	Transforming growth factor-β
TH	T helper
Tip DC	TNF-iNOS-producing DC
TIR	Toll-IL-1 receptor
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-α
TOLLIP	Toll-interacting protein
TRAF	TNF receptor-associated factor
TRAIL	TNF-related apoptosis-inducing ligand
TRAILR	TNF-related apoptosis-inducing ligand receptor
TRAM	TRIF-related adaptor molecule
Treg	Regulatory T cells
TRIF	TIR domain-containing adaptor
TSLP	Thymic stromal lymphopoietin

1 INTRODUCTION

The human immune system efficiently protects the host from exogenous pathogens such as bacteria, virus, parasites and endogenous pathogens such as tumors and damaged cells.

Based on the functional mechanisms, the immune system can be divided into the innate and adaptive parts. The innate immune system, found originally in most primitive unicellular organisms, provide the first line of defense through physical barriers, antimicrobial peptides and germ-line encoded receptors. Receptors of the innate immunity recognize evolutionarily conserved molecules and patterns in microbes. Cells of the innate arm respond quickly upon receptor engagement by phagocytosis or secretion of inflammatory mediators.

As an outcome of long term evolution, the adaptive immune system is a strong complementation to the non-specific, non-memory generating innate immune system. It is developed mainly in jawed vertebrates, and mediates specificity and memory to the immune response through the diversity of T- and B- cell receptors and clonal expansion of antigen recognizing lymphocytes, when any foreign peptide and/or structure is recognized.

DCs, as highly professional antigen presenting cells (APC), have a unique ability to connect the innate and adaptive immune system. When pathogens appear, DCs recognize them and activate innate responses, and subsequently process them and present them to lymphocytes for activating the adaptive immune system and causing an anti-pathogenic response. Based on the unique ability in immune system, DCs draw a huge interest for the potential usage as anti-tumor vaccines in the clinic. However, the mechanisms regulating the DCs biological processes and functions are still far from being understood.

This thesis deals with the regulation of DC differentiation, maturation and activation in connection to SOCS proteins and chemotherapeutic drugs.

1.1 INNATE IMMUNITY AND ADAPTIVE IMMUNITY

During evolution, the human body has developed many defensive mechanisms against invading pathogens, including bacteria, viruses, fungi and parasites, as well as protection against internal harmful components, such as tumors and damaged cells. For the invading pathogen, the keratinized epithelium forming skin on the body surface and the epithelia lining mucosae on the internal surfaces of the respiratory, gastrointestinal and urogenital tracts form the physical barrier to repel most mediocre attacks. At the same time, the epithelia secret mucus containing glycoproteins, proteoglycans and enzymes to form the chemical barrier to protect the epithelial cells from damage and limit the infection. These secreted substances work in different ways for this purpose. The enzyme lysozyme which is present in tears and saliva is an antibacterial substance. The mucus in the respiratory tract is continually propelled upwards, to discard harmful microorganisms that are breathed in. The acidic environments created by mucus on the surface of the stomach, vagina and skin prevent pathogenic growth. Through these physical and chemical barriers most microbes are prevented from gaining access to the cells and tissues of the body, however, they are not generally considered as a part of the immune system proper. When these barriers are overcome we are dependent on the immune system for our defense [1].

The immune system of jawed vertebrates is composed of innate and adaptive immune parts. They are distinguished mainly through the types of receptors used for pathogen recognition [2]. The innate immune system includes neutrophils, monocytes, macrophages, DCs, natural killer cells, mast cells, basophils and eosinophils cellular component [3]. The innate immune system causes immediate, unspecific responses to the invading pathogens, and the responses can be divided into two parts: pattern recognition and effector mechanism. The innate immune system specifically recognizes conserved, invariant and common structural patterns, known as pathogen-associated molecular patterns (PAMPs) [2] or danger-associated molecular patterns (DAMPs) [4] through distinct pattern recognition receptors (PRRs), which are germ-line encoded.

A variety of PRRs can be broadly categorized into secreted, trans-membrane and cytosolic classes. Secreted PRRs, which include collectins, ficolins, and pentraxins, mainly bind to microbial cell surfaces, activate the complement system and opsonize pathogens for phagocytosis [5]. The trans-membrane PRRs include the toll-like receptor (TLR) family, which are involved in bacterial product recognition or viral nucleic acid recognition [6] and will be further described in section 1.3, and the C-type lectins family, of which two members, dectin-1 and -2, are involved in anti-fungal immunity [7,8,9]. The cytosolic PRRs include Retinoic-acid-inducible gene (RIG)-I-like receptors (RLRs), leucine-rich repeat-containing receptors (NLRs), melanoma differentiation-associated gene 5 (MDA5) and DNA-dependent activator of interferon-regulatory factors (DAI). Members of RLRs and RIG-I both recognize single-strand RNA (ssRNA) and some dsDNA viruses, whereas MDA5 recognizes long double-stranded RNA (dsRNA) structures [2,10], and viral DNA is detected by DAI [11]. Based on the N-terminal domains, NLRs are further categorized into three subfamilies: nucleotide-binding oligomerization (NOD), NACHT, LRR and PYD domains-containing protein (NALP) and the NAIP subfamily. They sense bacterial peptidoglycans, microbial products, metabolic stress forms and noninfectious crystal

particles [12]. Parasite recognition is not well understood for the invertebrate phyla. Chitin, a main parasite-associated cell wall component, was found to induce eosinophil recruitment [13]. In addition, an indirect recognition system was proposed, and it is based on parasite released enzymes, which are used for invading and degrading tissues that could be recognized later by innate system [14].

The pattern recognition leads to endocytosis by effector cells, and internalized pathogens are destroyed in the phagosome. Phagocytes also produce several important mediators of innate immunity, such as cytokines and chemokines, to interact with or induce other cells for an enhanced inflammatory response. Meanwhile, the binding of PAMPs to PRRs convert phagocytes into APCs such as DCs and increase the expression of co-stimulatory molecules on the cell-surface to initiate the adaptive immune responses. Thus, the activation of adaptive immunity depends on induced molecules as a consequence of the innate immune recognition of pathogens [3,5,15].

The adaptive immune system is characterized by the ability to generate specific immune responses and memory responses; the latter is used as the base for vaccination. Compared to minutes or hours, the starting time for the innate immunity response, the adaptive immune system needs several days to start to become effective. The major reason is the specific selection process of clones recognizing the antigen and subsequent clonal expansion. The adaptive immune system includes two type cell components: B and T cells. The cell-surface receptors on those lymphocytes which recognize pathogens are called the B cell receptor (BCR) and the T cell receptor (TCR), respectively. The receptors are encoded by the genes assembling through V(D)J recombinase rearrangement with random joining of DNA gene fragments from the *Ig* and *Tcr* loci. Thus, each T and B cell has a unique antigen-binding receptor to recognize almost any antigenic molecule [16,17]. However, after the positive and negative selection in the thymus/bone marrow (BM), the T cells and B cells with a TCR/BCR that bind self-antigens are regarded as harmful and are to a large extent deleted (negative selection). The remaining and inactivated lymphocytes are called naïve lymphocytes [3].

Different activating mechanisms exist between T and B cells. The activation of naïve T cells requires recognition of antigenic peptides presented in the major histocompatibility complex (MHC) molecule on APCs. Based on which MHC is presenting the peptide, $CD8^+$ cytotoxic T cells (if the antigen is presented on MHC-I) or $CD4^+$ T helper (TH) cells (if the antigen is presented on MHC-II) are activated. However, a second signal from co-stimulatory molecules on the surface of the APCs is also required to finish the process, establishing the link to the innate immunity and preventing the reaction to self-tissue and the development of autoimmunity [18]. Naïve B cells can bind directly to antigen through their BCR. In addition most B cells need a signal from antigen activated T helper cells through the up-regulated cell surface molecule CD40L to become an effector cell [19]. Once naïve T and B cells are activated, they start to proliferate and expand to a large population with the same antigen specificity, and the term "clonal expansion" is used to describe this process. Upon formation in the BM, B lymphocytes express a unique antibody on its surface. When these naïve B cell re-encounter the same specific antigen, they rapidly

differentiate into memory B cells, which will be responsible for an immediate response upon a second encounter with the antigen [1,3].

1.2 DENDRITIC CELLS

In the late nineteenth century, Paul Langerhans first described Langerhans cells, a specialized DC type, in the skin. The term "dendritic cells" was utilized for the first time in 1973 by Ralph M. Steinman and Zanvil A. Cohn since the dendrite-like extensions they grow during cell culture [20]. DCs have been recognized for their key immune functions on pathogen recognition, activation of immediate and long-term immunity, and preservation of tolerance to self-antigens [21,22].

1.2.1 DC types

1.2.1.1 In vivo

Most studies investigating DC subsets *in vivo* have used mice. Based on cell surface marker protein expression, anatomical location, and functional responses, two major mouse categories of DCs have been demonstrated in steady state conditions (table 1): (1) nonlymphoid tissue migratory and lymphoid tissue-resident DCs and (2) plasmacytoid DCs (pDCs) [23,24,25].

The nonlymphoid tissue migratory and lymphoid tissue-resident DCs can be grouped into resident DCs and migratory DCs two main categories based on the paths they follow to access the lymphoid organs [24,25].

Resident DCs, also termed ‘conventional DCs’, exist in lymph nodes, thymus and in lymphoid tissues such as the spleen. Three mouse resident DC subsets are identified by the surface markers: $CD8\alpha^+$ DCs, $CD4^+$ DCs and $CD4^-CD8\alpha^-$ (double negative) DCs [23,24].

Migratory DCs, also referred to as ‘tissue DCs’, are found in non-lymphoid tissue of organs including skin, lung, and intestine. The migratory DCs develop from precursors in peripheral tissues and travel through the afferent lymphatics to reach the local draining lymph nodes [24]. Skin contains three DC subsets: Langerhans cells, dermal $CD103^+$ DCs and dermal $CD103^-$ DCs [26,27,28]. Subsets with similar marker characteristics are found in the intestine and in the lung, liver and kidney, for example, in the intestine $CD103^+CD11b^{lo}$ (Peyer’s patches), $CD103^-$ $CD11b^{hi}$ and $CD103^+$ $CD11b^+$ (lamina propria) [29,30], and in the lung, liver and kidney $CD103^+$ $CD11b^+$ and $CD103^-$ $CD11b^{hi}$ [31].

pDCs are characterized by producing a host of inflammatory chemokines [32] and cytokines including type I interferon (IFN) [33,34] when exposed to viruses [35], bacteria [36] and certain TLR agonists [34]. Recently, they have also been proposed to have a role in tissue repair processes implicated in wound healing [37]. Mouse pDCs are defined by their surface phenotype of $CD11c^+CD11b^-B220^+PDCA1^+SiglecH^+$ [35,38]. They reside in BM, blood, thymus, and in T cell rich areas of lymphoid organs in steady state conditions, but they can also be localized to skin and other tissues in inflammation or autoimmunity [34,36,39].

In humans, DC subtypes are less characterized due to poor accessibility to human spleen and lymphoid tissue, the absence of key murine DC subtype markers on human DC, and poor reagents to dissect the human DC subtypes. Human pDCs are phenotypically characterized as $CD11c^{-}CD45RA^{+}CD123^{+}CD4^{+}BDCA2^{+}$ $BDCA4^{+}$, and they are found in steady-state BM, spleen, thymus, lymph nodes, and the liver [40]. Human Langerhans DCs were found to express high levels of markers, such as CD1a, langerin and E-cadherin, and reside in the epidermis of skin [41]. CD8α is not expressed on human DCs, but with the new BDCA markers available, a rare $BDCA3^{+}$ DC was identified in human blood that resembles the mouse CD8α DCs [42,43]. Like murine $CD8^{+}$ DCs, they were also $CD11c^{+}MHCII^{+}CD2^{-}CD13^{+}CD16/32^{-/lo}CD33^{+}CD162^{high}CD11b^{-}HLA\text{-}DO^{+}$ and in the T-cell areas of the spleen [44,45].

Table 1 Mouse DC subsets

DC subsets			**Cell surface markers**	**location**
nonlymphoid tissue migratory and lymphoid tissue-resident DCs	Resident DCs	$CD8^{+}$ DCs	$CD11c^{+}CD11b^{-}MHC\ II^{+}$ $CD205^{+}CD4^{-}CD8\alpha^{+}$	Spleen T cell zones, lymph nodes
		$CD4^{+}$ DCs	$CD11c^{+}CD11b^{+}MHC\ II^{+}$ $33D1^{+}CD4^{+}CD8\alpha^{-}$	Spleen marginal zones, lymph nodes
		Double negative DCs	$CD11c^{+}CD11b^{+}$ $CD4^{-}CD8\alpha^{-}$	Spleen, lymph nodes
	Migratory DCs	Langerhans cells	$CD11c^{+}CD205^{+}langerin^{+}$	Skin, lymph nodes
		$CD103^{+}$ DCs	$CD103^{+}langerin^{+}CD11b^{lo}$	Skin, lamina propria peyer's patches, lung, liver, kidney, lymph nodes
		$CD103^{-}$ DCs	$CD11c^{+}CD11b^{hi}CD103^{-}$ $langerin^{-}$	Skin, lamina propria, lung, liver, kidney, lymph nodes
Plasmacytoid DCs			$CD11c^{+}CD11b^{-}B220^{+}$ $PDCA1^{+}SiglecH^{+}$	BM, blood, thymus, lymphoid organs, inflammation sites

1.2.1.2 In vitro

Most studies have been performed with *in vitro*-generated DCs. In some respects, DCs cultured *in vitro* do not show the same behavior or capability as DCs isolated *ex vivo*. Nonetheless, they are often used for research as they are still much more readily available than genuine DCs.

There are two major classifications regarding *in vitro* culture systems [46]. One is termed granulocyte-macrophage colony-stimulating factor (GM-CSF)-derived DCs. Human monocytes or mouse BM cells are cultured with GM-CSF and other cytokines such as IL-4 and IFN-α in order to get potent antigen-presenting cells, which express high levels of CD11c and MHC II and low levels of CD86 [47,48]. This culture method is widely used to generate DCs for biological investigations and immunotherapy trials [49]. The GM-CSF-derived DCs have often been referred to as the equivalent of lymphoid $CD8^-$ conventional DCs having the similar expression of markers. However, recent findings have revealed that monocytes are not the major precursors for steady-state DCs and readily convert to DCs in inflammatory situations instead. The GM-CSF-derived DC is the *in vitro* equivalents of TNF-iNOS-producing DC (Tip DC), which has a phenotype expressing $CD11c^{int}CD11b^{hi}MAC\text{-}3^+$ [50].

Mouse DCs can also be produced *in vitro* from BM cultures in the presence of FMS-like tyrosine kinase 3 ligand (Flt3L), which is a growth factor for DCs *in vivo* [51]. The subsets of DCs obtained from Flt3L cultures more closely resemble DCs in classical lymphoid tissue, such as $CD8^+$ and $CD8^-$ DCs subsets in the spleen. They show similar cell surface marker expression, dependence of IFN regulatory factor (IRF)-8, ability to produce interleukin (IL)-12, and antigen presentation function [51].

1.2.2 DC differentiation

The recent finding that Flt3 is expressed in DC progenitors reveals the process of DC differentiation as a clue [52]. In BM, hematopoietic stem cells develop a common precursor for monocytes, macrophages and classical DCs named macrophage-DC progenitor (MDP), which are $Lin^-CX3CR1^+CD11b^-CD115^+cKit^+CD135^+$ and account for 0.5% of all BM mononuclear cells in mice. MDP subsequently split into monocyte and common-DC progenitor (CDP) (Lin^- $CD115^+Flt3^+CD117^{lo}$), the latter has been proven to produce pDCs and precursor DCs (pre-DCs) ($CD11c^+MHCII^-SIRP\alpha^{lo}$). Until now, the cells produced in the BM including pre-DCs, pDCs and monocytes migrate from the BM to the peripheral tissues and organs through the blood. The monocytes could be activated and developed into a DC subset named Tip-DC during infection. pDCs reside in peripheral tissues. With a very short time circulating in the blood (1 hour), pre-DCs migrate to different peripheral lymphoid organs and tissues, and continually differentiate into several different subsets of DCs in lymphoid and non-lymphoid tissues [46]. The differentiation of monocyte-derived Tip-DCs can be mimicked in BM cultures with GM-CSF ± IL-4 or ± TGF-β. In addition Pro-DC ($CD11c^-MHCII^-$) differentiate into pre-DCs ($CD11c^+MHCII^-$) en route for the DC subtypes generation, which can be mimicked in flt3 ligand BM cultures [50].

1.2.3 DC maturation

At steady-state, tissue-resident DCs are immature and part of the innate immune system, thus called immature DCs (iDCs). iDCs use specific receptors to detect PAMPs or DAMPs and act as immunological sensors, these ‘danger’ signals trigger signaling cascades in iDCs that result in their maturation, a profound phenotypic and functional metamorphosis driven by changes in gene expression [53,54]. During the maturation process, iDCs loose the adhesive structures and phagocytic receptors [55], but up-regulate co-stimulatory molecules, such as CD40, CD80, and CD86 [56], and translocate MHC class II compartments to the cell surface [57], simultaneously they secrete cytokines, which differentiates and polarizes and attracts immune effector cells [58,59]. The signals also trigger a profound change in iDCs to acquire a high cellular motility, such as up-regulation of the chemokine receptor CCR7, enabling DCs in

peripheral tissues to access local lymph vessels and migrate to the draining lymph nodes to activate T lymphocytes [60]. Thus, DC maturation plays a key role in initiating and controlling the magnitude and the quality of the adaptive immune response. The phenotypic diversity of the DC family is reflected in distinct functional properties, and the latter is reflected partly in the expression of different PAMP and DAMP receptors, divergent antigen presentation and cross presentation capacities, as well as differential propensities to induce tolerance and regulatory T cells (Treg) differentiation.

1.2.4 DC induced T cell activation

Matured and activated antigen-loaded DCs migrate to the T cell zone of secondary lymphoid organs and become immunogenic APCs competent to sustain the expansion and differentiation of antigen-specific T cells into appropriate effector cells [61,62].

The three signals delivered from the activated APC are believed to determine the fate of naïve T cells [63] (Figure 1). PAMP contact activates DCs and increase expression of MHC molecules carrying processed pathogen-derived peptides. The peptide-MHC complex engage T cell receptors on naïve pathogen-specific T cells, this first activating signal to the T cell is therefore referred to as 'signal 1'. $CD8^+$ T cells can interact with peptides (9-11 amino acids in length) bearing MHC class I. These MHC class I-restricted peptides are called 'endogenous antigens' as they are generally produced from proteins translated within the cell and encoded either in the host genome or by infecting viruses or other pathogens replicating intra-cellularly. $CD4^+$ T cells can interact with peptides bearing MHC class II. In contrast to MHC class I molecules, which are constitutively expressed on all nucleated cells, MHC class II molecules are present on APCs and are also inducible by innate immune stimuli, such as TLR ligands on certain other cell types. If signal 1 is not combined with the other two types of signals (2 and 3 below) but occurs on its own, it is also proposed to promote naïve T cell inactivation by anergy, deletion or co-option into a regulatory cell fate, and thus leads to 'tolerance'.

PAMP activation also increases a variety of co-stimulatory molecules on the surface of DCs, such as CD80 and CD86, which trigger CD28 on the T cell [64]. These co-stimulatory molecules can engage counter-receptors on T cells and transmit 'signal 2' which is important for T cell proliferation and survival. Signal 2 is thought to be an accessory signal with signal 1 together for inducing 'immunity'. PAMP activated DCs also produce mediators to promote the T cell and determine its differentiation into an effector cell, this is referred to as 'signal 3'. The signal 3 instructed differentiation can drive T cells to differentiate towards TH1 cells, TH2 cells or cytotoxic T lymphocytes (CTLs) dominated responses [65,66]. IL-12 is a typical mediator that delivers a signal 3 to promote TH1-cell or CTL development [67]. Notch ligand is suggested to deliver a signal 3 for TH2-cell development [68]. The integration of these three classes of signals delivered from DCs to the T cell determines its subsequent type of full effector generation.

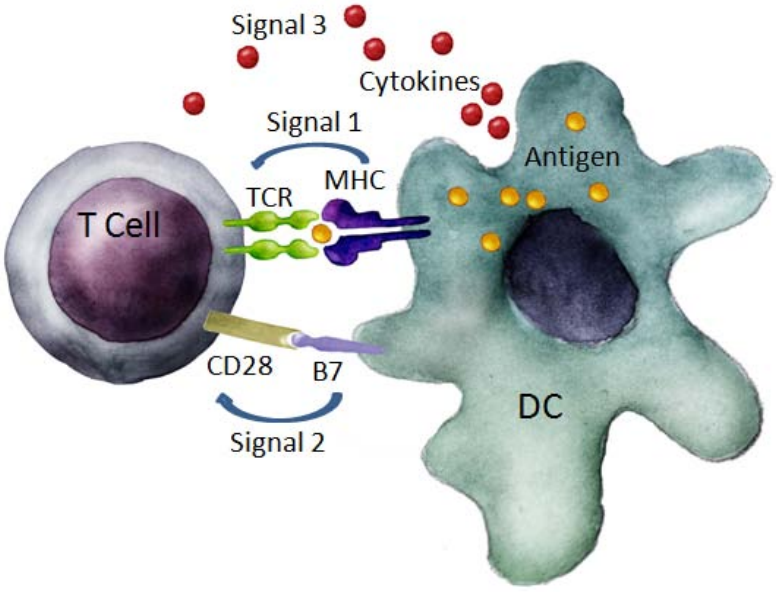

Figure 1. APC delivered signals for T cells activation. Binding of T cell receptor (TCR) to the antigen-MHC complex on the DC delivers signal 1, signal 2 is given by binding of CD28 to B7 co-stimulatory molecules, and signal 3 is the mediators produced by DCs when activated by PAMP.

1.3 TLRS AND TLR SIGNALING

During the past decade, rapid progress has been made in the understanding of innate immune recognition for microbial components through PRRs to initiate the first line of host defensive responses for killing of infectious microbes. In addition, PRR signaling simultaneously induces maturation of DCs, which is subsequently activating the second line of host defense, the adaptive immune response [3,6].

1.3.1 TLRs

Among PRRs, TLRs were the first PRRs to be identified in the mid-1990s. They are also the best characterized and recognize a wide range of PAMPs [2,69,70]. TLRs are type I trans-membrane proteins and are comprised of an ectodomain containing leucine-rich repeats that mediate the recognition of PAMPs, trans-membrane domains, and the intracellular Toll-IL-1 receptor (TIR) domains required for downstream signal transduction.

To date, 10 and 12 functional TLRs have been identified in humans and mice, respectively. TLR1-TLR9 are conserved in both species, mouse TLR10 has no function because of a retrovirus insertion, and TLR11, TLR12 and TLR13 are absent in the human genome. Each TLR recognizes distinct PAMPs, these include lipids, lipoproteins, proteins and nucleic acids derived from viruses, bacteria, mycobacteria, fungi, and parasites [71]. Once recognizing their respective PAMPs, TLRs recruit a specific set of adaptor molecules containing the TIR domain, such as myeloid differentiation factor (MyD) 88 and TIR domain-containing adaptor (TRIF), and initiate downstream signaling events, which cause the secretion of inflammatory cytokines, type I IFN, chemokines, and antimicrobial peptides [6]. These responses lead to the recruitment and activation of phagocytes, induction of IFN-stimulated genes

and result in direct killing of the infected pathogens (Figure 2). Furthermore, TLR activation induces maturation of DCs contributing to the induction of adaptive immunity.

Based on their cellular localization and recognition of PAMP ligands, TLRs are divided into two subtypes [72]. One group, TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11, are localized on the cell surface and mainly recognize microbial membrane components such as lipids, lipoproteins and proteins. TLR2 generally forms heterodimers with TLR1 or TLR6. Specifically, the TLR2-TLR1 heterodimer recognizes triacylated lipopeptides from Gram-negative bacteria and mycoplasma [73], while the TLR2-TLR6 heterodimer recognizes diacylated lipopeptides from Gram-positive bacteria and mycoplasma [74]. TLR4 recognizes and responds to bacterial lipopolysaccharide (LPS) as a long-sought receptor, LPS is a component of the outer membrane derived from Gram-negative bacteria, and TLR5 recognizes the flagellin protein component of bacterial flagella [69]. For mouse TLR11, it has been suggested to recognize uropathogenic bacterial components and the profilin-like molecule derived from Toxoplasma gondii [75,76].

The second group, TLR3, TLR7, TLR8, and TLR9, are expressed within intracellular vesicles such as the endoplasmic reticulum, endosomes, lysosomes and endolysosomes, and recognize microbial nucleic acids. In addition to polyinosinic-polycytidylic acid (poly(I:C)), which is a synthetic analog of dsRNA, TLR3 recognizes the genomic RNA of reoviruses, dsRNA derived from the replication of ssRNA, viruses and certain small interfering RNA (siRNA)s [77]. TLR7 recognizes imidazoquinoline derivatives such as imiquimod and resiquimod (R-848) and guanine analogs such as loxoribine, it also recognizes ssRNA derived from RNA viruses, as well as synthetic poly (U) RNA and certain siRNAs [69,78]. Human TLR8 also recognizes R-848 and viral ssRNA like TLR7 [69]. TLR9 recognizes cytidine-phosphate-guanosine (CpG) DNA motifs that are generally derived from bacteria and viruses but rarely from mammalian cells, and also directly recognizes the insoluble crystal hemozoin, a byproduct of the detoxification process after digestion of host hemoglobin by Plasmodium falciparum [69,79].

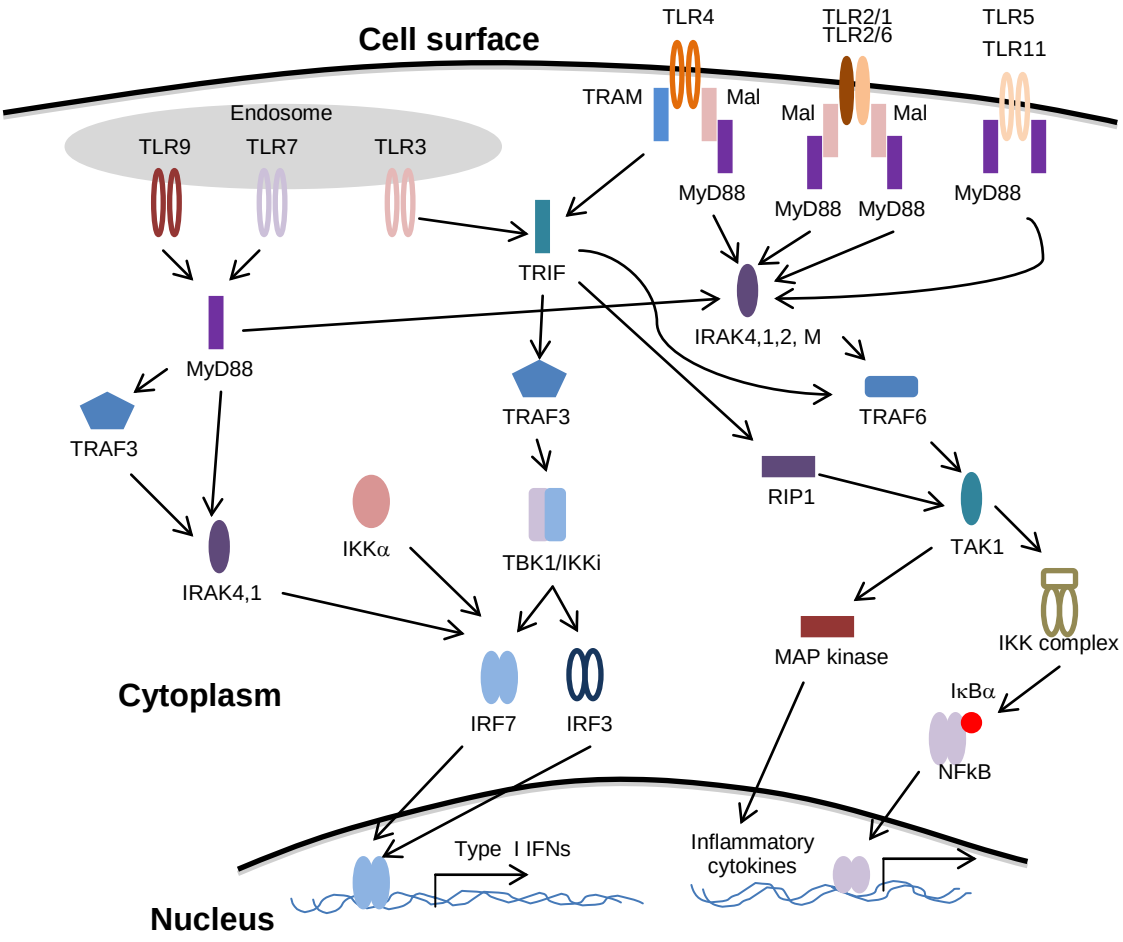

Figure 2. TLRs and TLR signaling in DCs. TLR2/TLR1, TLR2/TLR6, TLR4, TLR5 and TLR11 are localized on the cell surface. TLR3, TLR7, and TLR9 are localized in the endosome. All TLRs, except TLR3, recruit MyD88, while TLR1, TLR2, TLR4 and TLR6 recruit the additional adaptor Mal to link with MyD88. TLR3 and TLR4 recruit TRIF, and TLR4 requires the additional linker adaptor TRAM for TRIF. In conventional DCs, the activation of TLR1, TLR2, TLR5, TLR6, and TLR11 initiate the MyD88-dependent pathway whereas TLR3 ligands initiate the TRIF-dependent pathway. TLR4 activates both MyD88-dependent and TRIF-dependent pathways. In the MyD88-dependent pathway, MyD88 recruits the IRAK family of proteins and TRAF6. In turn, TRAF6 activates TAK1. The activated TAK1 activates MAPKs and the IKK complex, which activates NF-κB. In the TRIF-dependent pathway, TRIF recruits RIP1 and TRAF6. Activated RIP1 and TRAF6 activate NF-κB and MAPKs. TRIF also interacts with TRAF3 and activates TBK1/IKKi, which activate IRF3 and IRF7. In plasmacytoid DCs, TLR7 and TLR9 stimulation activates NF-κB and MAPKs via the MyD88-dependent pathway. MyD88 associates with the IRAK family and IKKα to activate IRF7 for type I interferons production. IRAK1 also interacts with TRAF3 and activates IRF7. The activated NF-κB subunits and IRFs are translocated to the nucleus. NF-κB and MAPKs initiate the transcription of inflammatory cytokine genes whereas IRFs initiate the transcription of type I interferons.

1.3.2 The MyD88-dependent pathway

After recognition of their respective PAMP, TLRs activate signaling pathways and trigger specific immunological responses. The specific response initiated by individual TLRs depends on the recruitment of TIR-domain-containing adaptor molecules, including MyD88, MyD88-adaptor-like (MAL), TRIF and TRIF-related adaptor molecule (TRAM), which are recruited by distinct TLRs and activate distinct signaling pathways. For example, TLR3 and TLR4 generate both type I IFN and inflammatory cytokine responses, whereas cell surface TLR1-TLR2, TLR2-TLR6 and TLR5 induce mainly inflammatory cytokines [80].

MyD88 is a member of the IL-1 receptor family and is universally utilized by all TLRs except TLR3. It transmits signals to induce nuclear factor (NF)-κB and mitogen-activated protein kinase (MAPK)s activation and the activation causes secretion of inflammatory cytokines. TRIF is used by TLR3 and TLR4 to activate an alternative pathway leading to the activation of NF-κB and IRF3 and the induction of type I IFN and inflammatory cytokine productions. MAL is utilized by TLR2 and TLR4 as an additional adaptor to recruit MyD88. Finally, TRAM acts as a bridge between TLR4 and TRIF. Thus, based on the used TIR-domain-containing adaptor molecules, TLR signaling pathways can be largely divided into MyD88-dependent pathways, which drive the induction of inflammatory cytokines, or TRIF-dependent pathways, which are responsible for the induction of type I IFN as well as inflammatory cytokines [69,80].

In the MyD88-dependent pathway, after the engagement of TLRs by their specific PAMPs, MyD88 recruits interleukin 1-associated kinase (IRAK) 4, IRAK1, IRAK2 and IRAKM, which are the IL-1 receptor–associated kinases, to form a complex. IRAKs activation results in an interaction with TNF receptor-associated factor (TRAF) 6 which is an E3 ligase catalyzing the synthesis of polyubiquitin on target proteins. Furthermore, TRAF6 activates transforming growth factor-β (TGF-β)-activated kinase 1 (TAK1) in complex with TAB2 and TAB3, and then activates the IKK complex consisting of NEMO and IKKαβ, which phosphorylates IκBα that in turn leads to their subsequent degradation and ultimately NF-κB activation. TAK1 activation simultaneously triggers MAPKs, which include Erk1, Erk2, p38 and Jnk, by inducing the phosphorylation of MAPKs, and then activate various transcription factors, including AP-1, as well as influencing translation [6]. Many genes are induced by activation of the MyD88-dependent pathway, and some of them have critical roles in modulating NF-κB-dependent transcription. The IκBα protein IκBζ works as an inducible co-activator for the NF-κB p50 subunit to facilitate IL-6 and IL-12p40 induction[81], C/EBPδ promote IL-6 production by acting together with NF-κB [82], IκB-NS modulates the DNA-binding activity of the NF-κB p65 subunit to inhibit the induction of both IL-6 and TNF [83], and ATF3 recruits histone deacetylase by restricting NF-κB activity [84].

1.3.3 The TRIF-dependent pathway

This pathway is also called ‘the MyD88-independent pathway’. The TRIF-dependent pathway is enable to activate both IRF3 and NF-κB [77]. TRIF recruits TRAF6 and activates TAK1 for NF-κB activation through ubiquitination-dependent mechanisms, which is similar to those of the MyD88-dependent pathway. Generally, TRIF forms a complex together with TRAF6, TRADD, Pellino-1 and RIP1 to activate TAK1, and then TAK1 further activates the NF-κB and MAPKs pathways. TRIF also recruits TRAF3, and then activates a signaling complex consisting of Tank-binding kinase 1

and IKKi, which trigger the phosphorylation of IRF3 and induce its nuclear translocation causing IFN-β transcription [85]. TRAF3 has been demonstrated to have a critical role for IFN-β induction when TLR3, TLR7, TLR9 and RLRs are activated by various nucleic acids [86].

The TLR4 signaling is the only one using both the MyD88-dependent and the TRIF-dependent pathway within all the TLR signaling. Besides being activated by the TRIF-dependent pathway, TRAF3 is also involved in the MyD88 complex during TLR4 signaling. TRAF3 degradation triggered by MyD88-dependent signaling results in translocation of the membrane-proximal signaling complex to the cytoplasm, and then leads to TAK1 activation [87]. These findings suggest that TRAF3 promotes IRF3 activation as well as inhibiting the MyD88-dependent pathway in TLR4 signaling. Thus, balanced production of inflammatory cytokines and type I IFN by these molecules might have key roles in controlling tumor cell growth and autoimmune diseases.

1.4 NEGATIVE REGULATION OF TLR-INDUCED INFLAMMATORY RESPONSES

The activation of TLR signaling by different PAMPs is a double-edged sword. On the one hand, it leads to the direct innate immune response and subsequent adaptive immunity against invaded pathogens. On the other hand, sustained and dys-regulated inflammatory signaling can lead to a variety of pathological conditions, such as septic shock, autoimmunity, atherosclerosis and metabolic syndrome [88]. One of the most debilitating diseases is the septic shock induced by LPS, a TLR4 ligand, and this severe and acute inflammation is originating from excessive production of pro-inflammatory cytokines. It induces vascular instability, leakiness and excessive clotting which stops the blood supply to tissues, and eventually leads to multiple organ failure and death [89,90]. Thus, even though the induction of an inflammatory response is essential for host defense during infection, timely resolution is also important to limit the detrimental effects of inflammation, to avoid host damage, particularly when the inflammation is inappropriately sustained or increased. By evolutionary development, the immune system has acquired mechanisms to regulate the inflammatory response at multiple levels.

The negative regulators of TLR signaling could be grouped into extracellular, trans-membrane and intracellular regulators based on their functional position (table 2) [91,92]. Soluble decoy TLRs (sTLRs) are currently identified as the only extracellular negative regulator. They are proposed to function by competing with TLR agonists and directly attenuate TLR signaling to prevent acute inflammatory responses [93]. The existence of several TLR4 isoforms has been shown in mouse and human models [94], and sTLR4 has been demonstrated to inhibit activation of NF-κB induced by LPS and TNF production in macrophages [95]. Six soluble forms for TLR2 have been identified [96]. They inhibit bacterial lipopeptide induced IL-8 and TNF production through the direct interaction with the co-receptor sCD14 [97].

Trans-membrane protein regulators are another important negative regulatory mechanism for TLR signaling, and include suppressor of tumorigenicity (ST) 2, single immunoglobulin IL-1 related protein (SIGIRR) and TNF-related apoptosis-inducing ligand receptor (TRAILR). These proteins inhibit TLR functions through sequestering the TLR adaptors or interfering with the binding of TLR ligands to their specific TLR. ST2 has two forms including ST2L and sST2. ST2L can inhibit IL-1 and TLR signaling by sequestration of MyD88 and Mal through its TIR domain in mouse macrophages [98]. Pro-inflammatory cytokines and LPS stimulation increase sST2 expression, and it in turn can significantly suppress the mRNA expression of TLR1 and TLR4 causing reduced pro-inflammatory production [99]. sST2 is also suggested to suppress IL-6, IL-1β and TNF-α production by reducing affinity of NF-κB to the IL-6 promoter and cause degradation of IκB [100]. SIGIRR belongs to the TIR family and has a single extracellular immunoglobulin domain [101]. It is suggested that SIGIRR inhibits the MyD88-dependent pathway, and act through the interaction with TLR4, IRAK and TRAF6 [102]. TRAILR is the receptor for TNF-related apoptosis-inducing Ligand and has been implicated to inhibit TLR signaling. TRAIL-deficient and TRAILR-deficient mice show increased levels of IL-12, IFN-β and IFN-γ secretion and enhanced clearance of mouse cytomegalovirus infection, and the respective ligands stimulation to TRAILR-deficient mice macrophages caused enhanced cytokine production after TLR2, TLR3 and TLR4, but not after TLR9 ligation [103].

Intracellular negative regulators form another line of defense against TLR-mediated over-response such as MyD88s, IRAKM, IRAK2, TRAF4, SOCS1, PI3K, TOLLIP, A20, TRIAD3A, TGF-β and IL-10.

MyD88s is a short form of MyD88, over-expression of MyD88s in IL-1 and LPS stimulated monocytes resulted in impaired activation of NF-κB [104], which was demonstrated through the inhibited activation of IRAK4 and the subsequent phosphorylation of IRAK1 [105]. Silencing SARM using siRNA resulted in elevated levels of cytokine production in response to polyI:C and LPS in the TRIF-dependent pathway [106], thus SARM has been implicated as negative regulator of NF-κB activation through TRIF.

IRAKM and IRAK2 belong to the IRAK family. IRAKM deficient mice showed elevated inflammatory responses to bacterial ligands and flawed LPS tolerance against endotoxic shock [107]. As the spliced isoforms of murine IRAK2, the over-expression of IRAK2c and IRAK2d were shown in fibroblasts to have inhibitory effects on NF-κB activation following LPS stimulation [108]. This suggests negative regulatory roles for IRAKM and IRAK2 within the TLR signaling pathways.

TRAF4 is a member of the TRAF family of proteins. HEK-293 cells were co-transfected with TLR-2, TLR-3, TLR-4 and TLR-9, and then stimulated with their appropriate ligands and increasing doses of TRAF4, this lead to a decrease in activation of both the NF-κB and the IFN-β promoter, but not to a decrease in TNF-α receptor-mediated signaling, as determined by luciferase reporter gene assays for the respective promoters. The suggested mechanism is that TRAF4 likely functions by interacting with TRAF6 [109].

Phosphatidylinositol 3´-kinases (PI3K) belong to a signal-transducing enzymes family, and it is a heterodimer that consists of a p85 regulatory subunit and a p110 catalytic chain. PI3K has been demonstrated to have a role of negative regulation in TLR signalling, which results in inhibition of IL-12 synthesis and prevents the overexpression of a TH1 response, and its regulating mechanism was suggested through the suppression of p38, JNK, ERK1/ERK2 and NF-κB [110].

Toll-interacting protein (Tollip) is a protein which interacts with the IL-1R accessory protein and is responsible for bringing IRAK to the receptor complex [111]. As over-expression of one member of the Tollip family, Tollip-1 leads to the subsequent inhibition of NF-κB activation, and Tollip also interacts with IRAK1 leading to a decrease in IRAK1 autophosphorylation and NF-κB activation, the suggested mechanism is that it allows the release of Tollip from the Tollip/IRAK1 complex resulting in the termination of its negative regulatory actions [112].

A20 is a zinc ring finger protein expressed in numerous cell types with a rapidly increased expression in response to LPS and TNF [113]. It has been identified as a negative TLR regulator in both the MyD88-dependent and TRIF-dependent signaling pathways. Studies in A20-deficient mouse macrophages show a significant increase in pro-inflammatory cytokines in response to TLR-2, TLR-3 and TLR-9 ligands, and A20 was suggested to work as a cysteine protease de-ubiquitinylating protein able to prevent TLR signaling via TRAF6 [114].

Triad3A belongs to Triad3 family that acts as an E3 ubiquitin protein ligase. It was found to bind the cytoplasmic domain and to promote the ubiquitylation and degradation in TLR9 and TLR4 but not TLR2. Overexpression of Triad3A results in the inhibition of TLR4- and TLR9-mediated NF-κB signal transduction, but did not affect TLR-2 or TLR-3-mediated signalling. Conversely, siRNA of Triad3A significantly increased TLR4 and TLR9 expression and the responses to their ligands in vitro [115,116].

TGF-β and IL-10 have been demonstrated to negatively regulate the expression and functions of TLRs. TGF-β1 inhibits TLR-4 expression by suppressing LPS-mediated responses and also can induces MyD88 degradation by the proteasome [117]. IL-10 is able to inhibit the production of pro-inflammatory cytokines through LPS, and suppress IL-12 production by TLR3 and TLR4 signalling in human dendritic cells [118].

Besides SOCS1, other members of the SOCS family have been implicated to have a regulatory role in TLR signaling, and this is discussed in detail in section 1.5.

Table 2 Negative regulators of TLR signaling

Negative regulator		Regulated TLRs	Proposed mechanism
Extracellular regulators	sTLR2	TLR2	TLR2 antagonist
	sTLR4	TLR4	Blocks interaction of TLR4 and MD2
Trans-membrane regulators	ST2	TLR2, TLR4, TLR9	Interact with MyD88 and MAL
	SIGIRR	TLR4, TLR9	Interacts with TRAF6 and IRAK
	TRAILR	TLR2, TLR3, TLR4	Stabilizes IκBα
Intracellular regulators	MyD88s	TLR4	MyD88 antagonist
	IRAKM	TLR4, TLR9	Inhibits phosphorylation IRAK1
	IRAK2	TLR4	Inhibits phosphorylation IRAK1
	TRAF4	TLR2, TLR3, TLR4, TLR9	Inhibits TRAF6, TRIF and IRAK1
	PI3K	TLR2, TLR4, TLR9	Inhibits p38, JNK and NF-κB function
	Tollip	TLR2, TLR4	Autophosphorylates IRAK1
	A20	TLR2, TLR3, TLR4, TLR9	De-ubiquitylates TRAF6
	Triad3A	TLR4, TLR9	Ubiquitylates TLRs
	SOCS1	TLR4, TLR9	Suppresses IRAK

1.5 SOCS PROTEINS AND IMMUNE REGULATION

The magnitude and duration of an immune response is determined by the integration of responses mediated by effector and regulatory T cells. Each activated signaling pathway has its own negative-feedback systems to avoid excessive cytokine production causing damage to the host. Among the negative regulators, suppressor of cytokine signaling (SOCS) proteins were initially identified as inhibitory regulators of the Janus kinase (JAK)–signal transducer and activator of transcription (STAT) pathway of cytokine receptor signaling. However, recent studies using genetically engineered mouse models have revealed an extended role for these proteins in regulating signaling pathways within the immune system and in other developmental systems [119].

1.5.1 SOCS protein structure and function

The SOCS family contains eight proteins consisting of SOCS protein 1–7 and cytokine-inducible Src homology 2 domain-containing protein (CIS). Their expression is induced in response to a wide range of cytokines, growth factors and hormones, and they inhibit cytokine signaling in a classical negative feedback loop [120,121].

All SOCS proteins share a three-part architecture: A central Src homology 2 (SH2) domain related to substrate binding through recognition of cognate phosphotyrosine motifs, a variable N-terminal region containing an extended SH2 subdomain that contributes to substrate interaction and a conserved C-terminal domain known as the SOCS box. In SOCS1 and SOCS3, the N-terminal region also contains a kinase inhibitory region (KIR) that is required for inhibition of JAK kinases [122,123,124] (Figure 3).

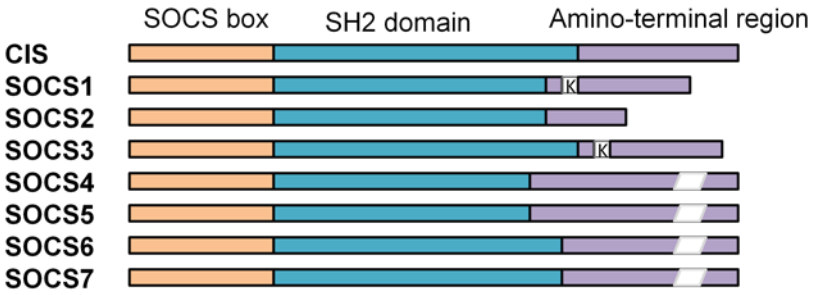

Figure 3. SOCS proteins structure. All SOCS proteins share a similar modular structure with a variable N-terminal domain, an SH2 domain and a SOCS box. Only SOCS-1 and -3 also possess a KIR domain in the N-terminal part.

SOCS proteins regulate cytokine receptor signaling through multiple complementary mechanisms. *1. Direct inhibition of JAK proteins kinase*. SOCS1 and SOCS3 proteins block the interaction of the JAK catalytic domain with their STAT protein substrates through KIR, thereby terminating signal propagation [124]. SOCS1 directly binds to the phosphorylated activation loop of JAK2, and SOCS3 shows only weak affinity for JAK2 that binds to the receptor in close proximity of the kinase [125,126]. *2. Binding competitors against STATs.* SOCS proteins are competitive inhibitors for binding to shared phosphorylated motifs of the activated receptor to suppress downstream signaling [127]. *3. Acting as ubiquitin ligases, thereby promoting the degradation of their partners*. SOCS proteins are components of an E3 ligase, and they can target their binding substrates to become ubiquitinated via the SOCS box which will lead to proteasomal degradation of the substrate [128]. Many investigations have been made to reveal the specific physiologic action for each of the SOCS proteins. In general, SOCS proteins are expressed at low levels in unstimulated cells. Upon stimulation of cells with a variety of cytokines, hormones and PAMPs, SOCS proteins are rapidly induced, and then they regulate intracellular signaling [129]. This thesis focuses on the role of SOCS proteins in the immune system, and specifically the role of SOCS in the regulation of DCs.

1.5.2 Regulation of TLR signaling by SOCS proteins

TLR ligands have been demonstrated to act as inducers for SOCS proteins in innate immunity. The stimulation of TLR9 by CpG-DNA or TLR4 by LPS resulted in SOCS1 and SOCS3 induction in macrophages [130,131], and except for TLR 5 and TLR9, the triggering of all TLRs induced SOCS2 expression in monocyte-derived DCs [132,133]. Among them, the role of SOCS1, SOCS2 and SOCS3 in regulating TLR signaling has been investigated [119,133,134].

1.5.2.1 SOCS1 and TLR signaling

SOCS1 is proposed to negatively regulate TLR signaling. SOCS1-deficient mice were hypersensitive to LPS, with an increase in cytokine secretion of TNF-α and IL-12 resulting in increased LPS-induced lethality [135,136]. In addition, the stimulation of macrophages, DCs and fibroblasts derived from SOCS1 knockout mice with TLR ligands *in vitro* resulted in the increased secretion of pro-inflammatory cytokines TNF, IL-12 and IFN-γ [135,136,137,138]. The effect of SOCS1 on TLR signaling is suggested to be mediated by direct and indirect pathways. Several mechanisms have been suggested for the direct negative regulatory role of the MyD88-dependent pathway by SOCS1. SOCS1 binds to the p65 subunit of NF-κB and facilitates ubiquitinylation and degradation of p65 [139]. SOCS1 also binds to tyrosine phosphorylated MAL protein and induces ubiquitinylation and degradation of MAL to suppress the MAL dependent activation of NF-κB [140]. In addition, SOCS1 might regulate the stress-activated MAPKs JUN N-terminal kinase (JNK) and p38 through binding to their upstream activator, apoptosis signal-regulating kinase 1 [141]. The indirect mechanism for SOCS1 effects is suggested to act via inhibition of the secondary activated JAK–STAT pathway that is activated by the TRIF-dependent pathway induced IFN-β [142]. Moreover, SOCS1 also is suggested to inhibit JAK2 activated by LPS with subsequently decreased IL-6 production [143].

1.5.2.2 SOCS2 and TLR signaling

Recent studies have revealed that SOCS2 is induced by TLR signaling in DCs and imply a potential regulatory role for SOCS2 in TLR signaling, however, subsequent investigations were contradictory [132,133,144]. Using siRNA knock-down experiments in human monocyte-derived DCs, our study indicates that SOCS2 reduction affected both MyD88-dependent and TRIF-dependent pathways after LPS stimulation, and this lead to reduced activation of NF-κB and IRF3, decreased pro-inflammatory cytokine gene expression and impaired DC maturation [144]. A recent report indicates that silencing of SOCS2 in DCs leads to increased IL-10 and IL-1β cytokine secretion, and the authors suggested that SOCS2 inhibits STAT3 working as an inhibitor for TLR ligand-induced DC activation [133]. However, the molecular mechanism for this inhibition is still not clear.

1.5.2.3 SOCS3 and TLR signaling

A negative regulatory role for SOCS3 in TLR signaling has been suggested with a mechanism involving both direct and indirect pathways. SOCS3 directly inhibits the activation of TRAF6 and TAK1, that both are crucial for TLR- and IL-1-induced responses [145]. For the indirect functional mechanism, although both IL-6 and IL-10 are induced in the presence of LPS, SOCS3 only inhibited STAT3 activated by IL-6 signaling through binding to the IL-6R subunit gp130, but no effect was seen on IL-10 signaling. SOCS3 knock-out mice also displayed increased STAT1 activation that implied a role for SOCS3 as an inhibitor of inflammatory responses [146,147,148].

1.5.3 SOCS proteins and DC physiology

SOCS proteins have also been demonstrated to be involved in DC physiology including the regulation of DC subsets, differentiation, maturation and antigen presentation. Most investigations are focusing on SOCS1 and SOCS3, however, recent finding also indicate effects of SOCS2 in this field.

SOCS1

SOCS1 has been found to be involved in the regulation of DC subsets, with regard to their differentiation, maturation, activation and antigen presentation. The number of $CD11c^{+}CD8\alpha^{+}$ DCs is increased in the spleen of SOCS1-deficient mice. SOCS1-deficient DCs are characterized by elevated expression of MHC class II and co-stimulatory molecules and in addition have an increased cytokine secretion of IFN-γ, IL-6, IL-12, and TNF in response to LPS and CpG-containing DNA [137,149]. Furthermore, SOCS1 has been implicated in the inhibition of the differentiation of human monocyte-derived DC (moDC)s following TLR signaling by suppressing GM-CSF signals [150]. SOCS1 also negatively regulates LPS and IL-4 induced DC maturation. SOCS1 expression is up-regulated during DC maturation, and SOCS1 is proposed to be involved in the switch from STAT6 to STAT1 expression, since the STAT6 signaling pathway is constitutively activated in iDCs and declines as these cells differentiate into mature DCs, whereas STAT1 is up-regulated during this process [151]. It has been demonstrated that ovalbumin (OVA)-pulsed SOCS1-siRNA-treated DCs can enhance proliferation and function of OVA-specific CTLs, and siRNA silenced SOCS1 enhanced antigen presenting ability of DCs that caused an increased antigen-specific antitumor immunity [152] and effective vaccination against HIV [153]. In addition, SOCS1-siRNA-treated DCs can cause autoimmune pathology by activating auto-reactive T cells. This brake of self-tolerance might be due to IL-12 hyper-production by SOCS1-siRNA-treated DCs [154], supporting a role for SOCS1 as an essential negative regulator for T cell tolerance.

SOCS2

Our findings and a recent publication revealed the involvement of SOCS2 in DC maturation [133,144]. Our results indicated a promoting role for SOCS2 in DC maturation. However, another group suggested an inhibitory function for SOCS2 in TLR ligand-induced DC activation. This issue will be discussed later in this thesis.

SOCS3

The function of SOCS3 in DCs has been related to the regulation of cytokine production directing Th2 or Treg cell differentiation programs. SOCS3-transduced DCs exhibited lower expression of MHC class II molecules and the co-stimulatory molecule CD86, when stimulated with LPS. In addition, an altered pattern of cytokine secretion compared with control DCs was noticed, characterized by high levels of IL-10 but decreased production of IL-12, IFN-γ, and IL-23. SOCS3-transduced DCs directed the T cell differentiation toward a Th2 phenotype, with increased levels of secreted IL-4 and IL-10 and decreased levels of IL-17. A similar Th2-skewed immune response was observed *in vivo* after adoptive transfer experiments. Thus, SOCS3-transduced DCs are highly effective inducers of Th2-cell differentiation *in vitro* and *in vivo* [155]. SOCS3-transduced DCs suppress the development of experimental autoimmune encephalomyelitis, an animal model of multiple sclerosis. The suggested mechanism in SOCS3-transduced DCs is that reduced Th17-cell differentiation causes reduced IL-23 production and a predominant induction of Th2 cells [155].

SOCS3 deficient DCs have a strong potential as forkhead box P3 $(FoxP3)^{+}$ T cell-inducing tolerogenic DCs. $SOCS3^{-/-}$ DCs also expressed lower levels of class II MHC, CD40, CD86, and IL-12 both *in vitro* and *in vivo*, and displayed constitutive

activation of STAT3. However, unlike SOCS3-transduced DCs, $SOCS3^{-/-}$ DCs are poor activators of effector $CD4^+$ T cells, but they selectively expand $FoxP3^+$ regulatory T cells, which can suppress experimental autoimmune encephalomyelitis. High levels of TGF-β produced in $SOCS3^{-/-}$ DCs is suggested to play an essential role in the expansion of $FoxP3^+$ Treg cells. These results indicate an important role for SOCS3 in determining immunity or tolerance by DCs [156].

1.6 THE EFFECT OF CHEMOTHERAPEUTIC DRUGS ON ANTI-TUMOR IMMUNE RESPONSE

Chemotherapy is one of the conventional therapies for tumor treatment, and it is traditionally assumed to suppress the immune system in two ways: First, chemotherapies cause apoptosis of target cells, and this model of cell death is immunologically regarded either as non-stimulatory or able to induce immune tolerance. Thus T cells can no longer respond to the presented antigen by mounting an immune response. Secondly, many anticancer drugs have the common side effect of inducing lymphopenia, which has also been assumed to be detrimental to any potential immune response. However, accumulating evidence has indicated that chemotherapy may in addition have immunostimulatory effects in the anticancer immune responses [157,158,159]. The suggested mechanisms are: 1, in the chemotherapy induced lymphopenia, regulatory T cells and tolerated T cells are depleted. After the lymphopenia, a homoeostatic proliferation occurs, and T cell numbers are restored. Thus active anti-tumor activity can be enhanced by removal of negative regulatory cells [160,161,162] and depletion of myeloid-derived suppressor cells [163]. Furthermore, lymphodepletion in combination with tumor vaccines has shown efficacy in mice and in human trials [164,165]. 2, chemotherapy causes increased antigen release and up-regulation of immunogenic surface molecules. It has been demonstrated that apoptotic tumor cell death increases the quantity of antigen release and augments cross-presentation by mature DCs [166] and increases immune activity through up-regulation of surface calreticulin [167,168]. 3, chemotherapy treatment may result in increased antigen presentation and priming of tumor-specific CD8 cells [169]. 4, Chemotherapy can sensitize target cells to subsequent elimination by immune cells, through up-regulation of death receptors such as Fas and TRAIL [170,171].

1.6.1 Chemotherapeutic drugs and dendritic cells

Recent studies indicate that chemotherapeutic drugs cause tumor or stromal cell death, and deliver signals to DCs for enhancing anti tumor responses by antigen uptake, processing and presentation [157,158].

Antigen uptake: the cell stress induced by chemotherapy can cause the transcriptional activation of a series of molecular chaperones. Following tumor insult by cytotoxic agents, tumor cells rapidly translocate intracellular calreticulin to the cell surface, which is a chaperone and acts as a mandatory ‘eat-me’ signal for DCs and induces immunogenic cell death. Calreticulin translocation on the cell surface is crucial for the recognition and engulfment of dying tumor cell by DCs. The cytotoxic agent anthracycline and γ-irradiation has a demonstrated effect inducing cell surface calreticulin expression resulting in immunogenic tumor cell death [167,172,173].

Antigen processing: the chemotherapeutic drugs including anthracycline, oxaliplatin and irradiation have been shown to inhibit tumor growth with a higher efficiency in immunocompetent mice compared to athymic littermates, revealing a role for the TLR4 and TLR4-MyD88 pathways as chemotherapy induced mechanism [174,175]. TLR4-deficient DCs have a normal ability to present antigen from soluble proteins taken up by pinocytosis, but they are unable to present antigen from dying cells taken up by phagocytosis. It has been suggested that the specific defect in antigen presentation after phagocytosis is caused by rapid lysosomal degradation of phagocytic material due to the TLR4 defect. High-mobility group box 1 protein (HMGB-1), which is released from dying cells after these chemotherapeutic drugs treatment, is demonstrated to act as a TLR4 ligand and is responsible for enhanced anti-tumor function.

Antigen presentation: several chemotherapeutic drugs have been demonstrated to enhance the antigen presentation ability of DCs. Paclitaxel can bind to mouse TLR4 and mimic bacterial LPS to activate mouse macrophages and DCs through the MyD88 dependent pathway [176]. The cytotoxic agent bortezomib can cause HSP90 appearance on the surface of human myeloma cells, and HSP90 is a chaperone serving as a contact-dependent signal to activate autologous DCs [177]. Furthermore, the chemotherapeutic drug gemcitabine induces tumor cell apoptosis, and then enhance the DC dependent cross-presentation of tumor antigens to T cells [169]. In support of these data, gemcitabine can enhance the function of CD40 stimulation of T cells to cure tumors in a mouse model [178].

1.7 CLINICAL APPLICATION

Based on the critical role of DCs as APCs in connecting innate and adaptive immunity, dysregulation of DCs may lead to the development of distinct types of diseases. Intrinsic dysregulation might lead to autoimmunity and allergy. Furthermore, the specific character of DCs makes them promising for anti-tumor immune vaccination therapies [179].

1.7.1 DCs and allergy

Myeloid DCs (mDCs) and pDCs are involved in the induction and maintenance of immune tolerance. When mDCs capture harmless environmental antigens, they can silence the corresponding T cells by inducing IL-10-producing Tregs through the interaction between the inducible co-stimulator and its ligand [180]. Inducible co-stimulator ligand is also expressed on activated pDCs and promotes the differentiation toward IL-10 producing Tregs [181]. In asthma, airway DCs has an essential role for controlling the Th2-dependent eosinophilic airway inflammation [182]. *In vivo* depletion of mDCs during allergen challenge abrogates the characteristic features of asthma [183], and depletion of pDCs in a mouse model for airway hypersensitivity resulted in the exacerbation of the development of asthmatic symptoms [184].

When skin and airways are exposed to external stimuli, the dysregulation of inflammatory responses may enhance the secretion of thymic stromal lymphopoietin (TSLP), which skews the T cell response towards Th2, from epithelial cells in individuals who have a genetic background increasing the risk for atopic dermatitis and asthma [185,186]. TSLP activates DCs to induce an inflammatory Th2 type cell response by producing the OX40 ligand instead of IL-12 [187]. The TSLP-based pathway has been implicated in atopic dermatitis in mice and humans [185,188] as well as in murine models of asthma [189].

1.7.2 DCs and autoimmune diseases

Autoimmune diseases are chronic inflammatory conditions that depend on inappropriate responses to self-antigens in persons with certain genetic backgrounds. It has been shown that DCs bearing self-antigens are involved in induction of autoimmune cardiomyopathy [190] and systemic lupus erythematosus (SLE) [191]. In psoriasis, mDCs secrete large amounts of TNF-α that result in pro-inflammatory effects through ectopic maturation of DCs that would otherwise control peripheral tolerance [192], and TNF-α is also proposed to have an essential role in rheumatoid arthritis as well as in several other autoimmune diseases [193].

Systemic lupus erythematosus (SLE) is proposed to be associated with an increased production of type I IFNs. Blood monocytes from SLE patients can induce the robust proliferation of allogeneic naïve $CD4^+$ T cells, and combination of type I IFN and GM-CSF results in the differentiation of monocytes into mature DCs that presents antigens from dying cells in an immunogenic rather than tolerogenic manner [194]. When treating SLE patients with high-dose glucocorticoids, it results in a total disappearance of pDCs from the circulation [195] and this is associated with IFN loss [196]. IFN is also shown to considerably accelerate the development of autoimmune symptoms in lupus-prone NZB/NZW mice [197]. These observations support the critical role for IFN in the lupus pathogenesis. For the mechanisms leading to the increased production of type I IFN, genetic alterations of TLR-7 or SOCS are suggested to induce an extended production of IFN by pDCs in response to viral encounters [198,199]. This effect may be further enhanced by the stimulatory effects of nucleic-acid-containing immune complexes internalized with viral nucleic acids and self-nucleoproteins to further activate pDCs through TLR-7 or TLR-9 [200,201,202]. SLE sera generated DCs also drive the differentiation of $CD8^+$ T cells toward fully active cytotoxic effector T lymphocytes, and then generate auto-antigenic fragments through the destruction of target tissues [194], [203]. These auto-antigens could be captured and presented by mDCs, thereby further broadening the autoimmune process. In addition, an alteration of the B cell pathway leading to breakdown of tolerance to nuclear antigens for SLE has also been proposed, since B cell depletion is emerging as a useful therapeutic alternative in SLE [204,205].

Type I IFN and pDCs are also involved in other autoimmune diseases, which include psoriasis, insulin dependent type 1 diabetes mellitus, dermatomyositis, and Sjögren's syndrome, as pathogenic factors. In psoriasis, pDCs accumulate in the inflamed skin and secrete type I IFN [206]. Elevated expression of IFN was also found in the pancreas of recently diagnosed patients who suffer from insulin-dependent diabetes mellitus [207]. In dermatomyositis patients, muscle biopsies show infiltration with pDCs as well as increased expression of IFN-inducible genes and protein expression [208]. Finally, in the salivary glands of Sjögren's syndrome patients, IFN-inducible genes are over-expressed, and pDCs infiltrate the gland [209].

1.7.3 DCs based anti-tumor vaccination

Tumor cells can suppress the host immune system and lead to prevention of specific immunity, induction of specific tolerance, and triggering of suppressive pathways. DCs are involved in this process by altered functions in their differentiation, maturation, and antigen presentation [210]. Tumors originating from hematopoietic and epithelial cells can constitutively activate STAT3 that in turn inhibits the production of pro-inflammatory cytokines and promotes the release of soluble factors to suppress DC

functions, these factors then further up-regulate STAT3 expression in DCs and result in the induction of anti-tumor tolerance rather than immunity [211,212,213]. Several cytokines such as vascular endothelial growth factor and IL-10 have also been implicated to interfere with DCs differentiation and maturation in diverse tumors [214,215,216]. Tumor cells can interfere with the DC antigen-capture and antigen-presenting pathways. Breast cancer cells can secrete tumor glycoprotein mucin-1 (MUC-1), which is endocytosed by DCs but inefficiently processed and presented to T cells causing lower frequency of MUC-1-specific effector cells [217,218]. Furthermore, MUC-1 inhibits the capacity of DCs to secrete IL-12, and then drives the development of T cells towards Th2 responses [219]. The immune response can simply be misled to promote cancer development at the early stage of disease. For example, DCs at tumor sites induce $CD4^+$ T cell differentiation toward T cells secreting high levels of IL-4 and IL-13, which promote early tumor progression [220]. DCs also directly interact with myeloma cells to enhance tumor progression and tumor cell survival [221].

Because of their unique role in linking the innate and adaptive immune systems, DCs have been a logical focus for novel immunotherapies [179,222]. Two approaches to DC-based antitumor vaccines are being developed: antigen-loaded *ex vivo*-generated DCs and *in vivo* DC targeting. For typical *ex vivo* DC vaccines, DCs are generated by culturing host monocytes with GM-CSF and IL-4 matured with a cocktail of pro-inflammatory cytokines, such as TNF and CD40 ligands or allogeneic tumor antigens, and then re-injected back the host to induce antitumor immune response against different forms of cancer [223,224,225,226]. In addition, blood DCs *ex vivo* loaded with different proteins have also been used as vaccines in trials [227,228,229]. These early phase I studies have concluded that DC vaccines are safe and can induce immune and some clinical responses. To reach a greater rate of clinical responses, many parameters need to be considered for assessing vaccine efficacy of future DC vaccine development, such as clinical endpoints and measurements. However, a major parameter may actually be the DCs themselves, since the DC vaccines matured with a variety of stimuli can cause diverse immune response outcomes [225,230]. For cancer vaccines, the desired DCs would preferentially induce high tumor-specific CTLs together with a strong Th cell activity to provide long-term memory, and limit humoral immunity and induction of Tregs [231]. Thus, further studies are necessary to select the most appropriate DC platform and optimal DC mature stimuli. Another important area of DC-based cancer vaccine development will be combination with therapies that block the suppressive environment created by the tumor. Chemotherapeutic drugs combined with DCs vaccines have been proven to induce an efficient anti-tumor immunity [177,232] via the mechanisms discussed in 1.6. Several other combination therapies are also considered to enhance DCs vaccine efficacy [194,233,234]. For targeting DCs *in vivo*, many DC surface molecules have been considered as targets. *In vivo* targeting of antigens to maturing DCs results in the generation of immunity against the antigen in mice [235,236,237] and in humans [238,239]. The approach is promising and has the potential of yielding a wealth of new vaccines.

2 AIMS

The overall aim of this thesis was to investigate the mechanisms regulating differentiation, maturation and activation of DCs.

The specific aims are:

1. To map SOCS expression and investigate the role of SOCS2 during DC maturation.

2. To investigate how diverse TLR ligands regulate SOCS2 and the mechanisms for LPS induced SOCS2 transcription during DC maturation.

3. To investigate how deficiency of SOCS2 influences DC differentiation and its ability to activate T cells.

4. To investigate effects of chemotherapeutic drugs on the differentiation and T cell activating ability of human monocyte derived DCs

3 METHODOLOGY

Detailed descriptions of the methods used in the included papers (I-IV) are found in the respective 'Materials and methods sections'.

Mice (II, III)
Knockout (MyD88, IRF3 [II] and SOCS2 [III]) and transgenic OT-II [III] mice on the C57BL/6 background were used.

Human monocyte and T cells isolation (I, II, IV)
Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donor's buffy coat by gradient centrifugation. $CD14^+$ monocytes (I, II, IV) or $CD4^+$ T cells (IV) were subsequently isolated from PBMC by magnetic sorting using bead conjugated antibodies.

Mouse splenocyte isolation (III)
$CD90^+$ splenocytes from OT-II mice were isolated by magnetic sorting using bead conjugated antibodies.

In vitro cells culture (I, II, III, IV)
Human moDCs (I, II, IV) were obtained by culturing $CD14^+$ monocytes in medium supplemented with GM-CSF and IL-4 for 6 days (I, II). The cells were harvested for FACS analysis as indicated in order to study moDC differentiation (IV).

Mouse bone marrow derived DCs (BMDCs) (III) were obtained by culturing BM cells in medium supplemented with GM-CSF and IL-4 for 7 days.

Mouse bone marrow derived macrophages (BMMs) (II) were obtained by culturing BM cells in medium supplemented with 20 to 30% L929 cell-conditioned medium (as a source of macrophage-colony stimulating factor) for 6 days.

In vitro cell stimulation (I, II, III)
Human moDCs or siRNA transfected human moDCs were stimulated with a variety of TLR ligands (LPS [I, II], Pam3CSK4, polyI:C, flagellin, imiquimod, ssRNA40/LyoVec, ODN 2336 [II]), cytokines IFNα, IFNβ1a, IFNγ and IFNλ1, anti-human IFNAR2 neutralizing antibodies (II). The cells were harvested for FACS measurement or to extract total RNA for real-time PCR analysis and proteins for western blotting analysis.

Mouse BMDCs were stimulated with LPS (III) and the cells were harvested for FACS measurement or to extract total RNA for gene expression analysis.

Mouse BMMs were stimulated with BCG (II) and the cells were harvested to extract total RNA for genes expression analysis.

Quantitative Real-time PCR (I-IV)
RNA was isolated with Trizol reagent and subsequent cDNA synthesis was performed by reverse transcription. Human CIS, SOCS1, SOCS2, SOCS3, SOCS4, SOCS5, SOCS6, CCL-4, IFN-β, CXCL-9, CXCL-10 (I), NF-κB, IRF1, IRF3, IFNγ, IFNλ1, STAT3, STAT5, STAT6 (II), TNF-α, IL1-β, IL6, (I, IV), IFN-α, IFN-β (II, IV), and mice SOCS2 (II), IFNβ, TNFα, IL-6, IFNγ (III) gene expression were measured by PCR amplification using specific primers.

Flow cytometry (I, III, IV)
Single cell suspensions of human moDCs and T cells (I, IV), mouse BMDCs and T cells (III) were prepared and extracellular proteins were labeled using fluorescent-conjugated antibodies. Samples were analyzed using a FACSaria or a CyAnTMADP instrument and FlowJo software.

Transfection (I, II)
Human iDCs were transfected with SOCS2 (I), NF-κB, IRF1, IRF3, STAT3, STAT5, and STAT6 (II) siRNAs using a commercial kit and a nucleofector machine (Amaxa Co., Köln, Germany). The transfection efficiency was measured with fluorescent-labeled control siRNA and FACS.

Cell nuclear and cytoplasmic protein and whole cell protein extraction (I, II)
Nuclear and cytoplasmic proteins were extracted from DCs using a commercial nuclear and cytoplasmic protein extraction kit (Pierce Biotechnology, Rockford, IL). Whole cell proteins were extracted with radio immune precipitation assay buffer.

Western blotting (I, II)
Proteins in cell lysates were separated by gel electrophoresis, transferred to protein binding membranes and detected using HRP-conjugated antibodies and ECL-reagents.

DC activated T cell proliferation assay by CFSE labeling (III)
Murine BMDCs were incubated with LPS and OVA323-339 peptide overnight, the next day $CD90^+$ splenocytes were incubated with media containing Carboxyfluorescein succinimidylester prior to stimulation and then kept with BMDCs for 5 days. Proliferation was evaluated by FACS.

T cell proliferation assay by [^{3}H] incorporation (IV)
[^{3}H] thymidine was added to cultures of activated T cells. Incorporation of thymidine was measured using a beta scintillation counter.

Flow cytometry analysis for blast transformation of T cells (IV)
Isolated $CD4^+$ T cells and $CD14^+$ monocytes were incubated separately with either supplemented medium alone or with one of the chemotherapeutic drugs Dexamethasone, Doxorubicin, Cisplatin or Irinotecan respectively. Next day the cells were mixed (1:2 ratio), and Staphylococcus enterotoxin B (SEB) was added and the cells were incubated for an indicated time period until FACS analysis.

Bioinformatic analysis (II)
Genomic sequence of human SOCS2 promoters was obtained from the UCSC Genome Bioinformatics Site (http:// genome.ucsc.edu). The prediction of transcription factor binding sites was performed using a binding site searching software (http://www.genomatix.de).

Statistical analysis (I-IV)
Independent two sample t-test was used to compare populations of unpaired samples. Dependent t-test or student's *t* test was used for the comparison of paired samples and Fisher's exact test was used to calculate the level of significance from SOCS2 siRNA knockdown DCs mRNA data.

Ethical considerations
These studies were conducted according to the regulations related to handing of laboratory animals and human material and were approved by the local ethical committees.

4 RESULTS AND DISCUSSION

4.1 SOCS2 INFLUENCES LPS INDUCED DC MATURATION

DCs serve as highly specific APCs and play a crucial role connecting the induction of innate immunity and the subsequent development of the adaptive immune response [69,240]. As one of the PRRs, TLR4 on the surface of DC can be activated by its ligand LPS, and cause DC maturation. SOCS1 and SOCS3, members of the SOCS family, have recently been demonstrated to negatively regulate TLR signaling in macrophage and DC maturation [92,119,136]. A recent study demonstrated that SOCS2 knock-out mice showed decreased microbial proliferation, leukocyte infiltration, production of pro-inflammatory cytokines, and a high mortality upon infection [132], this implies that SOCS2 may have an important role in the regulation of immune responses to diverse infectious agents. We therefore set out to investigate the role of SOCS proteins and TLR4 signaling pathways in DC maturation.

CIS, SOCS1, SOCS2 and SOCS3 gene expression is regulated by LPS stimulation in mouse DCs or macrophages [151,241]. We first studied the gene expression of all SOCS members during human DC maturation. SOCS2, SOCS3 and SOCS6 expression level changed significantly after LPS treatment, whereas the expression of the other SOCS family members did not (Figure 4). SOCS2 expression increased significantly after 4h treatment and this suggests that SOCS2 has a delayed induction during LPS induced DC maturation.

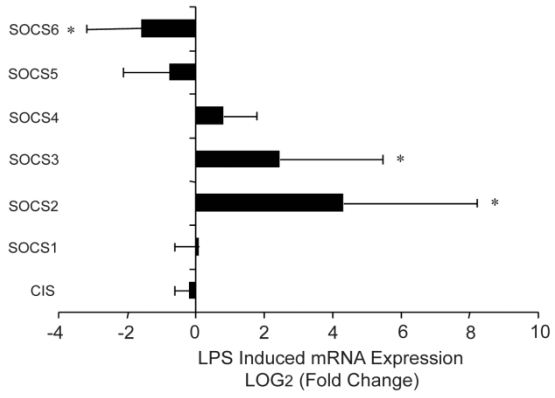

Figure 4. mRNA expression of SOCS family members during LPS induced human monocyte derived DC maturation

The downstream actors of the TLR4 signaling pathway include the MyD88-dependent and the TRIF-dependent pathways [242]. To study the effect of SOCS2 on the TLR4 signaling pathway, we silenced the SOCS2 gene with siRNA, and after LPS stimulation, separately measured the activation of SAKP/JNK, P38, ERK and IκBα and nuclear translocation of NF-κB, which all are part of the MyD88-dependent pathway downstream kinases, and the activation of IRF3, which is a TRIF-dependent pathway downstream kinase. The results revealed that SOCS2 silencing inhibits both the MyD88-dependent and TRIF-dependent signaling pathway in human DCs by interrupting the normal SOCS2 dependent kinase cascades. Activation of the TLR4 signaling pathway induces TNF-α, IL-6, IL-1β and CCL-4 downstream genes via the MyD88-dependent pathway [243,244] and IFN-β, CXCL-9, and CXCL-10 downstream

genes via the TRIF-dependent pathway [245,246]. Furthermore, we found that SOCS2 silencing decreased the expression of all these downstream genes. Taken together, these findings suggest that SOCS2 influences TLR4 signaling via both the MyD88-dependent and TRIF-dependent signaling pathways.

As LPS induced DC maturation is mediated via TLR4 signal transduction, we decided to investigate a possible role for SOCS2 in DC maturation. The result showed that the expression of the marker for mature DCs, CD83 and co-stimulatory molecules CD40 and CD86 were significantly decreased in SOCS2 silenced DCs (Figure 5). This supports that the elimination of SOCS2 leads to impairment in LPS induced DC maturation.

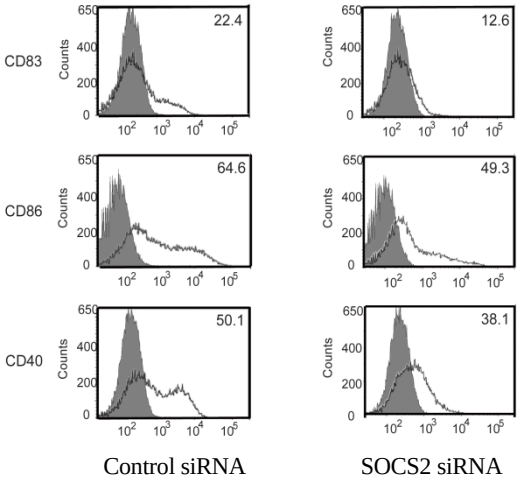

Figure 5. Effect of SOCS2 siRNA in DC maturation. The histogram shows changes of indicated surface molecules in DCs from the different groups. Matched isotype controls are presented as solid histograms. Percentages of positive cells are indicated in the upper right corner of each histogram.

Our data confirm and further extend the knowledge of SOCS involvement in DC maturation and indicate that SOCS2 is an important SOCS family member involved in the maturation process. Several hypotheses for the regulation mechanism could be proposed. It seems plausible that SOCS2 targets act early on the signaling pathway such as SOCS1 or the Tyro3/Axl/Mer receptor family. Because both the MyD88 and TRIF dependent pathways are affected by SOCS2 knock-down, other SOCSs can inhibit TLR signaling [140,145] and be negatively regulated by SOCS2 [247], the Tyro3/Axl/Mer receptor family can be tyrosine phosphorylated and act as negative regulators of TLR signaling [248]. It is also possible that SOCS2 has separate targets for the MyD88- and TRIF-dependent pathways. In this case several negative regulators such as Src homology 1-containing tyrosine phosphatase 1 [249], SH2-containing tyrosine phosphatase [250] and MAPK phosphatase-1 [251] might be some of the possibilities. However, it is important to note that SOCS2 is known to have stimulatory activity on certain cytokine signaling pathways like IL-6 signaling [129], thus SOCS2 may also have a positive feedback on the activity of cytokines produced DC maturation.

In conclusion, our results indicate that SOCS2, SOCS3 and SOCS6 are regulated significantly after LPS treatment, whereas the other SOCS family members CIS, SOCS1, SOCS4 and SOCS5 did not change at the expression level. SOCS2 positively regulates maturation of human DCs *in vitro*. This is achieved by promotion of TLR4 signaling through both the MyD88-dependent and TRIF-dependent signaling pathways.

4.2 LPS REGULATES SOCS2 TRANSCRIPTION IN A TYPE I IFN DEPENDENT AUTOCRINE-PARACRINE LOOP

To avoid detrimental tissue damage, evolution has developed several lines of negative regulatory mechanisms to keep TLR and ensuing inflammatory responses at adequate levels. These involved negative regulators are divided into two groups: signal-specific regulators that inhibit signal transduction by TLRs such as SOCS proteins and gene-specific regulators that modulate gene expression [91]. SOCS2 has been suggested to have a role as an important modulator for immune responses [252], and can be induced in the TLR2 and TLR4 signaling in DCs [132,144], but the regulation of SOCS2 expression has not been studied extensively in the cells of the immune system. This led us to further investigate the transcriptional regulation of SOCS2 expression in TLR4 signaling.

TLRs recognize microbial patterns, and can be arranged into three major families by their ability to bind lipids (TLR2 and TLR4), proteins (TLR5) and nuclear acids (TLR 3, 7, 8 and 9) [253]. We first investigated which TLR signals could affect SOCS2 expression in human DCs. The results revealed that TLR2 and TLR4 stimulation caused the highest induction of SOCS2 expression, where the induction was moderate in TLR3, TLR5, TLR8 and TLR 9 stimulated cells. No increase was found in TLR7 and this finding was expected, since TLR7 is reported to be expressed in mouse myeloid DCs [254] but only in human plasmacytoid DCs [255]. The results suggest that TLR signaling by lipid ligands induce SOCS2 expression, with the dominant inducer being LPS.

Since LPS was found to be the main inducer of SOCS2 expression we next studied SOCS2 transcriptional regulation by TLR4 signaling comprehending both MyD88-dependent and -independent pathways [253]. The transcription factors P65NF-κB and IRF1, which are involved in the MyD88-dependent pathway, and IRF3, which is part of MyD88-independent pathway, were activated and translocated from the cytoplasm to the nucleus after LPS treatment. However, P65NF-κB knock-down by siRNA had no effect on SOCS2 induction, but IRF1 and IRF3 silencing decreased SOCS2 induction significantly at the 24h time point. We concluded that IRF1 and IRF3 are major inducers of SOCS2 suggesting that SOCS2 is affected by IFN signaling.

The IFNs are classified into type I IFNs including α, β etc more than 20 other subtypes, the single type II IFNγ and three type III IFNλs [256,257]. We first investigated which type of IFNs that may be involved in SOCS2 induction after TLR4 signaling in human moDCs. After LPS treatment, IFNβ and IFNλ1 mRNA were induced dramatically after 2h, IFNα showed a late increase at 8h instead of 2h, and IFNγ was only slightly induced by LPS treatment. Furthermore, treatment of cells with IFNα resulted in a small increase of SOCS2 expression, treatment with IFNλ1 did not induce SOCS2, but both IFNβ and IFNγ showed a rapid and substantial induction of SOCS2 expression after 1h stimulation. Taken together LPS induced IFNβ and IFNβ has the direct ability to stimulate SOCS2 expression in human moDCs. Subsequently, addition of anti-human IFNAR2 antibody blocked the SOCS2

mRNA induction in response to LPS treatment (figure 6). This suggest that endogenous type I IFN mediates increased SOCS2 expression in response to LPS.

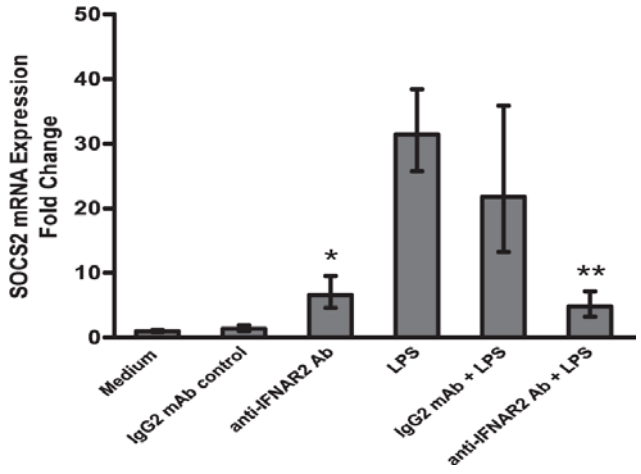

Figure 6. The effect of neutralizing anti-IFNR2 antibodies or IgG_{2a} isotype control mAb for LPS induced SOCS2 mRNA expression in DCs

Based on the time lapsed after LPS stimulation, TLR4 signaling activates primary response genes within 0.5-2h, secondary response genes are induced after 2-8h, and specific gene expression is induced after a longer period of time [258]. Since SOCS2 mRNA levels increased significantly after 4h LPS stimulation and within 1h after IFNβ stimulation, we hypothesized that SOCS2 was induced by type I IFN as a secondary response gene after LPS stimulation. STATs are known to be essential components of the type I IFN receptor signaling cascade [256,259], and by using computer prediction software, we found several putative binding sites for STAT3, 5 and 6 in the promoter regions of human SOCS2 gene. Thus, STAT3, 5 and 6 were identified as putative SOCS2 regulating transcriptional factors for type I IFN signaling in human DCs for later investigation. The STAT3, 5 and 6 transcription factors were all found to translocate into the nucleus 30 minutes after IFNβ stimulation, however, the LPS induced translocation occurred at 2h for STAT3 and STAT5 and only weakly for STAT6. Furthermore, when STAT3, STAT5 and STAT6 expression was silenced, a significant reduction of LPS induced SOCS2 mRNA expression was observed for STAT3 and STAT5 but not STAT6 knockdown. Thus, STAT3 and STAT5 are most likely the main transcription factors regulating SOCS2 induction after TLR4 signaling.

BCG stimulates MyD88-dependent and -independent pathways by engaging TLR2 and TLR4 receptors [135,136]. SOCS2 mRNA expression was abrogated about 45% in BMM from $MyD88^{-/-}$ mice and almost completely eliminated in BMM derived from $IRF3^{-/-}$ mice. These results indicated that SOCS2 induction is dependent on the IRF3/IFN pathway and that both MyD88-dependent and –independent pathways are involved.

Our data suggest that an autocrine/paracrine type I IFN loop is required for LPS to stimulate SOCS2 expression (Figure 7). A recent publication suggested that SOCS2 is a direct downstream target of TLR ligation [133]. However, the effect of IFN on SOCS2 induction was not investigated. We clearly demonstrate that the

inhibition of IRF1, IRF3 and INFAR2 signaling severely impairs SOCS2 induction. Furthermore, in contrast to IRFs, NF-κB silencing only marginally affected SOCS2 expression in our study. In IRF3 and MyD88 deficient mice experiments, while MyD88 signaling only partially affects SOCS2 induction, SOCS2 induction was almost completely abolished in IRF3 deficient macrophages. These data demonstrate the importance of type I IFN for the LPS induced SOCS2 in an indirect loop way.

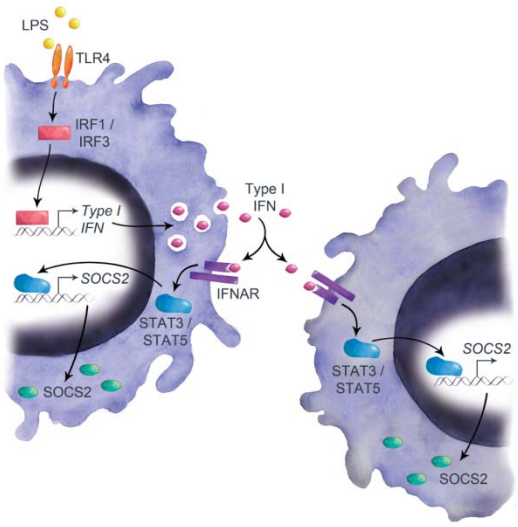

Figure 7. The proposed mechanism for SOCS2 transcriptional regulation in TLR4 signaling.

Both IFNα- and IFNβ-dependent signals can activate STATs [256]. STAT5 has previously been demonstrated by our group to regulate mouse and human SOCS2 genes via binding to their promoter regions [260]. In our study, binding sites of STAT5 in the human SOCS2 gene can be predicted and may explain how SOCS2 is activated by IFNβ in human cells, and we also show that STAT3 is activated by IFNβ and is required for TLR-dependent SOCS2 expression. Thus, IFNAR activation in the presence of LPS stimuli leads to translocation of STATs, particularly STAT3 and STAT5 followed by increased SOCS2 expression.

The present study as well as a previous report [133] show that signaling via other TLRs besides TLR4 increase SOCS2 expression. TLR3, 8 and 9 agonists all enhance type I IFN secretion by human DCs [261,262,263], providing a plausible explanation for SOCS2 induction by those TLR signals. However, TLR2 and TLR5 activation, which do not activate IFN secretion, also induced SOCS2 expression in our study. It implied that besides IFN signaling additional signals may be involved, and may also explain why MyD88$^{-/-}$ BMM partly affect SOCS2 expression after BCG infection.

In conclusion, we demonstrate that various TLR ligands induce SOCS2 gene expression in human DCs, and we propose that SOCS2 regulation in late phase by TLR4 signaling is dependent on an autocrine/paracrine type I IFN loop that activates SOCS2 via STAT3 and STAT5.

4.3 SOCS2 POSITIVELY REGULATES DC ANTIGEN PRESENTING TO $CD4^+$ T CELLS

DCs have the ability to initiate T cell responses, and several factors are likely to influence this T cell response quality at the level of DCs, such as antigen dose, microbial stimuli [264], secreted cytokines [265], increasing cell surface expression of molecules [266] and co-stimulatory molecules [267,268]. After a T cell response the activation of DCs needs to be terminated to avoid harmful inflammation or autoimmune disease. We have demonstrated that SOCS2 influences LPS induced DC maturation *in vitro* [144]. Thus, we further investigated to see if SOCS2 has a role in terminating DC action by influencing the DC antigen presenting ability.

Growth factors involved in the production of DCs, such as GM-CSF and IL-4, are known to be regulated by SOCS proteins [134]. Therefore, we started to examine whether SOCS2 regulates DC development *in vivo*. Flow cytometry analysis revealed that DCs from $SOCS2^{-/-}$ mice were similar to WT DCs in their distribution of the splenic CD11c high population and different DC subsets for $CD4^+CD8\alpha^-$, $CD4^-CD8\alpha^+$ and $CD4^-CD8\alpha^-$ CD11c high DC subpopulations. We conclude that SOCS2 does not regulate murine lymphoid DC differentiation *in vivo*.

We then further studied whether SOCS2 had an effect on murine BMDC differentiation *in vitro* using GM-CSF and IL-4. Although CD11c expression for DCs was detected before day 3 in $SOCS2^{-/-}$ mice, it was reduced significantly at later time period when compared with WT cells. There were no apparent differences in the expression of the co-stimulatory CD80 and of MHC II from $SOCS2^{-/-}$ mice DCs compared to WT. However, the expression of the co-stimulatory molecule CD86 was significantly reduced on $SOCS2^{-/-}$ mice BMDC. The findings suggest that SOCS2 is necessary for the differentiation of GM-CSF and IL-4 induced BMDCs.

To test the possible effect of SOCS2 on BMDC maturation, we stimulated GM-CSF and IL-4 treated BMDCs from $SOCS2^{-/-}$ or WT mice with LPS. MHC II expression for mature $CD11c^+$ BMDCs is not different between WT and $SOCS2^{-/-}$ mice. However, in $SOCS2^{-/-}$ BMDC, CD80 expression on $CD11c^+$ BMDCs was significantly reduced, and the CD86 marker was slightly reduced in MFI. The results demonstrate that SOCS2 elimination influenced expression of some co-stimulatory molecules during myeloid DC maturation *in vitro*.

We further investigated the role of SOCS2 for the pro-inflammatory cytokine response during BMDC maturation. BMDCs from $SOCS2^{-/-}$ mice did not change their mRNA expression profile of IFN-β and TNF-α . However, the expression of IL-6 and IFN-γ mRNA were significantly increased in $SOCS2^{-/-}$ BMDCs. Taken together, the findings demonstrate that SOCS2 is necessary for the regulation of pro-inflammatory cytokine production during BMDC maturation.

Finally, we studied the effect of $SOCS2^{-/-}$ DC on T cell activation. We found that untreated BMDCs, or BMDCs treated with LPS were not able to activate naïve OT-II T cells from WT and $SOCS2^{-/-}$ DCs. However, with the addition of a specific antigen OVA peptide, $SOCS2^{-/-}$ BMDCs were not able to induce T cell proliferation as

compared to WT BMDCs (Figure 8). Thus, SOCS2 acts as a positive regulator of DCs in their capacity to activate naïve T cells.

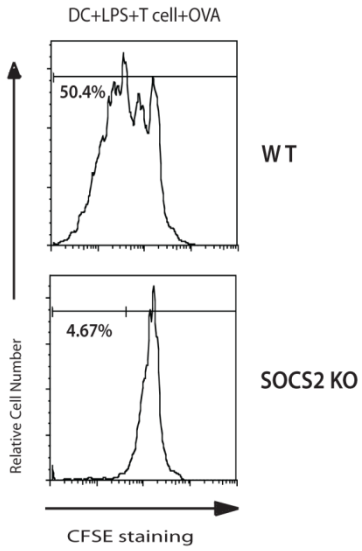

Figure 8. Proliferation of $CD4^+$ T antigen specific cells is decreased when co-cultured with $SOCS2^{-/-}$ mice BMDCs. Percentages denote the fraction of $CD4^+$ T cells.

The final outcome of a T cell response to DC antigen presentation is affected by multiple factors. Since the expression of MHC II on $SOCS2^{-/-}$ DCs was unaffected it is likely that the co-stimulatory molecules with decreased expression might contribute to the decreased $CD4^+$ T cell proliferation. Sung-Joo et al demonstrated that IL-6 controls and decreases LPS induced DC maturation and their ability to activate OT I and OT II transgenic T cells *in vitro* and *in vivo* [269]. This suggests that the increased production of IL-6 seen in $SOCS2^{-/-}$ BMDCs may contribute to a decreased ability to activate T cells. On the contrary, IFN-γ alone can efficiently up-regulate the class II antigen presenting pathway and thus promote peptide-specific activation of CD4+ T cells [270,271]. However, based on our result showing the impaired capacity for $SOCS2^{-/-}$ BMDCs to activate naïve $CD4^+$ OT-II T cells, it is tempting to speculate that the increased IL-6 expression by $SOCS2^{-/-}$ DCs is the main contributor to the decreased activation of $CD4^+$ T cells.

For murine BMDC culture models *in vitro*, it has been demonstrated that the differentiation of myeloid-committed precursors to myeloid DCs mainly depend on GM-CSF influences [272,273]. However, GM-CSF [274] and GM-CSF receptor β chain [275] deficient mice display only a modest decrease in the DC number indicating that *in vivo* differentiation of DCs is only partially dependent on GM-CSF signaling, a finding in line with our data demonstrating a normal DC population in $SOCS2^{-/-}$ mice. It is known that GM-CSF is completely dependent on STAT5 when directing inflammatory DC production from BM lin-$Flt3^+$ progenitors *in vitro* [276]. Furthermore, SOCS2 gene expression is induced in BM cells after GM-CSF stimulation [277]. Though SOCS2 over-expression partially inhibits the phosphorylation and DNA-binding capacity of STAT5 *in vitro* indicating a negative regulatory role for SOCS2 in GHR signaling [278], some studies indicate that SOCS2 have dual effects on GH-induced STAT5 activation *in vitro* [279] and *in vivo* [280]. So, it is likely that SOCS2 positively regulates STAT5 activation in the GM-CSF driven BMDC differentiation in our murine system.

In conclusion, our results demonstrate that SOCS2 positively regulates mouse BMDC differentiation *in vitro* but not *in vivo*, and that SOCS2 is a complex regulator of DC maturation and DC effector functionality, with an overall positive regulatory function on T cell activation.

4.4 CISPLATIN INHIBITS THE DIFFERENTIATION, BUT ENHANCES THE ANTIGEN PRESENTATION ABILITY OF DCs

Chemotherapeutic treatments for solid tumors are traditionally considered to cause lymphopenia and impair host immunity [158,281]. However, recent studies indicate that some chemotherapeutic agents increase the anti-tumoral immune response and cause tumor regression [158,282]. Simultaneously, immunotherapy is becoming an attractive approach to additional treatment of cancer, and DCs have been used in many tumor immunotherapy trials and been shown to induce anti tumor immunity [283]. However, there is evidence that chemotherapeutic agents may induce suppression of DCs. Thus, chemo-immunotherapy programs in clinical oncology ought ideally to be initially evaluated for the effects on immune function. For future DC chemo-immunotherapy in clinical usage, we therefore set out to investigate the effect of three conventional chemotherapeutic drugs; Doxorubicin, Cisplatin and Irinotecan on human monocyte differentiation to DCs and on DC functionality.

We optimized the individual concentrations of the tested drugs based on the dose resulting in 50% inhibition of $CD4^+$ T cells proliferation. Firstly, we studied the effect of the chemotherapeutic drugs on the differentiation of human monocyte derived DCs. Dexamethasone was included as a positive control in the experiment. It significantly delayed the down-regulation of the monocyte marker CD14 and caused a significant smaller fraction of DCs to express CD1a, as a sign of delayed differentiation. No effect of Dexamethasone was seen on the fraction of cells expressing CD80, but the fraction of cells expressing the co-stimulatory molecule CD86 and MHC II were significantly increased. Doxorubicin treatment showed a mild inhibitory effect. Although the fraction of $CD14^+$ cells was significantly decreased, no obvious change in the expression of CD1a and CD86 was observed. Interestingly, the expression of CD80 and MHC II were enhanced. Cisplatin and Irinotecan showed a similar moderate inhibitory effect with regards to the proportion of cells expressing CD14, CD1a and MHC II, but these compounds did not induce any CD86 expressing. The CD80 expression was inhibited by Irinotecan, whereas no such effect was shown for Cisplatin. For the mean fluorescence intensity (MFI) measurement, the mean expression levels of CD14 and CD1a mirrored the effect for all the tested drugs. However, none of the tested drugs had an obvious effect on CD80 and CD86 expression. For the MHC II expression, Dexamethasone induced a significant increase, whereas the remaining drugs did not have any effect. We conclude that chemotherapeutic drugs change the differentiation of human moDC.

To study the functional consequences of the chemotherapeutic drugs, monocytes or lymphocytes treated with chemotherapeutic drugs were co-cultured and activated with the super antigen staphylococcus enterotoxin B (SEB) followed by lymphoblast development measurements. Pretreatment of $CD4^+$ lymphocytes with

chemotherapeutic drugs seems to have a slight effect on T cell activation. However, $CD4^+$ lymphocytes cultured in the presence of monocytes pre-treated with Cisplatin resulted in a significantly increased activation of T cells (Figure 9). Dexamethasone, Doxorubicin and Irinotecan treated monocytes did not lead to an increased activation of T cells. These results demonstrate that Cisplatin enhance the ability of monocyte-derived DCs to stimulate T cell activation.

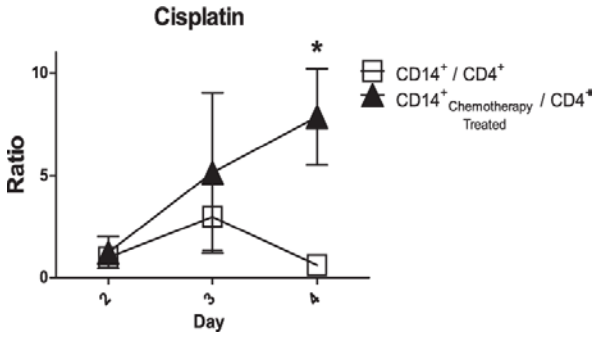

Figure 9. The effect of Cisplatin treated monocyte derived DCs to the $CD4^+$ T proliferation in human

Then we set out to dissect the mechanism of Cisplatin enhanced DC function by measuring the production of pro-inflammatory cytokines during DC differentiation. Cisplatin treatment did not regulate IL-1β, TNF-α, IL-6 and IFN-α mRNA expression, but the expression of the IFN-β was significantly up-regulated. Thus, IFN-β is a likely candidate explaining the enhanced DC function after Cisplatin treatment.

It has been demonstrated that Cisplatin treatment enhanced the antigen presenting ability of macrophages and increased KLH-primed T cells proliferation in a biphasic manner [284]. In our study, Cisplatin showed a similar ability to increase DC function causing T cell activation but no significant effects on co-stimulatory molecules CD80 and CD86. DC antigen presentation to T cells requires the combined action of signal 1, 2 and 3. The fraction of MHC-II positive cells increased after Cisplatin treatment, therefore the increased presence of MHC expression (signal 1) in combination with the increased expression of the pro-inflammatory cytokine IFN-β (signal 3) may explain the mechanism behind Cisplatin induced T cell activation in our SEB system.

Dexamethasone has been demonstrated to inhibit human DC differentiation, maturation and antigen presenting function [285,286], and in our experiments the results are consistent with earlier findings. Doxorubicin was revealed to have dual effects on DC differentiation; enhancement at low concentration, but inhibition at a higher concentration [287,288]. This might explain our experimental results, that when 0.2μM Doxorubicin was used, the differentiation of human monocyte-derived DC was slightly inhibited, but the antigen presentation ability was not affected.

Investigations for the Irinotecan effect on DCs function are rare. Carole et al demonstrated that in a mouse colon carcinoma model the combination of DC based immunotherapy with Irinotecan did not affect its efficacy, and furthermore strongly

decreased the toxicity of chemotherapy [289]. In our study, though Irinotecan moderately inhibits the DC differentiation, the treatment did not affect antigen presenting ability, and the results may explain the functional experiments partly.

A novel understanding of the chemotherapeutic effects on the immune system may potentially lead to new and optimized treatment strategies. Though several studies have shown that the combination of chemotherapy and immunotherapy may have synergistic effects, setting the time- and dose-schedule is critical, since chemotherapy may have devastating effects upon the immune system when administered in a vulnerable phase of leukocyte activation, differentiation and proliferation.

In conclusion, chemotherapeutic drugs; Dexamethasone, Doxorubicin, Cisplatin and Irinotecan inhibit the differentiation of human monocyte-derived DCs to various extents. Cisplatin treatment of human DCs leads to increased T cell activation, a beneficial effect of Cisplatin mediated by increased expression of IFN-β cytokines by DCs.

5 GENERAL CONCLUSION AND FUTURE OUTLOOK

In this thesis, we found that SOCS2 positively regulated LPS induced DC maturation and antigen presenting ability leading to activation of $CD4^+$ T cells. Furthermore, TLR signaling induces SOCS2 gene expression and the transcriptional regulation of the SOCS2 gene in TLR4 signaling was related to a type I IFN dependent autocrine/paracrine loop. In addition, SOCS2 positively regulated DC differentiation during GM-CSF and IL-4 cultures *in vitro* but not *in vivo*. All tested chemotherapeutic drugs Dexamethasone, Doxorubicin, Cisplatin and Irinotecan inhibited monocyte derived DC differentiation *in vitro*, but only Cisplatin treatment enhanced the DC antigen presenting ability with further induction of T cell proliferation.

The positive regulatory role of SOCS2 in TLR4 signaling has been demonstrated both in human and mouse DC systems. It is important to obtain a detailed understanding of the regulatory mechanisms in order to evaluate the potential for future clinical use. Further future investigations need to be focused on the finding and identification of the targets for SOCS2 binding within the TLR4 signaling pathway and thus aim to clarify the exact functional mechanisms for the actions of SOCS2. We believe that it is important to further explore related molecular actions of SOCS2 to form an intact clearer view of SOCS2 function in innate immune responses.

SOCS2 deficient mice have an impaired immune system with decreased microbial proliferation, leukocyte infiltration, production of pro-inflammatory cytokines, and a high mortality upon infection [132]. Our results further extend the role of SOCS2 to modulation of DC biology in human and mouse. Recently, a patient was diagnosed with a SOCS2 gene deficiency where special endocrine symptoms was found [290]. However, the immune effects of the SOCS2 deficiency in the patient were not reported. It would be of great interest to expand this study to involve the clinical impact of SOCS2 deficiency on the immune system.

We found an IFN autocrine/paracrine loop regulating the SOCS2 gene transcription after TLR4 signaling. IFNβ induced STAT3 and STAT5, were identified as important SOCS2 regulatory transcriptional factors. However, further experimental confirmations for the exact role of STAT3 and STAT5 need to be performed, such as the demonstration of direct STAT binding to the SOCS2 promoter region. In paper II, besides the type I IFN autocrine/paracrine loop, the MyD88-dependent pathway was also shown to be involved in inducing SOCS2 expression. This raises the interesting question how SOCS2 is induced via the MyD88-dependent pathway and spurs further questions what is the biological meaning of this pathway leading to SOCS2 induction during infection.

Although SOCS2 can not affect mouse DC differentiation *in vivo*, it was implied to influence mouse BMDC differentiation *in vitro* by regulating GM-CSF signaling since GM-CSF was shown to induce SOCS2 expression and the differentiation of myeloid-committed precursors to myeloid DCs mainly depend on GM-CSF influences [272,273]. However, further confirmation for the role of SOCS2 in

regulating GM-CSF signaling needs to be performed, and the mechanism for how SOCS2 regulates the signaling remains to be elucidated.

The result from our paper IV revealed that chemotherapeutic drugs Dexamethasone, Doxorubicin, Cisplatin and Irinotecan inhibit the differentiation of human monocyte-derived DCs. However, only Cisplatin can enhance the DC antigen presenting ability, leading to T cell proliferation. This finding may be promising as a basis for future tumor vaccination studies where Cisplatin could be used in the clinic to enhance the effect of DC based anti tumor vaccines. However, pre-clinical studies to optimize the dose and pre-treatment time points are called for when taking such DC vaccine protocols into consideration. A clinical trial can be performed when all conditions meet the requirement, and this may lead to the development of a new anti tumor chemo-immunotherapy concept.

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