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ROS-producing Macrophages in immune modulation

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ROS-producing Macrophages in immune modulation

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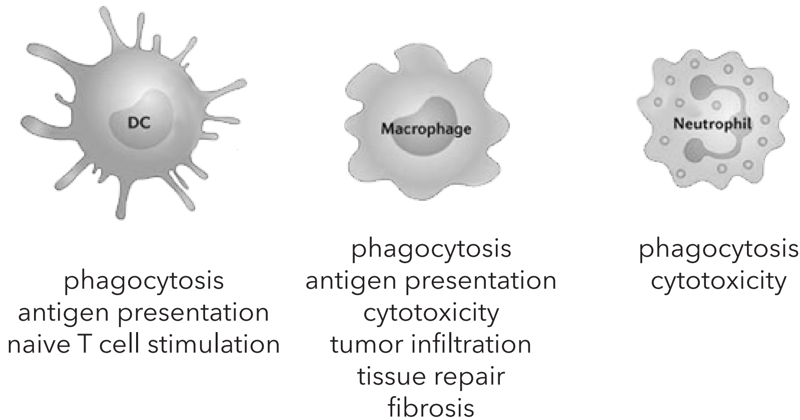
Chapter 1

General introduction

Introduction

Summary
Macrophages (Mph) are major effector cells of the innate immune system. Mph are broadly divided into two types: pro-inflammatory Mph1 and anti-inflammatory Mph2. A distinctive feature described between human Mph1 and Mph2 is the high reactive oxygen species (ROS) production by Mph1, whereas Mph2 are being described as a non-ROS producing cell. ROS generated by Mph are usually associated with oxidative stress. However, ROS produced in the immunological synapse or produced in lower amounts can inhibit T cell activation. In addition, ROS serve as second messenger in various signaling pathways by changing specific proteins. These redox changes are in many cases associated with altered activity and effects on signaling pathways or cellular processes. ROS are thus being produced as a result of normal cell physiology or due to ageing or stress, but can also be produced in the context of immunological defense by a specific enzymatic system. A naturally occurring polymorphism in the NADPH oxidase complex member *Ncf1* was found to regulate the severity of arthritis in rats. This polymorphism caused reduced ROS production and promoted the activation of arthritogenic T cells. The main producers of phagocytic NADPH oxidase (NOX2)-derived ROS are neutrophils and Mph. It was shown using transgenic mice expressing a functional NOX2 restricted to Mph that Mph could inhibit T cell responses in a ROS-dependent fashion. Thus NOX2-derived ROS are involved in immune modulation and cellular signaling as well. The scope of this thesis was to obtain more insight in the role of ROS being produced by the human M-CSF-differentiated anti-inflammatory Mph2 in immune modulation.

Macrophage differentiation
In the bone marrow, hematopoietic progenitor cells give rise to neutrophils and cells from the mononuclear phagocyte system, including circulating blood monocytes (Mn), tissue macrophages (Mph) and dendritic cells (Figure 1) (1,2). Mn are released into the circulation upon maturation where they remain for a few days before migrating into all the tissue compartments in the body. There the Mn will differentiate into resident Mph and by adapting to their local environment, Mph such as Kupffer cells (liver), alveolar Mph (lung), and microglia (central nervous system) develop. These Mph types are morphologically and phenotypically very different, underscribing the plasticity of Mph. Mph are major effector cells of the innate immunity; they engulf and kill pathogens, but are also involved in tissue repair and resolution of inflammation. Under the influence of colony stimulating factors (CSF), including macrophage (M)-CSF and granulocyte-macrophage (GM)-SCF, Mn are differentiated from progenitor cells. This became evident when observing osteopetrotic mice having a genetic defect that makes them M-CSF deficient (3). These mice have severe reductions in circulating Mn, and almost complete absence of various myeloid cells including osteoclasts and resident peritoneal Mph. The majority of the tissue



phagocytosis
antigen presentation
naive T cell stimulation

phagocytosis
antigen presentation
cytotoxicity
tumor infiltration
tissue repair
fibrosis

phagocytosis
cytotoxicity

Figure 1: Functions of meyloid cells: DC, Mph, and neutrophil

Mph depend upon signaling through the CSF-1R for their migration, survival and (re)population (4).
Since M-CSF is constitutively present in blood, it is thought that an anti-inflammatory Mph phenotype is maintained under homeostatic conditions (5). However, under the influence of environmental factors, including signals like cytokines, chemokines, and TLR ligands, resident Mph can undergo phenotypic and functional differentiation, thereby resulting in different Mph populations (6-8). This was shown in an elegant study by Arnold et al. using *in vivo* tracing methods where inflammatory Mph switched to an anti-inflammatory Mph phenotype in response to a changed microenvironment (9).

Macrophage plasticity during inflammation

In response to various inflammatory and immune stimuli, tissue-resident Mph undergo local activation. In addition, Mn are recruited causing an accumulation of tissue Mph with a pro-inflammatory phenotype (Mph1) contributing to inflammation. Mph1 phagocytose debris, invading micro-organisms and opsonized particles, thereby triggering a pro-inflammatory response with production of pro-inflammatory mediators, including Interleukin (IL)-1β, IL-6, IL-12, and tumor necrosis factor-α (TNF)-α (10). During the phagocytosis of apoptotic cells, Mph dampen their pro-inflammatory activities, acquiring characteristics of anti-inflammatory Mph2; Mph that can initiate tissue healing and repair by producing the anti-inflammatory mediators TGF-β, IL-10, and prostaglandin E2 (11,12).
In a model of glomerulonephritis in the kidney, ablation of Mn and Mph diminished the amount of tissue injury (13,14). In addition, Kupffer cell depletion in a model of liver injury using clodronate liposomes attenuated liver damage (15). Mph depletion in a model of autoimmune diabetes blocked disease, and Mph inactivation led to increased graft survival in a model of islets of Langerhans

transplantation in the rat (16,17). This implicates that under several conditions Mph depletion could be of therapeutic interest. However, eliminating Mph can also have disadvantages. It has been demonstrated that hepatic Mph were necessary for matrix regression and regulating cell proliferation following liver injury (18). Similarly, in muscle Mph are needed to enhance myogenic growth by releasing trophic factors (19). These Mph depletion studies confirm *in vivo* that different Mph types exist: depletion does not only affect the pro-inflammatory function of Mph but also the wound healing function.

Macrophages in chronic inflammatory diseases

Lately there has been an increasing interest in the role of Mph in chronic inflammatory diseases. In atherosclerosis a persistent recruitment of Mn into atherosclerotic lesions is observed, where a variety of pro- and anti-inflammatory Mph can be found (20). A damaging role for Mph1 has been indicated in atherosclerosis, whereas Mph2 were shown to be protective (21,22). In addition, Mph play also a central role in the pathogenesis of rheumatoid arthritis (RA). In steady-state conditions a balance is established that allows normal joint function. This balance can shift towards an inflammatory pathway by increased production of growth factors that promote Mph1 development and increased production of pro-inflammatory cytokines such as TNF-α and IL-6 further favouring Mph1 progression (23). Furthermore, in obesity-induced inflammation Mph1 are considered to be the major inflammatory cell in adipose tissue, whereas normally resident adipose tissue Mph are non-inflammatory and regarded as Mph2 (24).

Macrophage 2-like phenotypes

Resident tissue Mph express the Mph2 marker CD163, indicating an Mph2 phenotype *in vivo* (25). Mph2 are also found in the lung where they function to protect against unwanted immune responses (26). They were also documented in a Th2-mediated inflammatory setting such as nematode infection (27). Embryonic Mph and Mph isolated from placentae and tumors also display characteristics of Mph2 (28-30). Additionally, coculturing monocytes with Tregs differentiated these cells toward Mph2 (31). Tumor-associated Mph (TAMs) also have Mph2 properties. TAMs originate from circulating monocytic precursors, which are recruited to the tumor in response to secreted chemokines and cytokines derived from cancer cells (32). They express high levels of the mannose receptor, are poor at presenting antigen and their cytokine profile consist of high IL-10 and low IL-12 production (33). The role of TAMs in tumors involves tumor progression and metastasis, immune suppression, and angiogenesis. Thus functionally different Mph2-like phenotypes have already been documented.

Although the results described above suggest fully polarized functions, Mph with overlapping phenotypes have been observed as well. In human Mn infected with cytomegalovirus and in adipose tissue Mph a mixed gene profile expressing both Mph1 and Mph2 genes was observed (34,35). In addition, oxidized phospholipid-treated murine Mph were identified as a new

Mph phenotype which is distinctly different from Mph1 and Mph2 (36). Thus ample Mph-like phenotypes have been observed, indicating the complexity of defining a specific Mph phenotype.

Macrophage activation

Interferon-γ (IFN-γ) was the first identified marker that could activate Mph (37). Later, LPS and GM-CSF were found to promote the generation of Mph1 (38). Factors that skew Mph towards Mph2 are M-CSF, IL-4, IL-13, immune complexes, Wnt5a, IL-10, or TGF-β, indicating great heterogeneity (39-43). These activators all induce a distinct, as well as partially overlapping, pattern of gene expression, thus presenting different Mph2 subsets. Mph2 are induced by M-CSF, Mph2a are induced by IL-4 or IL-13, Mph2b are induced by exposure to immune complexes, agonists of TLRs or IL-1R, and Mph2c are induced by IL-10 and glucocorticoid hormones (39). Functionally, Mph2a are responsible for wound healing and Mph2b together with Mph2c for immune regulation. Th2 driven inflammation is induced by Mph2b, whereas Mph2c are predominantly involved in deactivating immunoregulation (39). Overall, Mph2 participate in Th2 responses, dampen inflammation, promote tissue remodeling, angiogenesis, tumor progression and immune regulation, whereas Mph1 participates in Th1 responses and are protective against tumors and organisms (44).
Recently other Mph subsets have been described. Mph2d are the results of switching from Mph1 as a response to adenosine A2A receptor signaling induced by TLR agonists (45). These Mph have decreased expression of pro-inflammatory cytokines, including IL-12 and TNF-α, while concurrently produce high levels of IL-10 and vascular endothelial growth factor. Mox Mph are induced by ox-PL 1-palmitoyl- 2arachidonoyl-sn-glycero-3-phosphorylcholine, resulting in a population of Mph that express an unique set of genes like heme oxygenase-1 (HO-1) and thioredoxin-reductase in a Nrf2 dependent manner (36). Mha macrophages are differentiated from monocytes using hapto-hemoglobin complexes or oxidized red blood cells leading to the up-regulation of CD163, HO-1 and IL-10 in an Nrf2-dependent manner, suggesting that these two subsets might be related (46). In addition, Mph4 have combined features of Mph1 and Mph2 and were shown to be CSF/CXCL4-dependent (47). These Mph have a weak phagocytic capacity, but their function remains poorly understood. Mox, Mha and Mph4 have until now been poorly characterized. Considering the heterogeneous expression of cell surface markers in Mph, the number of Mph-subpopulations could be infinite (2).

Distinguishing between Mph1 and Mph2 subsets

Considering the great heterogeneity, it is a major challenge to distinguish different Mph subsets. This characterization is mainly based on the expression of cell surface markers as well as their cytokine and chemokine production profile (Table 1). Specific Mph2 markers in mice, arginase-1, FIZZ1, Ym1, Ym2 and MRC1 have been identified both *in vivo* and *in vitro* (1,48), whereas establishing specific markers for human Mph2 is still an ongoing challenge. Candidates like CD163, CD206 (mannose receptor), HO-1, folate receptor-β,

	Mph1 (IFN-g/LPS/GM-CSF)	Mph2 (M-CSF)	Mph2a (IL-4)	Mph2c (IL-10)
markers	MHC class II CD80, CD86 CD16, CD32, CD64 CD206	CD16, CD32, CD64 CD163 MHC class II CD80, CD86	MHC class II CD80, CD86 CD163, CD206 CD16, CD32, CD64	CD16, CD32, CD64 CD163, CD206 MHC class II CD80, CD86
cytokines	TNF-a IL-1, IL6- IL-12 IL-18, IL-23 IL-10	IL-10 TNF-a IL-1, IL-6, IL-18	IL-10, IL-1ra TGF-b TNF-a IL-1, IL-6, IL-18, IL-12	IL-10, IL-1ra TGF-b TNF-a IL-1, IL-6, IL-18, IL-12
chemokines	CCL2,3,4,5,8,15,19,20 CXCL9,10,11,13,16 CX3CL1 CCR7 CCL17,18,22,24 CCR2	CCL2 CCL17,22	CCL2,13,14,17,18 CCL22,23,24,26 CXCL4,8 CXCR1,2 CCL5 CXCL9,10	CCL2,16, 18, 23 CXCL4,13 CCR2,5 CCL5 CXCL9,10
other	high ROS (NOX2) IRF5 STAT5 pathway Th1 response	IRF4 Th2 response	low ROS (NOX2) STAT6 pathway Th2 response	low ROS (NOX2) STAT3 pathway Th2 response

Table 1: Characteristics of Mph1, Mph2, Mph2a, and Mph2c regarding markers, cytokines, chemokines and miscancellous

and CCL18 have been described (1,49-51). In general, the high expression of cell surface markers CD163 and CD206 on Mph2 is used to distinguish Mph1 and Mph2, although different levels have been reported depending on the Mph2 subtype (52-55). Mph1 and Mph2 produce distinct cytokines: Mph1 show high TNF-α, IL-6, IL-12, and IL-23 production and low IL-10 levels, whereas Mph2 typically produce high levels of IL-10 and TGF-β (40,41,53,55,56) (Table 1). In addition, the chemokine production of Mph1 and Mph2 is different. Mph1 produces inflammatory CC chemokines and IFN-γ-responsive chemokines CCL5 (RANTES), CXCL9, CXCL10 (IFN-γ-IP-10), and CXCL16 that will recruit Th1, Tc1, and NK cells and thus a type 1 immune response (39,56). IL-4 and IL-10 differentiated Mph2 will inhibit the expression of most of these chemokines, and thereby limiting inflammation. M-CSF, IL-4, and IL-10 all induce CCL2 (MCP-1). IL-4 induces CCL17, CCL24, and CCL22, which will be inhibited by IFN-γ differentiated Mph1 (39,57). CCL22 binds to CCR4 which is expressed on Th2 lymphocytes, thereby amplifying the Th2 response (58). Taken together, the cytokine and chemokine production of Mph1 will lead to a Th1 response, whereas that of Mph2 will lead a Th2 response.
The transcriptional regulation during differentiation is also different in Mph1 and Mph2 (Table 1). The transcription factor PU.1 is essential to Mph lineage

commitment and Mph-specific gene expression (59). Transcription factors are also involved in the Mph polarization. The transcription factors involved in Mph1 polarization are NF-κB, activator protein-1, CCAAT/enhancer-binding protein-α (C/EBP-α), and interferon regulatory factor (IRF)5, whereas STAT6, peroxisome proliferator-activated receptor γ (PPARγ), IRF4, C/EBP-β, and Krüppel-like factor 4 (KLF4) are associated with Mph2 polarization (60). STAT6 induces the transcription factor PPARγ, which acts together with STAT6 to regulate Mph2-specific genes and polarization (61). KLF4 was found to cooperate with STAT6 to induce an Mph2 profile, while inhibiting Mph1 targets (62). IRF4 has been implicated in Mph2 polarization, whereas IRF5 participates in the activation of genes encoding pro-inflammatory cytokines and represses the gene encoding IL-10, resulting into Mph1 activation (56,63).

ROS producing capacity of Mph1 and Mph2

A distinctive difference described between Mph1 and Mph2 is the high reactive oxygen species (ROS) production by Mph1, while IL-4 differentiated Mph2 are described as non-ROS producing cell (40,53,57,64). ROS generated by Mph are usually associated with oxidative stress (65). However, it has been shown that ROS produced in the immunological synapse or produced in lower amounts can inhibit T cell activation, or can serve as second messenger in various signaling pathways (66-69). These data indicate that the exact role of Mph-derived ROS in the immune system needs to be resolved.

Reactive oxygen species

Important insights into the potential role of ROS in immune regulation were obtained in experimental models of autoimmunity, where Dark Agouti (DA) rats were found to be more prone to develop RA compared with other rat strains. Genetic studies identified a naturally occurring polymorphism in *Ncf1*, component of the NADPH oxidase complex, linked to the severity of arthritis in rats (70). This polymorphism resulted in a reduced ROS production and promoted the activation of arthritogenic T cells.

Phagocytic NADPH oxidase complex

ROS are produced by the mitochondrial electron transport chain, peroxisomes, xanthine oxidase, endoplasmic reticulum and the NADPH oxidases. There are seven family members of the NADPH oxidases, namely NOX complexes 1-5, and DUOX 1 and 2. The main producers of phagocytic NOX2-derived ROS are PMN and Mph. The NOX2 complex is responsible for the oxidative burst, causing a sufficient concentration of ROS in the phagosome, required to kill pathogens. NOX2 consists of multiple components: the membrane-bound cytochrome-b558 consisting of $gp91^{phox}$ and $p22^{phox}$, and the cytosolic components $p47^{phox}$ (*Ncf1*), $p67^{phox}$, and $p40^{phox}$, and the small GTPase Rac. Upon phosphorylation $p47^{phox}$, together with $p67^{phox}$ and $p40^{phox}$, form the NOX2 complex in the membrane by interacting with $p22^{phox}$ and $gp91^{phox}$, resulting in the production of superoxide. This will dismutate to hydrogen peroxide, and

is then further processed to generate more reactive metabolites like hydroxyl radical, after interaction with transition metal ions, and hypochlorous acid, which is catalyzed by myeloperoxidase. NOX2-derived ROS are generated in the plasma membrane, but ROS production can also occur within intracellular organelles, although the exact compartment is yet to be determined (71).
Chronic granulomatous disease (CGD) is a disease that develops when mutations in one of the NOX2 components occur, resulting in defective ROS production. At this moment, mutations in the CYBB (gp91^{phox}), CYBA (p22^{phox}), NCF1 (p47^{phox}), NCF2 (p67^{phox}), and NCF4 (p40^{phox}) genes have been described (72,73). The predominant form of CGD, comprising about 70% of cases, results from mutations in gp91phox which are inherited in an X-linked recessive manner, whereas mutations in the other genes are inherited in an autosomal recessive manner (73). CGD patients have a ROS production of 0.1 – 27% compared to normal levels (74). The X-linked recessive cases are more severe than autosomal recessive cases, since these patients have a lower residual ROS production. In addition, with a modest residual ROS production there is a greater probability of long-term survival compared with those having little residual ROS levels (74). The consequences of the diminished or abrogated oxidative burst by the NOX2 complex in CGD patients are recurrent bacterial and fungal infections, as well as granuloma formation (75). In addition these patients suffer more often from autoimmune diseases such as RA or systemic lupus erythematosis compared to the normal population (76,77). Since a hyperinflammatory phenotype is presented under non-infectious conditions with suboptimal ROS production, this indicates that normal phagocytic ROS production suppress pro-inflammatory gene transcription and preserve homeostasis (71). Interestingly, CGD patients have defective apoptosis after phagocytosis, as a result of reduced induction of the pro-apoptotic protein BAX (78). This study indicates that ROS are involved in the resolution of inflammation as well.

Redox signaling

ROS are being produced as a result of normal cell physiology or due to ageing or stress. As defense against ROS, cells have anti-oxidants, like glutathione, Vitamins C and E, superoxide dismutases, catalase, peroxidases, thioredoxins, glutaredoxins, and peroxiredoxins (79). Oxidative stress generally involves non-specific oxidation of a wide variety of molecules and is associated with higher oxidation states like sulfinic and sulfonic acid, causing irreversibly damage. There are strong indications that oxidative stress is an important pathogenic mechanism in various disorders, including cancer, ageing, atherosclerosis, diabetes, and ischemia-reperfusion injury (80). Thus eliminating ROS by anti-oxidants would potentially be beneficial. However, it has been demonstrated that antioxidants could not improve the disease course of RA or atherosclerosis (81,82). Even more so, the use of pro-oxidant phytol, causing increased ROS production, actually decreased inflammation in animals with arthritis (83).
ROS serve as second messenger in various signaling pathways by changing

specific proteins. Hydrogen peroxide oxidizes thiol groups of protein cysteine residues forming disulfides either with other protein cysteines or with low-molecular weight thiols like glutathione, or forming sulfenic acids. Only a subset of cysteine residues in proteins is susceptible to oxidation, indicating the specificity of the reactions. These redox changes are in many cases associated with altered activity and effects on signaling pathways or cellular processes. In bacteria, the transcriptional activator OxyR, is directly oxidized in response to hydrogen peroxide (84). Even though both the oxidized and reduced OxyR are able to bind DNA, only the oxidized form can activate the transcription of antioxidant genes. In eukaryotes, proteins susceptible to thiol oxidation are protein tyrosine phosphatases (PTPs), kinases, and some transcription factors (85,86). PTPs are inactivated after oxidation, resulting in the activation of downstream signaling pathways. The transcription factor family NF-κB is also under redox control, although both oxidation and reduction by ROS are implicated in the activation process (87). In addition, the stress-responsive transcription factor Nrf2 is activated by the oxidation of its inhibitory partner Keap1, thereby disassociating Nrf2 from the complex, leading to the activation of target genes like HO-1 and superoxide dismutase (88). Since the above mentioned transcription factors also control the expression of genes involved in immunity and inflammation, understanding redox signaling is of great importance.
Effects of oxidation on signaling via the T cell receptor (TCR) has also been reported. In RA patients T cell hyporesponsiveness was dependent on the translocation of linker for activation (LAT) of T cells induced by oxidative stress, which impaired the TCR signaling (89). Also TCR zeta and $p56^{lck}$ signaling molecules have been indicated as redox targets after structural changes were observed after oxidation (90). The integrin VLA-4 was identified as redox target, which is a co-stimulatory molecule in the immunologic synapse (91). These data indicate an important role for ROS in modifying the T cell response, directly associating redox-dependent TCR signaling regulation and T cell function. This was also shown in a rat model where decreased ability to produce ROS in DA rats was associated with increased numbers of thiol groups present on the cell surface of their T cells (92). In addition, T cells from rats with normal ROS production could only transfer arthritis after increasing the number of thiol groups, whereas T cells from DA rats became less arthritogenic after decreasing the thiol groups (92).

ROS producing capacity of myeloid cells

During phagocytosis of pathogens, neutrophils produce NOX2-derived ROS to kill microorganisms (3). Interestingly, when comparing neutrophils from CGD patients with healthy controls increased expression of genes encoding for inflammation and host defense were observed, whereas anti-inflammatory mediators were down-regulated (78). A more recently discovered process stimulated by ROS is the formation of neutrophil extracellular traps (NETs) (93). Stimulated neutrophils generate these NETs in order to catch and kill pathogens. Interestingly, CGD patients cannot form NETs, further implicating

NOX2-derived ROS in the antimicrobial response (93).
Myeloid-derived suppressor cells (MDSC), a recently identified population of myeloid cells, continuously produce inflammatory mediators, like IL-1, IL-6, and nitric oxide, but also ROS (94,95). MDSC are predominantly found in tumour bearing patients where they proliferate and stay activated due to chronic inflammation in the tumour environment. ROS production of MDSC is mediated through increased activity of NOX2. The role of ROS in T cell suppression was confirmed when MDSC from gp91$^{-/-}$ mice were not able to induce T cell tolerance (96). MDSC, via generation of ROS and peroxynitrite (O2·− and NO interaction), induced the modification of the peptide-MHC binding of T cells rendering them unresponsive to antigen-specific stimulation (96).

ROS producing capacity of Mph

The *Ncf1* gene, encoding the NOX2 protein p47^{phox},was responsible for DA rats being more prone to develop RA or multiple sclerosis compared with other rat strains (70). This indicates that a lack of ROS production in these rats caused a higher susceptibility to disease. A mutation in the *Ncf1* gene in the mouse had similar results in models of RA and multiple sclerosis showing increased susceptibility and severity due to reduced oxidative burst (97). Interestingly, in rats with a reduced ROS producing capacity the increased susceptibility to pristane-induced arthritis was mediated in a T cell-dependent fashion (98). However, T cells have a very low NOX2 expression production, if any, suggesting that other cells were involved. Among murine APCs, Mph were the highest ROS producing cells compared with B cells and DCs (99). In addition, mice expressing functional p47phox only in their Mph were similarly protected against collagen induced arthritis compared to fully functional wild type mice (99). These data indicate that ROS derived from Mph inhibit T cell responses. Mn/Mph NADPH oxidase was also identified playing a central role in controlling fungal infecting and limiting lung inflammation in a mouse model (100). Furthermore, mice with Mn/Mph expressing functional *Ncf1* were protected against bacterial infections (101). These data indicate an anti-inflammatory role for Mph-derived ROS. However, IL-4 differentiated Mph2 have reduced respiratory burst, whereas Mph1 are known for their ROS-producing capacity (45,59,68,83). Mph-derived ROS are associated with oxidative stress (84), and therefore linked to Mph1, but apparently there is a dual role for Mph-derived ROS. The function of ROS in the immune system is thus not simply antimicrobial, but NOX2-derived ROS are also involved in immune modulation, cellular signaling, chemotaxis, antigen cross-presentation and in inducing autophagy (102).

Scope of thesis

ROS are implicated in cell signaling and also play a role in immune-modulating processes. Anti-inflammatory Mph could inhibit T cell responses in a ROS-dependent fashion, which was shown using transgenic mice expressing functional *Ncf1* restricted to Mph. However, there is limited information on the T cell inhibitory role of Mph-derived ROS in the human setting. For that reason the scope of this thesis was to obtain better insight in the role of

ROS being produced by anti-inflammatory Mph2 in immune regulation. First we investigated the human anti-inflammatory Mph2 for their functional capacity in a ROS-dependent fashion in **Chapter 2**. The anti-inflammatory potential of the ROS-producing capacity of Mph2 led us to investigate the possibility of using Mph2 as cellular therapy, therefore **Chapter 3** focuses on the effect of several commonly used immunosuppressive drugs on Mph1 and Mph2 regarding ROS production and the subsequent effect on T cell responses. Since inhibition of T cell activation in a ROS-dependent fashion was observed, we investigated a potential mechanism by which Mph2-derived ROS could affect T cell function in **Chapter 4**. Since an observed protective role of macrophages-derived ROS in autoimmune diseases was observed, in **Chapter 5** we investigated the impact of ROS production on the inflammatory response in a renal allograft transplantation model. **Chapter 6** summarizes the studies presented in this thesis and discusses the implications of these findings as well as some unpublished data, and further discusses the role of Mph2-derived ROS in immune modulation.

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Chapter 2

Induction of regulatory T cells by macrophages is dependent on production of reactive oxygen species

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Abstract

The phagocyte NAPDH-oxidase complex consists of several phagocyte oxidase (^{phox}) proteins, generating reactive oxygen species (ROS) upon activation. ROS are involved in the defense against microorganisms but also in immune regulation. Defective ROS formation leads to chronic granulomatous disease (CGD) with increased incidence of autoimmunity and disturbed resolution of inflammation. Because regulatory T cells (Tregs) suppress autoimmune T-cell responses and are crucial in down-regulating immune responses, we hypothesized that ROS deficiency may lead to decreased Treg induction. Previously, we showed that in p47^{phox}-mutated mice, reconstitution of macrophages (Mph) with ROS-producing capacity was sufficient to protect the mice from arthritis. Now, we present evidence that Mph-derived ROS induce Tregs. *In vitro*, we showed that Mph ROS-dependently induce Treg, using an NADPH-oxidase inhibitor. This was confirmed genetically: rat or human CGD Mph with mutated p47^{phox} or gp91^{phox}, displayed hampered Treg induction and T-cell suppression. However, basal Treg numbers in these subjects were comparable to controls, indicating a role for ROS in induction of peripheral Treg. Induction of allogeneic delayed-type hypersensitivity with p47^{phox}-mutated Mph confirmed the importance of Mph-derived ROS in Treg induction *in vivo*. We conclude that NAPDH oxidase activity in Mph is important for the induction of Tregs to regulate T cell-mediated inflammation.

Introduction

Reactive oxygen species (ROS) are not only harmful and mediators of oxidative stress, but also have immune regulatory functions, especially when produced in lower amounts (1,2,3). The mechanisms of how ROS affect the immune system are just beginning to become clear. For example, mitochondrial ROS oxidize released HMGB-1 during apoptosis, thereby preventing immune activation and allowing induction of tolerance (4), and myeloid-derived suppressor cells suppress antitumor T-cell responses in a phagocytic NADPH-oxidase (Nox2) complex-dependent way (5). This Nox2 complex consists of multiple components (i.e. the membrane-expressed cytochrome-b558 consisting of gp91^{phox} and p22^{phox} and the cytosolic components p47^{phox}, p67^{phox} and p40^{phox}). Chronic granulomatous disease (CGD) develops when any of these components is absent or functionally hampered and ROS production is defective. CGD is characterized by recurrent bacterial and fungal infections and abnormal granuloma formation. These granulomas are mostly sterile and often respond to steroid therapy without antibiotics (6). In addition, CGD patients suffer more frequently from autoimmune diseases as compared to the healthy population (7,8). These features point to a defect in immune regulation due to the absence of ROS.

We observed previously that mice and rats with alleles of the neutrophil cytosolic factor 1 (*Ncf1*) gene (encoding p47^{phox}) that encode a less functional Nox2 and thus lower ROS production are more susceptible to induced autoimmune diseases than are their wild-type littermates (9,10). This observation is in line with observations in CGD patients. Interestingly, the reduced ROS-producing capacity in our congenic rat model mediated higher susceptibility to pristane-induced arthritis in a T-cell-dependent fashion (9,11). However, T cells express no or only very low levels of Nox2, suggesting that other cells determine the T-cell response by producing ROS (12). One study, however, does show low levels of Nox2 in T cells (13). Because antigen-presenting cells (APC) interact with T cells during antigen presentation, and APC express Nox2 they may affect T-cell responses via ROS production. Indeed, ROS produced during antigen presentation affect the immune response (14,15) by interfering in signal transduction (16,17). In addition, ROS generated in phagosomes/endosomes determines the ability to cross-present antigen, both in mice and humans (18,19). We showed that amongst murine APCs, macrophages (Mph) were most efficient at producing ROS (20). Transgenic mice expressing functional p47^{phox} only in Mph on a p47^{phox} mutated background were equally protected against collagen-induced arthritis (CIA) as their fully wild-type littermates. These observations indicate that Mph-derived ROS are sufficient to inhibit T-cell responses (20).

Regulatory T cells (Tregs) can suppress activation and proliferation of effector T cells and thereby diminish immune responses. Autoimmunity can therefore be the result of a defective Treg system (21) and successful treatment of autoimmune disease with Tregs has been reported in mouse models (22,23). We hypothesized that if Mph-derived ROS prevent T-cell-mediated immune responses, it could do so by inducing Tregs. Previously we showed that antiinflammatory Mph can induce potent Tregs, in contrast to proinflammatory Mph (24). Here we here investigated whether Mph-derived ROS influence induction of Tregs in humans *in vitro* as well as in rats *in vivo*. We observed that Mph can induce Tregs in a ROS-dependent fashion *in vitro* and, more importantly, that Mph from ROS deficient CGD patients are significantly less efficient in inducing Tregs. We conclude that Mph can modulate T-cell responses by producing ROS and induce Tregs in a ROS-dependent fashion.

Results

Human Mph produce ROS upon stimulation

To investigate the role of ROS in antigen presentation by different APC, the expression of two Nox2 members was determined: p47^{phox} (*Ncf1*) and gp91^{phox} (*Cybb*) in human dendritic cells (DC) and in Mph colony-stimulating factor (M-CSF)-differentiated Mph. mRNA expression for both p47^{phox} and gp91^{phox} was about 30 times higher in Mph as compared to DC (Fig 1A). By intracellular

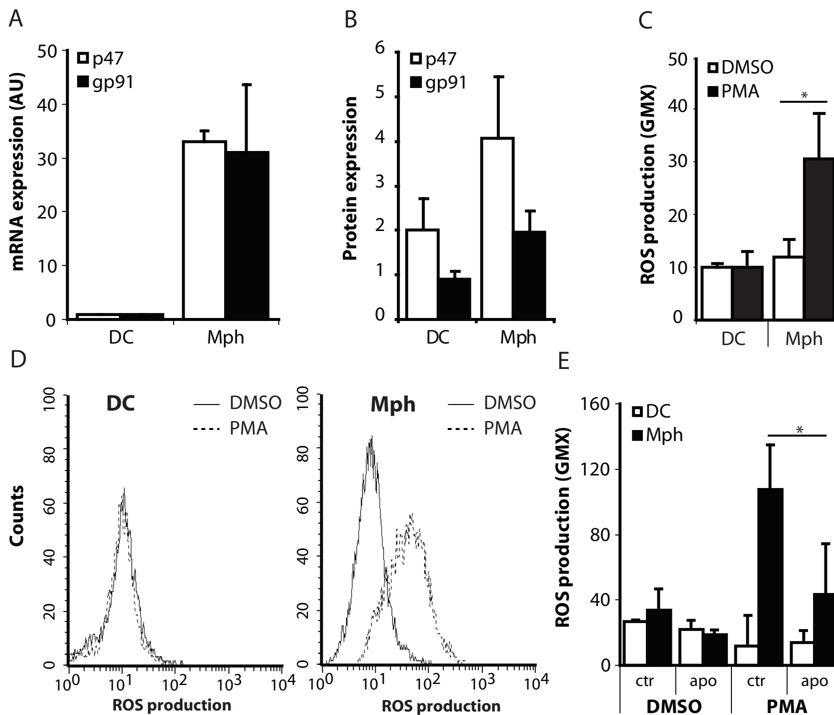

Figure 1: Mph produce ROS. (***A***) mRNA and (***B***) protein expression levels of the two most important NADPH oxidase complex components, p47^{phox} and gp91^{phox}, determined in DC and Mph by RT-PCR and flowcytometry, respectively. In (A) mRNA expression levels were corrected for GAPDH expression and expression levels of DC were set to 1. (***B***) shows the ratio between specific staining and isotype control. Shown are average + SD of the relative expression levels of 4 experiments with cells from 4 different donors. (***C***) The capacity of DC and Mph to produce ROS was measured after PMA stimulation. Shown are average + SD of 6-10 independent experiments; the conditions after PMA stimulation or stimulated with vehicle (DMSO) are shown. (***D***) Representative examples of FACS histograms of ROS production, measured by DHR123 fluorescence, by DC (left) and Mph (right) after PMA (dotted line) or DMSO (ctr; solid line) stimulation. (***E***) Production of ROS by DC and Mph was measured after PMA or vehicle activation and in the absence or presence of the specific p47^{phox} inhibitor apocynin (1 mM). Average + SD of 3-4 independent experiments are shown, * = $p<0.05$.

FACS analysis we observed that the expression of gp91^{phox} protein was very low in DC, whereas p47^{phox} was clearly present. In contrast, Mph showed significant expression of both gp91^{phox} and p47^{phox} (Fig 1B). Functionally, human Mph efficiently generated ROS upon phorbol 12-myristate 13-acetate (PMA) stimulation (Fig 1C,D). In contrast, DC hardly produced ROS, in line with the low of gp91^{phox} expression. Mph differentiated in GM-CSF (proinflammatory Mph) produced only marginal amounts of ROS (Fig S1A) The ROS production by Mph could be blocked to background levels with apocynin; an inhibitor of Nox2 that binds p47^{phox} and prevents translocation to the membrane (Fig 1E).

Mph suppress T-cell responses by producing ROS

To investigate the suppressive capacity of ROS produced by APC on T-cell responses, purified T cells were activated with anti-CD3/CD28 mAbs in

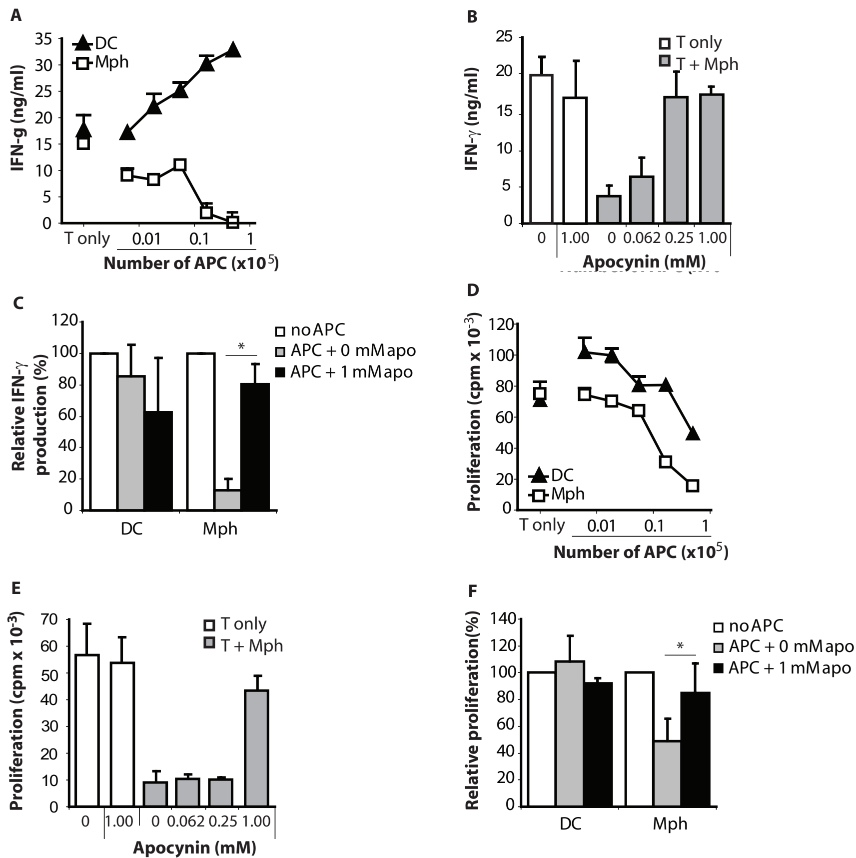

Figure 2: Mph suppress T cell activation in a ROS-dependent fashion. (***A***, ***D***) T cells (150.000) were activated with anti-CD3/28 Ab and Mph or DC were added in increasing numbers (x-axes). After 5 days of coculture IFN-γ production (A) and proliferation (D) were determined by ELISA and 3H thymidine incorporation, respectively. Shown are the average + SD of triplicates within one representative experiment. (***B***, ***E***) 150.000 T cells and 25.000 Mph were cocultured in absence or presence of different concentrations of apocynin (x-axis) to study the effect of ROS on Mph mediated T cell suppression. After 5 days of coculture IFN-γ production (B) and proliferation (E) were determined by ELISA and ^{3}H thymidine incorporation, respectively. Representative experiments are shown. (***C***, ***F***) Similar experiments as in A,B,D,E, but the average with SD of 3-4 independent experiments are shown for an APC:T ratio of 1:6. Values shown are relative to the conditions without APC (100%, white bars). * = $p<0.05$

the presence of increasing numbers of Mph or DC. IFN-γ production and the proliferative response were determined at day 5. IFN-γ production (Fig 2A,C,and Fig S1B) and, to a lesser extent, T cell proliferation (Fig 2D,F) were suppressed by Mph but not by DC. The Nox2-inhibitor apocynin abrogated the Mph-mediated suppressive effect in a dose-dependent manner (Fig 2B, C, E and F). GM-CSF differentiated proinflammatory Mph slightly suppressed T-cell activation, but this suppression was not reversible by apocynin and thus was not ROS-dependent (Fig S1C).

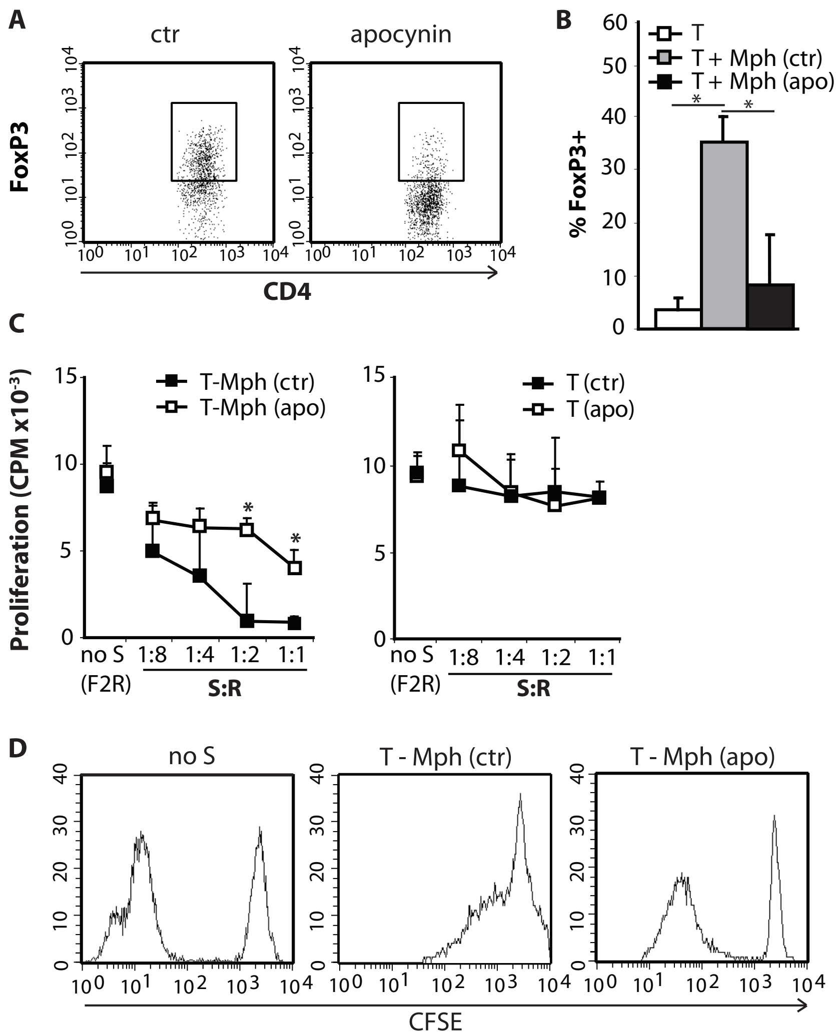

Figure 3: Mph induce Tregs via ROS. (***A***) Expression of FoxP3 within CD3⁺CD4⁺CD25⁺ population of T cells primed with Mph in absence (left) or presence (right) of apocynin. (***B***) Priming of CD4⁺CD25⁻ T cells with Mph in the presence or absence of apocynin for 7 days, followed by the analysis of the percentage of FoxP3⁺ cells amongst CD3⁺CD4⁺CD25⁺ cells. The white bar represents CD4⁺CD25⁻ T cells cultured similarly, but without Mph. The average + SD of 3 experiments is shown. (***C***) T cells were primed with (left panel) or without (right panel) Mph in presence or absence of apocynin (1 mM). After 5 days, these T cells were used as suppressor cells (S) and combined with responder T cells (R) and irradiated feeder cells (F). Proliferation of R was assessed by ^{3}H thymidine incorporation (C) or by determining CFSE dilution (D). In C, average + SD of 4 experiments are shown. In D, the 1:1 ratio from a representative experiment out of 3 is shown, as well as the control condition without S, but with two times responders (F2R) to correct for crowding effects. * = $p<0.05$

Mph induce Tregs in a ROS-dependent fashion

Mph differentiated with M-CSF induce $CD4^{+}CD25^{+}FoxP3^{+}$ Tregs (24). The observed ROS-dependent suppression of T-cell activation by Mph could be mediated, at least in part, via induction of Tregs. To check this possibility,

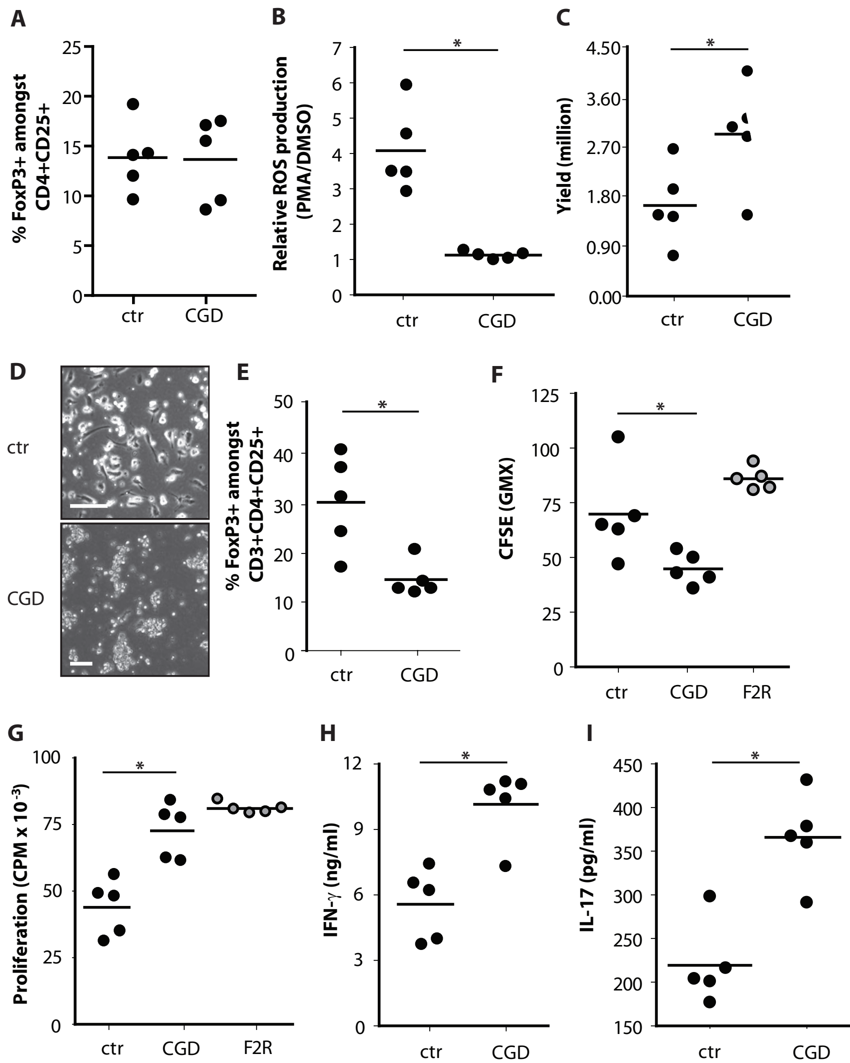

Figure 4: CGD macrophages induce less Tregs. (***A***) The % of FoxP3+ cells amongst CD4+CD25+ in peripheral blood of CGD patients and healthy controls. (***B***) ROS production by Mph differentiated from healthy control or CGD monocytes, as determined by flow cytometry after DHR123 staining. The ratio of ROS production after stimulation with PMA or DMSO is depicted. (***C***) 106 CD4+CD25- T cells per condition, from a single allogeneic donor, were primed with Mph from either CGD patients or controls in presence of anti-CD3/28 Ab. After 5 days the number of viable T cells was determined. (***D***) Represenative picture showing the T cell clustering observed after activation with anti-CD3/CD28 in presence of ctr Mph (upper) or Mph derived from CGD patients (lower). The bars represent 25 μm. (***E***) T cells primed with Mph derived from CGD patients or controls were analyzed for the percentage of FoxP3+ cells amongst CD4+CD25+ by flow cytometry. (***F***) CFSE-labeled responder cells were cocultured with the Mph-primed T cells and irradiated feeder cells in presence of anti-CD3/28 and dilution of CFSE was measured by flow cytometry after 4 days. (***G***) In parallel experiments, ^{3}H thymidine incorporation was determined. Grey dots are control conditions in the absence of Mph-primed suppressor T cells but with double amounts of responder cells (F2R), to correct for crowding effects. In the supernatants of this suppression assay IFN-γ (***H***) and IL-17 (***I***) levels were determined. * = $p<0.05$

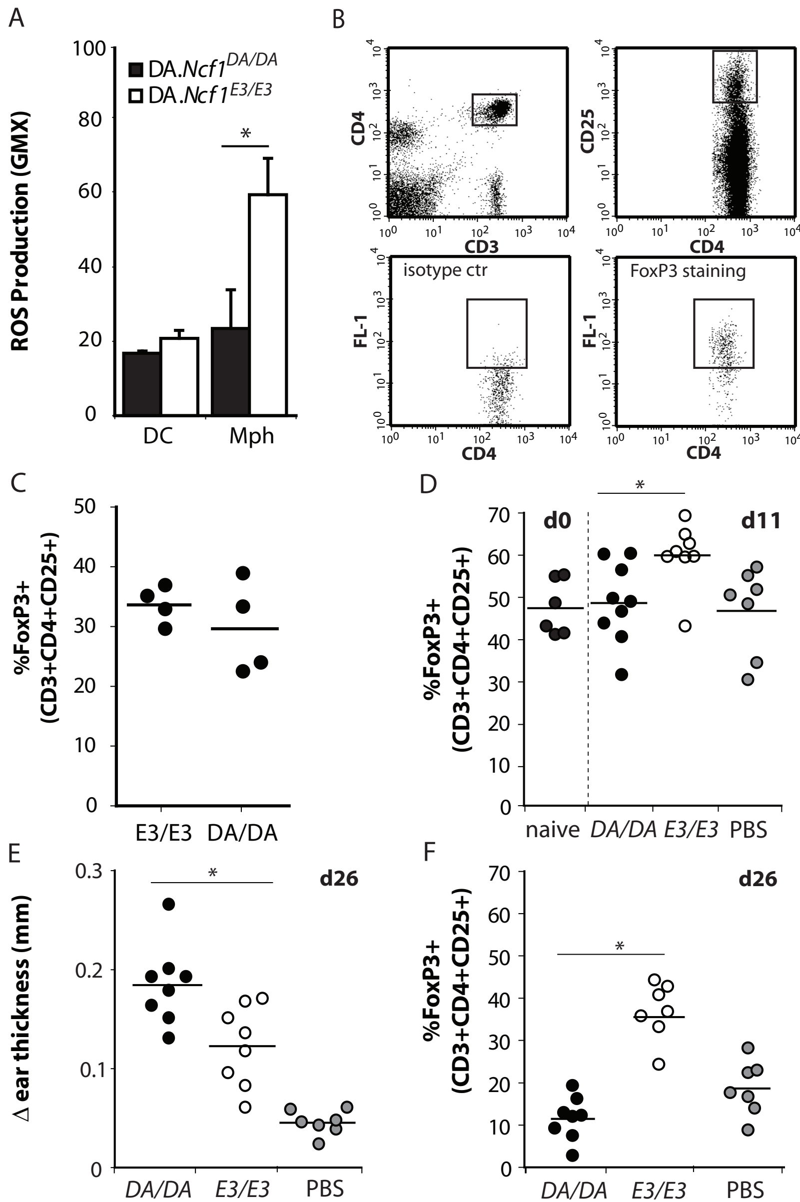

Figure 5: Mph suppress DTH reponses in vivo in a ROS-dependent fashion. (***A***) Rat BM cells were cultured with rat GM-CSF and IL-4 or human M-CSF to obtain DC and Mph, respectively. ROS production by PMA-stimulated DC and Mph generated from DA.*Ncf1*$^{DA/DA}$ or congenic *Ncf1*-wildtype DA.*Ncf1*$^{E3/E3}$ rats was measured by DHR123 staining. Average + SD of 4 experiments are shown. (***B***) Treg gating strategy on peripheral blood. (***C***) The % of FoxP3$^+$ cells amongst CD3$^+$CD4$^+$CD25$^+$ in peripheral blood of naïve DA.*Ncf1*$^{E3/E3}$ ($^{E3/E3}$) rats or DA.*Ncf1*$^{DA/DA}$ ($^{DA/DA}$) rats. (***D***) Lewis rats were primed with Mph from either DA.*Ncf1*$^{DA/DA}$ ($^{DA/DA}$) or DA.*Ncf1*$^{E3/E3}$ rats ($^{E3/E3}$) or PBS at day 0. At day 11, the % of FoxP3$^+$ amongst CD3$^+$CD4$^+$CD25$^+$ T cells was determined and compared to the levels in naïve Lewis rats. (***E***) After immunization at day 11 and challenging in the ear at day 25 with irradiated $^{DA/DA}$ splenocytes, the difference in ear thickness, as a measure for the DTH reaction, was determined at day 26. (***F***) The percentage of FoxP3$^+$ amongst CD3$^+$CD4$^+$CD25$^+$ T cells at day 26 after priming. * = $p<0.05$

$CD4^+CD25^-$ T cells were cocultured with Mph and anti-CD3/28 for 5 days and the percentage of $FoxP3^+$ cells amongst $CD3^+CD4^+CD25^+$ cells was assessed (Fig 3A left, 3B). The percentage of $FoxP3^+$ cells amongst $CD3^+CD4^+CD25^+$ was increased upon coculture of T cells with Mph, confirming their Treg inducing capacity. Addition of apocynin to these cultures significantly reduced the number of Tregs (Fig 3A right, 3B), demonstrating the ROS dependency of Treg induction. To determine the functional capacity of these Tregs, suppression assays were performed. $CD4^+CD25^-$ T cells were primed by Mph for 5 days, in presence of anti-CD3/28 and in absence or presence of apocynin. These primed T cells were used as suppressor cells and combined with $CD4^+CD25^-$ allogeneic carboxyfluorescein succinimidyl ester (CFSE)-labeled responder T cells and irradiated feeder cells. After 5 days, T cell proliferation was assessed by ^{3}H thymidine incorporation and CFSE dilution. T cells primed with Mph suppressed proliferation of responder T cells in a dose dependent fashion. In line with the observed ROS dependence of FoxP3 induction, priming in presence of apocynin prevented this suppressive activity (Fig 3C, D). ROS dependency of this effect was confirmed further by the observation that GM-CSF differentiated Mph that hardly produced ROS did not induce Treg (Fig S2A, B).

Mph from CGD patients show disturbed Treg induction

To obtain genetic proof of the ROS-dependency of Treg induction, similar experiments were performed with Mph from CGD patients. The percentages of $FoxP3^+$ cells amongst $CD4^+CD25^+$ cells were determined in peripheral blood of CGD patients and from healthy controls. No differences were observed (Fig 4A). To study the role of ROS in induction of Treg by Mph, monocytes from CGD patients and controls were isolated and cultured in M-CSF to obtain Mph. Mph from CGD patients and healthy controls showed similar morphology and expression levels of CD14 and CD163 (25), whereas the capacity to produce ROS was completely absent in CGD Mph (Fig 4B). Upon priming of $CD4^+CD25^-$ T cells from one donor with either CGD or control Mph in presence of anti-CD3/28, we observed that CGD Mph allowed significantly more T cell activation and expansion (Fig 4C and D). Moreover, CGD Mph induced significantly lower numbers of $FoxP3^+$ T cells (Fig 4E) than did control Mph. In a suppression assay, we observed that T cells primed by CGD Mph showed reduced inhibition of responder T cell proliferation than did cells primed by control Mph (Fig 4F, G). In line with this observation, the levels of IFN-γ and IL-17 produced in these assays were significantly higher when suppressor cells were primed with CGD Mph than with control Mph (Fig 4H, I). These results provide genetic confirmation in human cells that production of ROS by Mph is involved in the induction of peripheral Tregs in human cells.

Mph induce Tregs in vivo in a ROS-dependent fashion

To investigate whether Mph induce Treg in a ROS-dependent fashion *in vivo*, we used the congenic rat model (9). Dark Agouti (DA).*Ncf1*$^{DA/DA}$ rats have a reduced ROS producing capacity due to SNPs (M106V and M153T) in *Ncf1*.

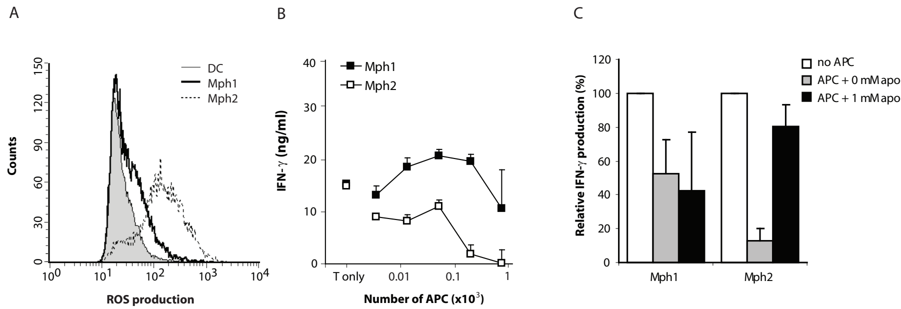

Figure S1: Anti-inflammatory Mph2 but not pro-inflammatory Mph1 suppress T cell activation in a ROS dependent fashion. (***A***) Proinflammatory Mph (Mph1) produce lower levels of ROS as compared to anti-inflammatory Mph2 as measured by DHR123 fluorescence by flow cytometry after stimulation with PMA. (***B***) Suppression of anti-CD3/28 Ab induced T cell IFN-γ production by pro-inflammatory Mph1 is less efficient as compared to suppression by anti-inflammatory Mph2. (***C***) Apocynin (apo) prevents inhibition of T cell activation by anti-inflammatory Mph2 but not by pro-inflammatory Mph1.

DA.$Ncf1^{E3/E3}$ congenic rats express the allelic variant which leads to higher ROS production. First ROS production by Mph and DC cultured from bone marrow of both strains was determined. Similar to the human and murine (20) situation, Mph of the DA.$Ncf1^{E3/E3}$ strain were able to produce significant levels of ROS after stimulation, whereas DC were far less efficient (Fig 5A). To compare circulating Treg numbers, FoxP3$^+$ cells amongst CD3$^+$CD4$^+$CD25$^+$ cells was measured, but no differences between the two strains were observed, similar to findings in CGD patients and controls (Fig 5B,C). To study the effect of Mph ROS on Treg induction and T cell responses *in vivo*, allogeneic Lewis rats were primed with Mph from either DA.$Ncf1^{E3/E3}$ or DA.$Ncf1^{DA/DA}$ rats. Eleven days later, all rats were immunized with irradiated splenocytes from DA.$Ncf1^{DA/DA}$ rats to boost the anti-DA response. At this time point Lewis rats primed with DA.$Ncf1^{DA/DA}$ Mph, had lower numbers of CD3$^+$CD4$^+$CD25$^+$FoxP3$^+$ cells than those primed with ROS-sufficient DA.$Ncf1^{E3/E3}$ Mph (Fig 5D), whereas the number of activated T cells (CD4$^+$CD25$^+$) cells was comparable (Fig S3A). Two weeks later, all rats were challenged with irradiated DA.$Ncf1^{DA/DA}$ splenocytes in the ear, to evoke a delayed-typer hypersensitivity (DTH) response. After 24 hours, rats initially primed with ROS producing DA.$Ncf1^{E3/E3}$ Mph showed a significantly less ear swelling as compared to rats primed with Mph from DA.$Ncf1^{DA/DA}$ rats (Fig 5F). Moreover, these rats still showed higher levels of FoxP3$^+$ cells in their peripheral blood (Fig 5G), whereas the number of activated T cells did not differ between groups (Fig S3B). These results indicate that Mph induce Tregs in a ROS-dependent fashion *in vivo*, thereby leading to lower T-cell responses.

Discussion

Here we show that, in both humans and rats, Mph-derived ROS suppress T-cell responses by induction of Tregs. This finding was confirmed by using Mph

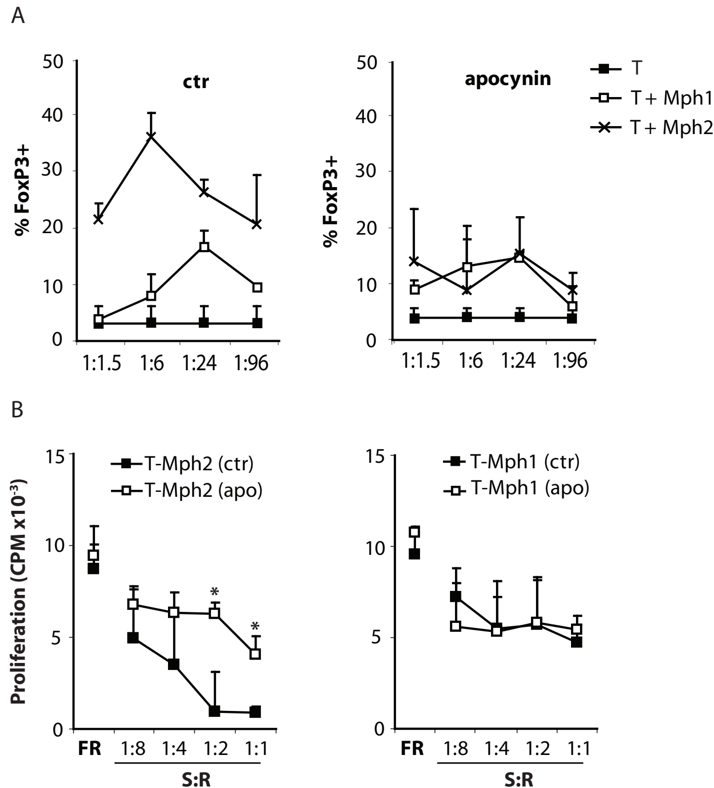

Figure S2: Mph2 but not Mph1 induce Treg in a ROS dependent fashion. (***A***) Priming of $CD4^+CD25^-$ T cells with anti-inflammatory Mph2 but not with pro-inflammatory Mph1 induces FoxP3 expression by these T cells. (***B***) T cells primed with Mph2 but not when primed with Mph1 suppress proliferation of responder T cells in a ROS dependent fashion. Apocynin (apo) was present during priming to inhibit the Nox2 complex.

from CGD patients. Importantly, we show that Mph induces Tregs in a ROS-dependent fashion both *in vitro* and *in vivo*.

ROS can inhibit T cell activation (14,15,26). For example, in cancer patients, granulocyte-derived H_2O_2 mediates impairment of T cell function (27). The mechanism how ROS affect T-cell responses is still quite unclear. Rats with defective ROS production because of an SNP in *Ncf1*, have more reduced proteins at their T-cell surfaces. The functional implications of these high cell-surface thiol levels were shown by arthritis transfer experiments; decreasing the number of thiols on $CD4^+$ T cell surfaces abrogated their ability to transfer disease from sick DA.*Ncf1*$^{DA/DA}$ rats to naïve DA.*Ncf1*$^{E3/E3}$ rats (12). Alternatively, ROS may pass the cell membrane and affect signal transduction proteins such as ζ-chain-associated protein kinase 70 (ZAP70) and linker of activation of T cell (LAT) (28). Another role for ROS has been suggested in the kynurenine pathway of tryptophan catabolism. In ROS-deficient mice with aspergillosis an O_2^- dependent step in the kynurenine pathway was blocked, contributing to the observed acute lung inflammation and unrestrained γδ T-cell activity (29).

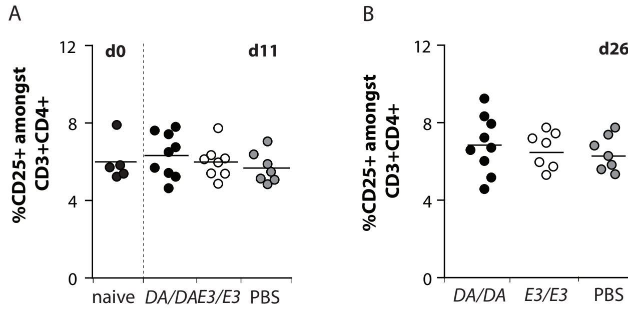

Figure S3: The number of activated T cells during DTH remains similar between groups. (***A***, ***B***) The percentage of CD25+ cells amongst CD3+CD4+ cells 11 days (A) or 26 days (B) after priming the rats with DA.Ncf1DA/DA Mph, DA.Ncf1E3/E3 Mph or PBS is comparable between groups.

On the other hand, CGD Mph previously have been shown to have a normal tryptophan metabolism (30) and recently the microsomal cytochrome b_5 rather than O_2^- was recently indicated to activate indoleamine 2,3 dioxygenase (31). (However, this may vary among species.) Finally, it has been reported that ROS can induce apoptosis in T cells (32,33), thereby decreasing the number of activated T cells. In our studies we did not see increased levels of apoptosis upon coincubation of Mph and T cells, so it is unlikely that increased apoptosis is the reason for the observed inhibition of activation. APC may affect T cells during antigen presentation through the production of ROS. This activitiy may take place in the immunological synapse, hence creating a micromilieu that allows oxidation of specific proteins; however, this notion has yet to be investigated. DC generally are considered to be the most powerful APC (34). We show here, as was shown previously (20)(35), that DC are not very efficient at producing ROS. In contrast, the Mph we investigated were very efficient in producing ROS. These Mph are differentiated from monocytes with M-CSF and have an antiinflammatory phenotype (36,37,38). Our Mph2 and Mph1 may not represent exactly all Mph occurring *in vivo*, but they provided good polar models to answer our research question (39). We already showed that these Mph, unlike proinflammatory type Mph, are able to induce potent Tregs (24). Tregs play a critical role in the prevention of autoimmunity and resolution of inflammation (40). Because Mph prevented T cell-mediated autoimmunity in the mouse by producing ROS, we here studies the effect of ROS produced by Mph on T cell activation and Treg induction, both in CGD patients and in an *Ncf1*-mutated rat model. Although ROS may have direct effects on T cells by oxidation of certain intracellular or membrane-bound proteins important for T cell signaling, ROS may also induce Tregs through Mph. We chose to use T-cell activation by Mph in an allogeneic setting, to exclude effects of ROS on antigen processing and presentation, which have been described before (19). We showed that Mph induce Tregs from a $CD4^+CD25^-$ population only when they were able to produce ROS. Both pharmacologic and genetic inhibition of ROS production abrogated the ability of Mph to induce Tregs. Interestingly, the percentages of Treg in peripheral blood of CGD patients vs control subjects or in DA.*Ncf1*$^{DA/DA}$

vs DA.$Ncf1^{E3/E3}$ rats were comparable. This observation suggests that, in contrast to peripheral Treg induction, the number of natural Treg induced in the thymus, is not affected by Mph ROS, although expansion of naturel Tregs in the Lewis rats in the DTH experiments could not be excluded. Previously we have shown that Tregs induced by antiinflammatory Mph use membrane-bound TGF-β for suppression (24). Although we did not address the role of TGF-β in this study, it has been reported previously that ROS can activate TGF-β (41,42). However, these ROS were not cell-derived, so it is unknown whether ROS produced by Mph upon interaction with a T cell has similar effects on membrane-bound TGF-β, and if such activation would affect Treg induction. It has also been shown that T cells themselves can produce ROS after anti-CD3/28 Ab activation and that these ROS activate TGF-β, leading to Treg induction (43). However, this ROS is produced intracellular and this mechanism probably is not comparable to our system. The exact mechanisms, however, still need to be investigated. It is an attractive idea that, as long as activation signals remain below a certain threshold level, Mph prevent unwanted inflammation and autoimmunity by regulating T-cell responses via the production of ROS (3). Upon potent immune activation (e.g. efficient antigen presentation by DC), the immune suppressive effect of Mph may be overwhelmed. This hypothesis is in line with the observation that both patients with CGD and mice with a nonfunctional Nox2 are more prone to develop autoimmunity (8,10,44,29,45). This observation suggests that Mph-derived ROS may protect against (auto-) immune activation. Indeed, altered monocyte function due to aberrant inflammatory gene expression has been observed in CGD patients (46,47). It would be interesting to investigate if the increased autoimmunity and defective granuloma resolution in CGD patients result from a defect in Treg induction. Recently, a role for Treg in granuloma clearing was described in Wegener's granulomatosis. Treg number and function were reduced in patients with this disease, and the reduction was most pronounced in subjects with most active disease (48).
To investigate if ROS production by Mph could also inhibit allogeneic T-cell responses *in vivo*, DTH experiments were performed in a rat model. These experiments showed that Mph can prevent T cell responses and induce Tregs in a ROS-dependent fashion *in vivo*, thereby decreasing the allogeneic response. We thus demonstrated that the ability to produce ROS by APC plays a critical role in determining whether these APC will activate or suppress T cells. In conclusion, we show that Mph, by producing ROS, suppress T-cell activation and induce Tregs both *in vitro* and *in vivo*.

Methods

Animals

Rats were from Harlan (DA or Lewis; Horst, the Netherlands) or own breeding (DA.$Ncf1^{E3/E3}$; founders originating from Medical Inflammation Research, Karolinska Institute, Stockholm, Sweden)(9). For DTH experiments littermates

were used (DA.*Ncf1*$^{DA/DA}$ and DA.*Ncf1*$^{E3/E3}$). Rats were used at 8-12 weeks of age and groups were sex and age matched. Rats were kept in polysterene cages and fed standard rodent chow. Animal experiments were approved by the committee of medical ethics (CEM) of the Leiden University Medical Center.

Patients

Peripheral blood was obtained from 5 CGD patients with mutations identified in the genes encoding p47^{phox} (AR-CGD, homozygous Δdeletion in NCF1) or gp91^{phox} (X-linked CGD, mutations in CYBB). Patients signed informed consent. PBMC were isolated as described below. The number of viable monocytes was not different from healthy controls.

Myeloid cell culture & T cell isolation

Human: Monocytes were isolated from buffy coats by positive selection of CD14^{+} cells from the ficoll interphase by MACS (Miltenyi)(38). Monocytes were cultured for 7 days in either 10 ng/ml IL-4 plus 5 ng/ml GM-CSF (both from Biosource) to obtain DC or 5 ng/ml M-CSF or GM-CSF (R&D systems) to obtain Mph (38). Cells were cultured at 1.5x10^{6}cells per well in 6 wells plates and medium containing cytokines was refreshed twice. For DC, the non-adherent cells were used. Macrophages were harvested by gentle scraping after short trypsin incubation (3 min, 37°C). T cells were isolated from buffy coats by sheep red blood cell rosetting. For Treg experiments CD4^{+} were isolated by MACS (negative selection kit, Miltenyi). CD25^{+} depletion was performed by panning on petridishes coated with goat-anti-mouse Ab and capturing T cells positively labeled with anti-CD25 Ab. These cells were >90% CD4^{+} and >99% CD25^{-}.
Rat: For rat, bone marrow cells were cultured in rat GM-CSF $^{+}$ rat IL-4 (Biosource) to obtain DC or in human M-CSF (5 ng/ml) to obtain Mph and refreshed every other day. The culture medium contained additional L-glutamine (2 mM) and fungizone but no β-mercaptoethanol. Cells were used after 7 days. T cells were isolated from spleens by magnetic sorting for CD3^{+} cells (Dynabeads).

MLR

150.000 allogeneic T cells were cocultured with irradiated (40 Gy) myeloid cells in an MLR in different ratios, with soluble anti-CD3 and anti-CD28 (IxE; 1 μg/ml and CLB-CD28/1; 0,25 μg/ml, both kindly provided by Prof L.A. Aarden). After 5 days, supernatant was assayed for IFN-γ production (eBioscience) and cells were cultured for 16 hours in presence of ^{3}H thymidine (0,5 μCi) and thymidine incorporation was determined as a measure for proliferation. Anti-CD28 alone had no effect and the Ab had no effect on myeloid cell proliferation in absence of T cells. Apocynin (Sigma) was used in concentration ranges up to 1 mM.

Flowcytometry

Expression levels of surface proteins were measured by flowcytometry (FACScalibur, BD) after staining with specific conjugated Ab or unconjugated Ab detected by conjugated secondary Ab. ROS production was determined

by incubating the cells with dihydrorhodamine123 (DHR123), 5 μM in RPMI^{++} at 37°C for 10 min. Subsequently, oxidative burst was induced by adding PMA (200 ng/ml for 20 min at 37oC). Human and rat Tregs were detected by staining with anti-CD25-PE, anti-CD3-PerCP and anti-CD4-APC (BD). Subsequently, cells were permeabilized, fixed (BD) and stained with anti-FoxP3-FITC, or FITC labeled isotype control, according to the manual to the used kit (eBioscience). The lymphocyte fraction was selected and the % of FoxP3^{+} amongst CD3^{+}CD4^{+}CD25^{bright} cells was determined. Apoptotic cells were determined by Annexin-V-FITC, propidium iodide (BD) double staining. Single and double positive cells were analyzed as being apoptotic and dead cells respectively. The expression levels of p47^{phox} and gp91^{phox} were also determined by intracellular staining as described above for FoxP3 staining using mouse-anti-human p47^{phox} and gp91^{phox} Ab (Santa Cruz Biotechnology), detected with goat-anti-mouse-PE (DAKO).

T cell suppression assay

CD4^{+}CD25^{-} T cells from one donor were cultured with macrophages from another donor for 5 days in a ratio of 6:1, in the presence of anti-CD3/28. After 5 days, T cells were harvested and depleted for HLA ClassII^{+} cells and used as suppressor cells (S) (<1% Mph contamination). Freshly isolated CD4^{+}CD25^{-} T cells were used as responders, whereas CD4- cells from the same donor were irradiated (40 Gy) and used as feeder cells. R:F:S was ranging from 8:16:32 to 8:16:1. A control condition with feeders and double the number of responder T cells (F2R) in stead of FR was taken along to correct for possible crowding effects in the absence of suppressor cells as present in experimental conditions. Cells were stimulated with a low dose of PHA (1 μg/ml) or anti-CD3/28 (49). After 5 days cells were labeled for 8-16 hr with ^{3}H Thymidine and incorporated radioactivity was determined. Supernatant was subjected to cytokine analysis by ELISA or Luminex bead-based assay. In some experiments, responder cells were labeled with 5μM CFSE, and proliferation was determined by FACS analysis, by gating on the CFSE labeled population and quantifying the CFSE dilution.

mRNA isolation and Q-PCR

mRNA was isolated using a Qiagen kit . After making cDNA, semi quantitative PCRs were performed on a Biorad Icycler machine and the following primers were used: p47^{phox} (Forward: cctgacgagacggaagac; Reverse: gggaagtagcctgtgacg), gp91^{phox} (Forward: tagtgggagcagggattg; Reverse: tcaaaggcatgtgtgtcc). The following GAPDH primers were used for normalization: Forward ttccaggagcgagatccct and reverse cacccatgacgaacatggg.

DTH experiments

Priming of Lewis rats at day 0 was done by i.v. injection of 5x106 Mph generated from either DA.*Ncf1*$^{E3/E3}$ or DA.*Ncf1*$^{DA/DA}$. Rats were immunized 11 days later i.p. with irradiated DA.*Ncf1*$^{DA/DA}$ splenocytes. At this day blood was drawn to

determine the percentage of Tregs. After another 14 days, at day 25, rats were challenged in the left ear with irradiated splenocytes in PBS. The right ears were injected with PBS only. Ear swelling was measured before challenge and after 24 hours and was expressed as the difference in thickness between the challenged and the control ear.

Statistics

Results of different independent, although similarly performed, experiments were pooled, and the averages of the values were subjected to statistical analysis. Significant differences were determined using the Mann Whitney U test. $P<0.05$ was considered as significantly different.

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2

Chapter 3

Dexamethasone increases ROS production and T cell suppressive capacity by anti-inflammatory macrophages

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// Abstract

Macrophages have been demonstrated to suppress T cell responses by producing Reactive Oxygen Species (ROS) leading to the subsequent induction of T regulatory cells in a ROS-dependent manner. Macrophages may therefore be instrumental in down-regulating T cell responses in situations of exacerbated immune responses. Here we investigated the effect of immunosuppressive drugs on ROS production by macrophage subsets and the subsequent effects on T cell activation. Macrophage type 1 and 2 were differentiated with GM-CSF or M-CSF, in presence or absence of dexamethasone, cyclosporine A, FK506, rapamycin, or mycophenolic acid. The ROS producing capacity of fully differentiated Mph was highest in anti-inflammatory Mph2 and not affected by exposure to immunosuppressive drugs. However, presence of rapamycin during Mph2 differentiation decreased the ROS production of these cells. In contrast, other immunosuppressive drugs, with dexamethasone being the most potent, increased the ROS producing capacity of Mph2. Intriguingly although the ROS producing ability of Mph1 was unaffected, dexamethasone strongly increased the ROS producing capabilities of dendritic cells. Both at the mRNA and protein level we found that dexamethasone enhanced the expression of NOX2 protein p47^{phox}. Functionally, dexamethasone further enhanced the capacity of Mph2 to suppress T cell mediated IFN-γ and IL-4 production. *In vivo*, only in rats with normal ROS production (congenic DA.*Ncf1*$^{E3/E3}$) it was observed that dexamethasone injection resulted in long-lasting upregulation of ROS production by macrophages and induced higher levels of Treg in a ROS-dependent manner. In conclusion, we show that the anti-inflammatory drug dexamethasone increases the ROS producing capacity of macrophages.

Introduction

In recent years it has become evident that macrophages (Mph) can efficiently suppress T cell responses (Mantovani et al., 2004; Ricardo et al., 2008). Recently, we have shown that an important mechanism of this Mph induced T cell suppression is the production of Reactive Oxygen Species (ROS) by the phagocyte NADPH oxidase complex (NOX2 complex) (Hultqvist et al., 2009). In a mouse model for arthritis it was shown that mice were protected when macrophages expressing a functional NOX2 complex were present. In contrast littermates that did not express a functional NOX2 complex developed clinically overt disease (Gelderman et al., 2007). Subsequently, we showed in the human situation, that Mph with a potent ROS producing capacity suppress allogeneic T cell responses and induce Treg in a ROS dependent manner (Kraaij et al., 2010). This work was recently repeated in the mouse by others (Lee et al., 2011). Importantly, in a rat model ROS-producing Mph could, *in*

vivo, attenuate allogeneic delayed type hypersensitivity responses (Kraaij et al., 2010), suggesting that these Mph might induce specific T cell suppression. Patients with Chronic Granulomatous Disease (CGD) have mutations in various components of the NOX2 complex and suffer from recurrent bacterial infections, underlying the importance of the NOX2 complex in innate immunity (Segal et al., 2011). Interestingly, these patients are also characterized by chronic inflammation, increased autoimmunity and a hampered ability to control inflammation, thus suggesting a regulatory role for the NOX2 complex and the subsequent ROS production.

In view of this unanticipated but critical role of NOX2 in immune regulation, it is important to investigate how the ROS producing capacity in Mph is regulated. Mph exist in different forms, including polarized Mph1 and Mph2 subsets with clearly distinct cytokine production and other functional characteristics (Mantovani et al., 2004; Ricardo et al., 2008). Monocytes differentiated in the presence of IFN-γ, LPS, or GM-CSF are known as the classical macrophages (Mph1) and are characterized by a high IL-12 production and the promotion of T helper cell type 1 (Th1) responses. On the other hand, Mph2 (alternatively activated macrophages) are induced by M-CSF, or IL-4/IL-13 among others, and generally have low pro-inflammatory cytokine levels and generate T helper cell type 2 (Th2) activation. Mph2 induced by M-CSF are known as anti-inflammatory Mph that express CD163 and downregulate costimulatory molecules upon activation (Verreck et al., 2006). Analysis of NOX2 expression and function in various Mph subsets will be relevant since ROS producing Mph may be instrumental in alleviating T cell responses in autoimmunity or by preventing allograft rejection in transplantation.

Immunosuppressive drugs (IS drugs) are commonly given to prevent T cell activation and graft rejection (Hansen et al., 2007; Nankivell and Chapman, 2006). However, the effects of these drugs extend far beyond suppressing T cells alone, and antigen presenting cells like dendritic cells and Mph have also been shown to be affected (Weichhart and Saemann, 2009). These IS drugs have severe side-effects including nephrotoxicity and an increased risk of infections and malignancies. Dosing should thus be minimized and the development of alternative therapies with fewer side-effects is essential. Cellular therapy, eg with myeloid cells such as dendritic cells and macrophages (Stax et al., 2008; Morelli and Thomson, 2007; van Kooten et al., 2011), or mesenchymal stromal cells (Reinders et al., 2010) could be such an alternative by inducing donor-specific immunosuppression. Since IS drugs may affect the immunosuppressive function of the cells administered, it is of major importance to unravel the effect of different IS drugs on potential therapeutical cells. In addition, these IS drugs may influence the phenotype and function of endogenous immunoregulatory cells and thereby affect the immune response (Hoogduijn et al., 2008).

In the current study we investigated the effect of several commonly used IS drugs on different macrophage types regarding ROS production and the subsequent effect on T cell responses. We show that dexamethasone increases the expression of NOX2 proteins and the ROS producing capacity of myeloid cells. In addition, dexamethasone has an enhancing effect on the

ROS producing capacity of monocytes/macrophages *in vivo*, associated with increased numbers of circulating Treg.

Materials and Methods

Animals

Littermates were obtained by intercrossing F1 animals from a DA (Harlan) x DA.*Ncf1*$^{E3/E3}$ cross (DA.*Ncf1*$^{E3/E3}$ founders originating from Medical Inflammation Research, Karolinska Institute, Stockholm, Sweden) (Olofsson et al., 2003). Animals were used at 8-12 weeks of age. Animal experiments were approved by the committee of medical ethics (CEM) of the Leiden University Medical Center. Dexamethasone (0.15mg) (Pharmacy, LUMC) was injected intraperitoneally at day 0 and 4. Heparinized blood was drawn from the tail for FACS-analysis. At day 22 the rats were sacrificed with CO2.

Macrophage / Dendritic cell culture

Monocytes were isolated from buffy coats by CD14+ MACS-bead selection (>95% purity) (Miltenyi Biotec) (Xu et al., 2007). Monocytes were cultured for 7d in 5 ng/ml GM-CSF (Biosource; Mph1), in 5 ng/ml M-CSF (R&D systems; Mph2), or in 5 ng/ml GM-CSF and 10 ng/ml IL-4 (Biosource; DC). Cells were cultured in 6 wells plates, $1.5x10^6$ cells per well in RPMI with 10% FCS and penicillin/streptomycin (RPMI++); medium and cytokines were refreshed twice. After short trypsinization, macrophages were harvested by gentle scraping. From the DC cultures only the floating cells were used. Cultures were performed with 1 µM dex, 5 nM rapamycin (Calbiochem), 100 ng/ml cyclosphorine (Novartis), 100 ng/ml FK506 (Tacrolimus, Astellas), 50 ng/ml mycophenolic acid (MPA, Sigma Aldrich, diluted in ethanol), or 50 µg/ml prednisolone (PharmaChem) for 7 days or for 48 hours starting at day 6. Drug concentrations were chosen based on other studies and on trough levels in patients (Heidt et al., 2010; Woltman et al., 2000). At day 6 cells were stimulated with 200 ng/ml LPS for 16 hours to assess IL-6, IL-10, TNF-α (Sanquin) and IL-12p40 (Biolegend) production by ELISA.

Flow cytometry

ROS production was determined by first staining with 5 µM dihydrorhodamine123 (DHR123) for 10 min at 37˚C in RPMI++ followed by stimulation with 200 ng/ml phorbol-12-myristate13-acetate (PMA) for 20 min at 37°C. Expression of CD14 and CD163 was determined, using the appropriate isotype control, with anti-CD14-PE (BD) or anti-CD163-PE (DAKO). Protein levels of p47^{phox} and gp91^{phox} were determined by intracellular staining. Cells were permeabilized, fixed (BD), and followed by staining with anti-p47^{phox} (Santa Cruz), anti-gp91^{phox}, or an isotype control (BD) which was detected with GαM-PE (DAKO). Cell surface molecule expression was assessed by flow cytometry using a FACSCalibur and BD CellQuest software (BD Biosciences).
Whole peripheral blood from rat was stained with OX42 (anti-CD11b)

and detected with GαM-Alexa647. $CD11b^{dim}$ cells were considered to be monocytes/macrophages and $CD11b^{bright}$ cells as neutrophils, this compared well with the ROS-producing capacity of these two populations. Rat Tregs were determined by staining with anti-CD25-PE, anti-CD3-PerCP and anti-CD4-APC (BD). Subsequently, cells were permeabilized, fixed (BD) and stained with anti-FoxP3-FITC, or FITC labeled isotype control (both eBioscience). Cells were gated on the lymphocyte fraction and the percentage of FoxP3+ amongst $CD3+CD4+CD25^{bright}$ cells was determined.

T cell suppression assays

T cells (150,000) were cocultured with different ratios of allogeneic Mph with 0.5 μg/ml anti-CD3 and 1 μg/ml anti-CD28 (IxE; 1 μg/ml and CLB-CD28/1; provided by Prof L.A. Aarden). After 5 days, the supernatants were harvested to evaluate IL-4 (Sanquin) and IFN-γ (Invitrogen) levels by ELISA. The cells were pulsed with 0.5 μCi ^{3}H thymidine overnight and T cell proliferation was determined by measuring thymidine incorporation.

3

mRNA isolation and Quantitative PCR

mRNA was isolated using a Qiagen kit. After making cDNA, quantitative PCRs were performed on a Bio-Rad Icycler machine using the following primers: $p47^{phox}$ (Forward: CCTGACGAGACGGAAGAC; Reverse: GGGAAGTAGCCTGTG ACG), $gp91^{phox}$ (Forward: TAGTGGGAGCAGGGATTG; Reverse: TCAAAGGCATGTGTGTCC). The following GAPDH primers were used for normalization: Forward TTCCAGGAGCGAGATCCCT and reverse CACCCATGACGAACATGGG.

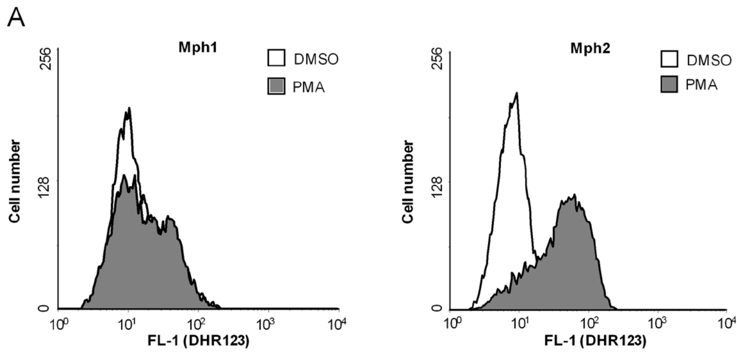
Statistics

Results of different independent, although similarly performed, experiments were averaged and subjected to statistical analysis. Significant differences were determined using the Mann-Whitney test.

Results

3.1 Anti-inflammatory macrophages produce ROS

Pro-inflammatory Mph1 and anti-inflammatory Mph2 were differentiated from CD14+ human monocytes by cultivation for 7 days in GM-CSF or M-CSF respectively. Their capacity to produce ROS was determined by flow cytometry after staining with DHR123. Upon PMA stimulation, and in a side by side comparison, Mph2 were more efficient in producing ROS as compared to Mph1 (Fig. 1A,B) (Kraaij et al., 2010). In line with previous data, these Mph2 express higher levels of CD14 and CD163 as compared to Mph1 (Fig. 1C), and produce significantly lower amounts of IL-6, IL-12p40 and TNF-α upon LPS stimulation (Fig. 1D) (Xu et al., 2007). No difference was observed in IL-10


A
Mph1
DMSO
PMA
256
128
0
Cell number
10^0
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10^4
FL-1 (DHR123)
Mph2
DMSO
PMA
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Cell number
10^0
10^1
10^2
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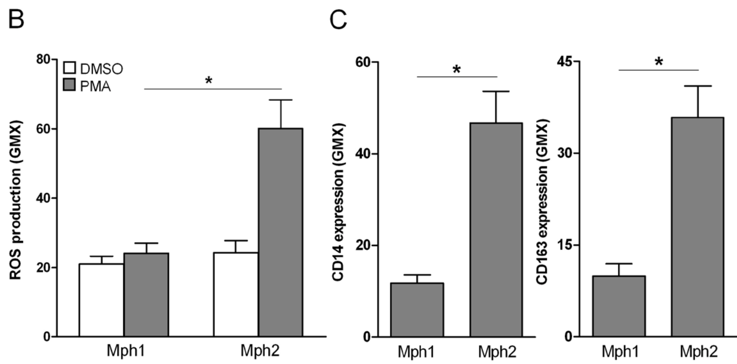
10^4
FL-1 (DHR123)


B
DMSO
PMA
*
ROS production (GMX)
80
60
40
20
0
Mph1
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CD14 expression (GMX)
60
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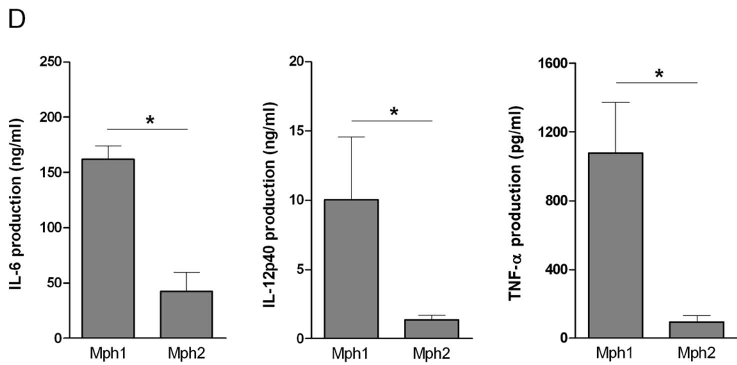
0
Mph1
Mph2
*
CD163 expression (GMX)
45
30
15
0
Mph1
Mph2


D
*
IL-6 production (ng/ml)
250
200
150
100
50
0
Mph1
Mph2
*
IL-12p40 production (ng/ml)
20
15
10
5
0
Mph1
Mph2
*
TNF-α production (pg/ml)
1600
1200
800
400
0
Mph1
Mph2

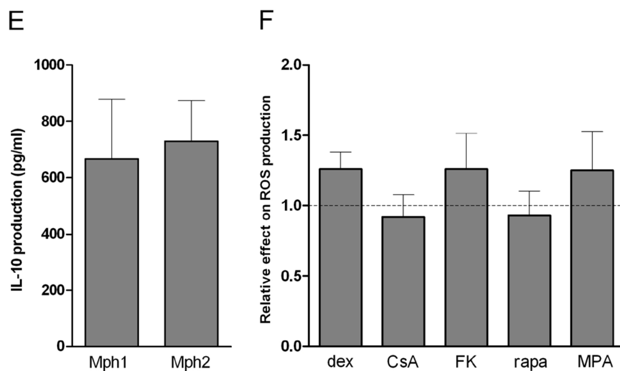

E
IL-10 production (pg/ml)
1000
800
600
400
200
0
Mph1
Mph2
F
Relative effect on ROS production
2.0
1.5
1.0
0.5
0.0
dex
CsA
FK
rapa
MPA

Figure 1: Anti-inflammatory Mph2 have higher ROS producing capacity. (***A***) FACS histograms of DHR123 fluorescence after staining of Mph1 (left) and Mph2 (right) to detect ROS production after PMA stimulation. (***B***) ROS production as induced by PMA or in absence of stimulation (DMSO; vehicle control) was determined for Mph1 and Mph2 by flow cytometry. The GeoMeanX of DHR123 fluorescence is depicted on the y-axis. (***C***) Mph1 and Mph2 were compared for the expression levels of CD14 and CD163 by flow cytometry. (***D***) IL-6, IL-12p40, and TNF-α production was determined for Mph1 and Mph2 by ELISA. (***E***) IL-10 production was determined for Mph1 and Mph2 by ELISA. (***F***) The effect of different immunosuppressive drugs (dexamethasone (dex), cyclosporine A (CsA), FK506 (FK), rapamycin (rapa), and mycophenolic acid (MPA)) were tested on Mph2; drugs were added for 48 hours after differentiation for 6 days. Mean and SEM of 17-25 (B), 3-6 (C,D,E), 2-8 (F) independent experiments are shown. * $P<0.05$.

production (Fig. 1E).

Next we investigated the effect of IS drugs on ROS production by Mph1 or Mph2. Differentiated Mph1 or Mph2 were exposed for 2 days to either dexamethasone (dex), cyclosporine A (CsA), FK506 (FK), rapamycin (rapa), or mycophenolic acid (MPA). The cell viability was not affected by these concentrations (not shown). Subsequently, PMA-induced ROS production was determined and directly compared with the ROS production by non-treated Mph. Of the drugs investigated, none elicited a remarkable effect on the ROS production by Mph2 (Fig. 1F). Also the low ROS producing capacity of Mph1 (Fig. 1A,B) was not affected by IS exposure (data not shown).

Immunosuppressive drugs influence the ROS producing capacity of Mph2

We further investigated whether the presence of these IS drugs during macrophage differentiation had an affect on their ROS. The amount of viable cells obtained after 7 days of culture was unaffected by the doses of drugs used in these experiments (not shown). On day 7, upon PMA stimulation, the ROS production by Mph 2 was ascertained. For all experiments described we made a direct comparison with Mph which were unexposed to the drugs. We observed a small enhancing effect of MPA on the ROS producing ability of Mph1 (Fig. 2A). It should be noted that this increase of MPA is minor in view of the already low ROS producing capacity of Mph1. Rapa was the only drug that significantly downregulated the ROS production by Mph2 (Fig. 2B). In contrast, the ROS producing capacity of Mph2 was significantly enhanced by dex, CsA, FK, and MPA, with dex being the most potent. A similar augumentary feature was observed for prednisolone, as cells cultured in the presence of this drug yielded a higher ROS production (data not shown). The expression of CD163 was increased when Mph2 were cultured in the presence of dex, as shown previously (Buechler et al., 2000), whereas no change was observed for CD14 levels (Fig. 2C). We also observed a decreased LPS-induced IL-6 production in the presence of dex (Fig. 2D). Thus, culturing monocytes in the presence of dex promoted the differentiation towards ROS-producing macrophages.

Previously we have shown that dexamethasone is a strong modulator of human monocyte-derived DC development, resulting in cells with tolerogenic properties (Woltman et al., 2000). Although immature DCs have a low ROS producing capacity upon PMA stimulation, a strong enhancement of ROS

production was observed in DCs when the cells were cultured in the presence of dex (Fig. 2E).

Dexamethasone increases the expression of components of the NOX2 complex

To explain the increased ROS producing capacity of Mph2 and DC, we investigated expression of the major components of the NOX2 complex. Generation of DC in the presence of dex was shown to increase mRNA expression of $p47^{phox}$ and $gp91^{phox}$, two essential NOX2 proteins (Fig. 3A). In line with this, we observed that also Mph2 cultured in the presence of dex showed increased expression of $p47^{phox}$ mRNA expression, although no change was observed for $gp91^{phox}$ (Fig. 3B). At the protein level we observed a trend with increased $p47^{phox}$ expression for both DC and Mph2 cultured in the presence of dex, although this was not statistically significant (Fig. 3C,D). No difference was observed with $gp91^{phox}$ protein levels (Fig. 3C,D). These results might contribute to the increased ROS producing capacity upon dex treatment.

Mph2 differentiated in presence of dex show decreased cytokine response

Previously we showed that Mph2 are able to suppress anti-CD3/28-induced T cell activation in a ROS-dependent manner (Kraaij et al., 2010). In view of the profound effects of dex we investigated if differentiation of Mph2 in the presence of dex affected their capacities to suppress T cells. T cells cocultured with increasing numbers of Mph2, showed slightly enhanced T cell proliferation at the lower ratios, whereas proliferation was suppressed by Mph2 at high ratios, as shown before (Fig. 4A) (Kraaij et al., 2010). However, this suppression was not amplified when Mph2 were generated in the presence of dex (Fig. 4A,D). In the same cocultures we also measured IFN-γ and IL-4 production. Mph2 inhibited IFN-γ and IL-4 production at high ratios, whereas at low ratios specifically the IFN-γ production was strongly increased. Mph cultured in dex did not show this increase in IFN-γ production (Fig. 4B) and showed more efficient inhibition of IFN-γ and IL-4 at higher ratios (Fig. 4B,C). Thus in contrast to the proliferative response, the T cell mediated IFN-γ and IL-4 production was significantly suppressed by Mph2 cultured in dex as compared to regular Mph2 (Fig. 4D).

Dex increases ROS production in vivo and induces Treg

We next studied the ability of dex to increase ROS production by Mph *in vivo*. We used the DA rat strain, which expresses two naturally occuring single nucleotide polymorphisms in the *Ncf1* gene encoding $p47^{phox}$, leading to a reduced ROS producing capacity (DA.$Ncf1^{DA/DA}$) (Hultqvist et al., 2011). We used these rats in comparison with its congenic strain which have a normal level of ROS production (DA. $Ncf1^{E3/E3}$) (Olofsson et al., 2003). Following injection of 0.15 mg dex (i.p.) at day 0 and day 4, the ROS producing capacity of monocytes/macrophages ($CD11b^{dim}$) in peripheral blood in DA.$Ncf1^{E3/E3}$

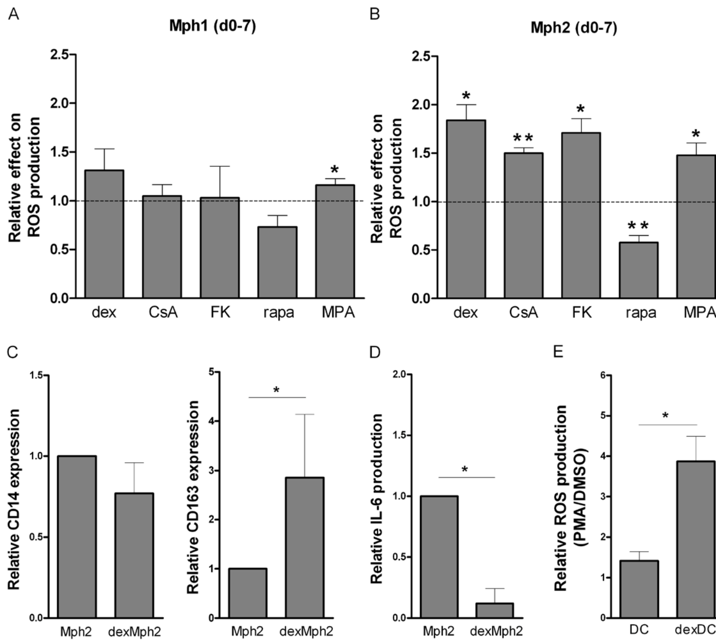


Figure 2: Dex increases ROS producing capacity. (***A***, ***B***) The effects of different immunosuppressive drugs (dex, CsA, FK, rapa, and MPA) during Mph differentiation were tested on ROS producing capacity. ROS production after PMA stimulation for both Mph1 (A) and Mph2 (B) is shown. Figures A and B both depict the relative effect of the drug(s): the ROS producing capacity of Mph differentiated in presence of drugs is divided by the ROS producing capacity of Mph differentiated in absence of drugs (no effect gives a value of 1). Mean with SEM are shown in all cases, of 2-8 independent experiments. (***C***) The effect of dex during Mph2 differentiation was tested for CD14 and CD163 expression by flow cytometry. (***D***) The effect of dex during Mph2 differentiation on IL-6 production was determined by ELISA. Figures C and D both depict the relative effect of dex: expression/production of Mph2 differentiated in presence of dex is divided by the expression/production of Mph2 differentiated in absence of dex. Mean with SD are shown of 4-6 independent experiments. (***E***) Relative ROS production induced by PMA or in absence of stimulation (DMSO; vehicle control) was determined for DC and dexDC by flow cytometry. Mean with SEM are shown in all cases of 3-5 independent experiments. * $P<0.05$, ** $P<0.005$.

rats was significantly increased at days 5 and 7 (Fig. 5A). This trend, although not significant, was observed until day 22, showing a long-lasting effect of dex on the ROS producing capacity of these cells (Fig. 5A). No change in ROS producing capacity was seen in the DA.*Ncf1*$^{DA/DA}$ rats (Fig. 5A). Interestingly, the ability of neutrophils to produce ROS at day 5 was unaffected by dex in the DA.*Ncf1*$^{E3/E3}$ rats (Fig. 5B).

Since we previously found that macrophage-derived ROS contributes to Treg induction (Kraaij et al., 2010), we investigated the percentage of CD3+CD4+CD25^{bright}FoxP3+ cells after injection with dex in both rat strains. We observed that at day 5 the percentage of Tregs was increased in rats with normal ROS production, but not in the rats with low ROS producing capacity (Fig. 5D). Thus increased numbers of Tregs upon administration of dex coincided with the dex-induced increase in the ROS producing capabilities of monocytes/macrophages and was only observed in the rats with normal ROS-producing abilities.

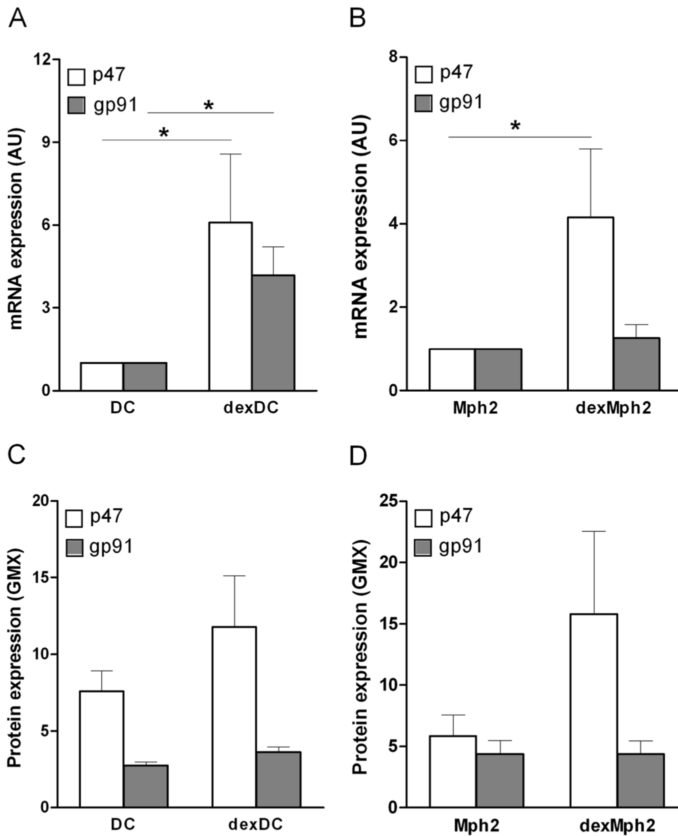


Figure 3: Dexamethasone increases the expression of components of the NOX2 complex. (***A***, ***B***) mRNA levels for p47^{phox} and gp91^{phox} as determined by qPCR. In every experiment, normalized mRNA expression for DC and Mph2 was put on 1.0 and dexDC/dexMph2 expression was related to that value. (***C***, ***D***) Protein levels for p47^{phox} and gp91^{phox} were determined by flow cytometry after intracellular staining. Mean with SEM are shown in all cases of 3-5 independent experiments. * P<0.05.

Discussion

In the present study we show that the corticosteroid dexamethasone, one of the strongest and widely used anti-inflammatory drugs, is able to enhance the ROS producing capacity of macrophages and dendritic cells. This increased ROS production was most pronounced in the anti-inflammatory Mph2 subset and was demonstrated in human cells *in vitro* as well as in rat monocytes/macrophages *in vivo*. Dex treatment coincided with an increased ability to suppress T cell activation *in vitro* and with the number of circulating Treg in rats. These findings provide an additional mechanism of the immunosuppressive action of steroids and further underline the recently described and unanticipated regulatory function of NOX2-mediated ROS when produced by antigen presenting cells

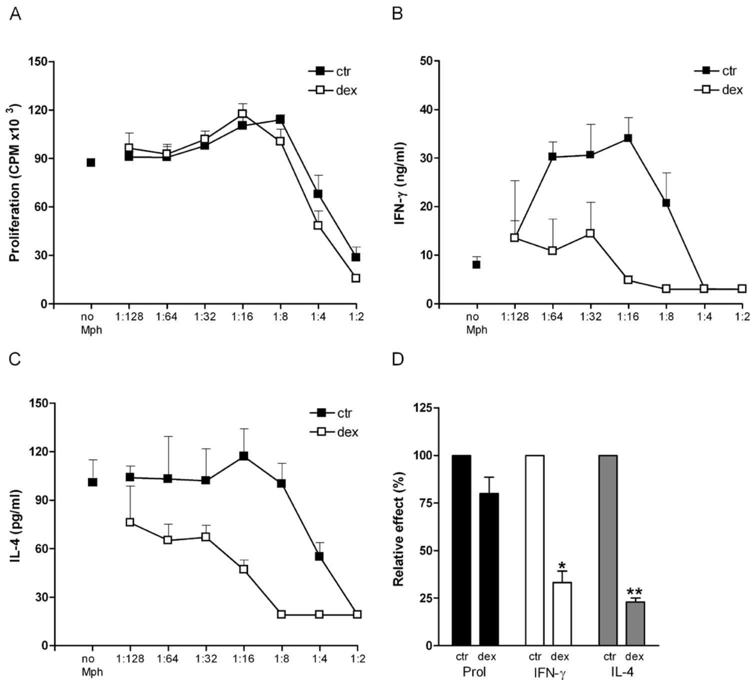


Figure 4: Mph2 differentiated in dex show decreased cytokine response. (***A***, ***B***, ***C***, ***D***) Allogeneic T cells (150,000) were stimulated with anti-CD3/28 Ab in combination with Mph2 differentiated in presence of dex or no drugs, and added in a dose-dependent fashion in ratios as depicted on the x-axis. (***A***) Proliferation, (***B***) IFN-γ production, and (***C***) IL-4 production, were determined in the same well for all conditions after 5 days of coculture. A representative experiment out of 4 is shown. Depicted are averages and standard deviations of triplicates within this experiment. (***D***) The relative effect of dex treated Mph2 on proliferation, IFN-γ and IL-4 production at a 1:8 ratio; mean and SEM of 3-4 experiments. * P<0.05, **P<0.005.

(Kraaij et al., 2010).

Excessive ROS, as produced during oxidative stress and inflammation leads to increased inflammatory responses and oxidative damage. However, relatively low amounts of ROS as produced by macrophages are instrumental in cell signaling and can suppress inflammation as shown previously (Gelderman et al., 2007;Kraaij et al., 2010;Segal et al., 2010). We have shown an immune-modulating function of ROS and that in particular anti-inflammatory Mph use this function to exert their T cell suppressive properties (Kraaij et al., 2010). Others have also shown a ROS-mediated T cell suppression and ROS-mediated induction of Treg, as these effects were abrogated when NOX2 was functionally inhibited (Efimova et al., 2011). Moreover, it has been shown that myeloid-derived suppressor cells can also induce Treg via the production of ROS (Lee et al., 2011). These data indicate that the production of ROS by certain myeloid cells can possibly be of clinical importance.

Here we demonstrate that IS drugs do exert an effect on the ROS producing capacity of the anti-inflammatory Mph2, but, interestingly, has no apparent effect on the pro-inflammatory Mph1. Several IS drugs, especially dex, increase

the capability of anti-inflammatory Mph2 to produce ROS. Intriguing, this effect was only observed when IS drugs were present during differentiation of monocytes towards Mph, but not when drugs were added to already differentiated Mph. This indicates that IS drugs do not negatively influence the phenotype and function of anti-inflammatory myeloid cells, which is of great importance when considering these cells for cell therapy. In addition, dex also increased the ROS producing capacity of DCs, whereas only very little ROS production is observed in normal DCs. Dex is known to provide DC with tolerogenic properties (Morelli and Thomson, 2007;Stax et al., 2008;van Kooten et al., 2011). In earlier studies it was shown that during cultivation in dex, DCs are frozen in an immature state that is associated with lower levels of pro-inflammatory cytokine production and lower levels of T cell activation (Woltman et al., 2000). Additionally these cells also yielded lower donor-specific T cell responses in a kidney transplantation model in the rat (Stax et al., 2008). Apparently dex diverts the DC towards an immune modulating cell with decreased pro-inflammatory properities, among them is an increase in ROS producing capacity which can be an additional immune regulatory mechanism. Furthermore, dex increased the T cell suppressive ability of Mph2 with regard to IFN-γ and IL-4 production, even though dex did not enhance T cell suppression by Mph2. Thus dex further modulates the T cell function, indicating that dex indeed aids in augmenting the T cell suppression by Mph2.

Rapamycin, an inhibitor of the mTOR kinase, is clinically used as an immunosuppressive drug due to its interference with IL-2 signaling and subsequent inhibition of T cell activation, while at the same time enriching for Treg (Weichhart and Saemann, 2009). However, in monocytes/macrophages mTOR signaling seems to limit the pro-inflammatory response and treatment with rapa results in an increased cytokine response, including IL-12, TNF and IL-6 (Weichhart et al., 2008). We observed a similar enhanced LPS-induced IL-6 production upon rapa treatment (data not shown). In contrast IL-6 production was inhibited by dex. Interestingly we found that rapa decreased the ROS producing capacity of the anti-inflammatory Mph2. This would be in line with the hypothesis that ROS acts as a regulatory molecule in Mph2, and that this process is counteracted by inhibition of the regulatory mTOR pathway. It has been reported previously that rapa increased monocytic NOX2-dependent ROS production, but these cells were only treated for a short period, thereby not providing the time for functional alterations (Engelbrecht et al., 1994).

The effect of dex on ROS production by different cell types and in different settings has been studied before, with different results. An increase in ROS production has been described in dex-treated monocytes stimulated with N-formyl-methionine-leucine-phenylalanine (fMLP), whereas suppression was seen when stimulated with microbial agents (Zhu and Mrowietz, 2005). In addition, an increase in ROS was shown in adipocytes and insulin-producing cells after treatment with dex, whose effects could be inhibited with the use of ROS scavengers (Houstis et al., 2006;Roma et al., 2009). This has been shown *in vivo* as well, where it was observed that injection of dex induced hypertension in a ROS-dependent manner in rats (Hu et al., 2006). In contrast, in PMNs it was

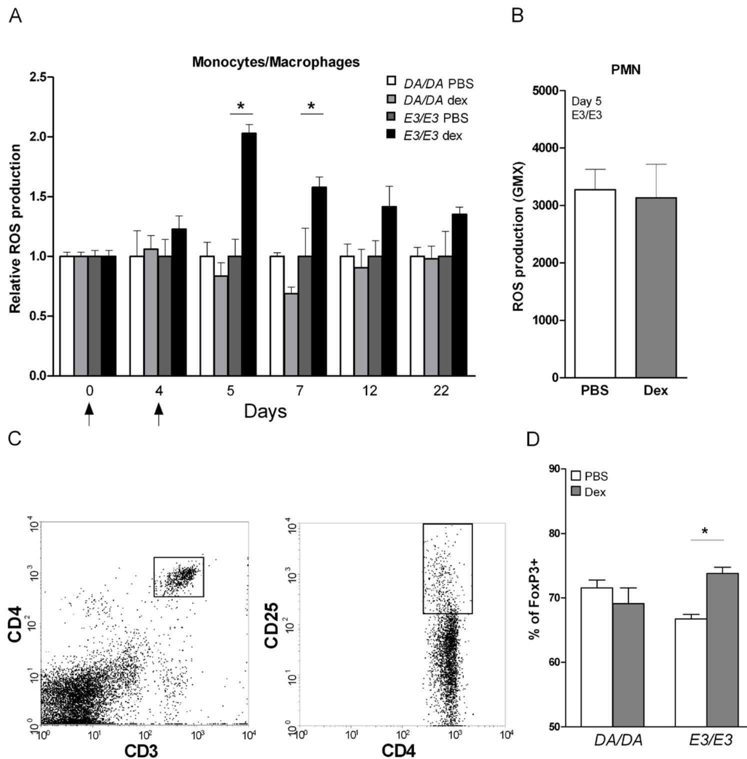


Figure 5: Dex increases ROS producing capacity and ROS dependent Treg induction *in vivo*. (***A***) DA.*Ncf1*$^{DA/DA}$ (DA/DA) rats that express alleles leading to low ROS producing capacity and DA.*Ncf1*$^{E3/E3}$ (E3/E3) rats that express alleles conferring normal levels of ROS production were injected with 0.15 mg dex or PBS as control at day 0 and day 4 (arrows) and the capacity to of their peripheral blood monocytes/macrophages to produce ROS was followed in time. The relative ROS producing capacity (PBS control on 1 for both groups) after PMA stimulation of circulating monocytes/macrophages is shown. (***B***) ROS producing capacity after PMA stimulation of neutrophils at day 5 in dex or PBS treated DA.*Ncf1*$^{E3/E3}$ rats. (***C***) Treg gating strategy on peripheral blood. (***D***) The percentage of FoxP3+ cells amongst CD3+CD4+CD25^{bright} cells in the blood of these rats at day 22. Averages with SEM of 5 rats per group are shown. *P<0.05.

shown that short term dex treatment had no effect on p47^{phox} mRNA expression, but reduced gp91^{phox} mRNA expression (Amezaga et al., 1992). Other studies using the monocytic cell line THP-1 showed that a short treatment with dex downregulated gp91^{phox} protein levels (Ahmed et al., 2003), or decreased both p47^{phox} and gp91^{phox} RNA expression in THP-1 cells differentiated in IFN-γ and TNF-α (Condino-Neto et al., 1998). The pro-inflammatory cytokines IFN-γ and TNF-α increased the expression of both p47^{phox} and gp91^{phox}, which was then attenuated by the anti-inflammatory dex. Interestingly, CGD patients are also treated with IFN-γ, which may have similar effects in patients with some NOX2 rest function (Segal et al., 2011; Weening et al., 1995).
We here show both *in vitro* as well as *in vivo* in a rat model that dex increases the ROS producing capacity of circulating monocytes/macrophages. Others have shown a decrease in ROS production in rat peritoneal leukocytes when

dex was administered in the drinking water for 48 hours (Roshol et al., 1995). Interestingly, we see our effect only in monocytes/macrophages with normal ROS producing capacity, whereas no effect was observed in monocytes/macrophages with low ROS producing capacity and neutrophils from rats with a normal ROS production. The effect of dex may thus, at least partly, be mediated via ROS production by monocytes/macrophages. It is possible that dex treatment influences the NOX2 complex itself. The gp91^{phox} mRNA level in Mph2 was not changed, but p47^{phox} was increased by dex. In DCs an increase for both p47^{phox} and gp91^{phox} was observed, although it should be noted that these levels were lower in DC compared to Mph2. Since dex apparently does not change the NOX2 protein level in Mph2, it is most likely that other levels of regulation are involved, including phosphorylation or subcellular localization of the NOX2 complex. Even though we cannot directly link the activity of p47^{phox} with the increased ROS producing capacity of dex, it is important to note that the increased ROS producing capacity by dex in *Ncf1*$^{E3/E3}$ rats was not observed in the *Ncf1*$^{DA/DA}$ rats which have a polymorphism in p47^{phox}, thereby suggesting a mechanism involving p47^{phox}.
In conclusion, we show that dex increases the ROS producing capacity of anti-inflammatory Mph *in vitro* as well as *in vivo*. Since Mph2 may be instrumental in down-regulating T cell responses via the production of ROS, the addition of dex may then amplify this response. Therefore we propose an additional mechanism in which dex enhances T cell suppressive capacity of myeloid cells.

Acknowledgements

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Chapter 4

Subsets of Human type 2 Macrophages show differential capacity to produce Reactive Oxygen Species

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Abstract

Reactive oxygen species (ROS) produced by macrophages have recently been shown to have immunosuppressive properties and induce regulatory T cells. Here we investigated the ROS producing capacity of well-defined human Mph2 subsets and studied the contribution of ROS in the Mph-T cell interaction. Mph were generated from monocytes using M-CSF (Mph2), IL-4 (Mph2a), or IL-10 (Mph2c). Upon PMA stimulation, Mph2 and Mph2c showed a high ROS producing capacity, whereas this was low for Mph2a. Mph2 and Mph2c displayed a reduced T cell stimulatory capacity compared to Mph2a. Addition of the ROS inhibitor DPI decreased the T cell proliferation and IFN-γ production. When testing directly on Mph, DPI dose-dependently decreased the IL-10 and IL-12p40 production of CD40L-stimulated Mph2 subsets. In conclusion, the ROS producing capacity is different among human Mph type-2 subsets. In all cases, DPI suppressed T cell proliferation and cytokine production, indicating a ROS-dependent mechanism of T cell activation.

Introduction

Macrophages (Mph) represent a heterogeneous group of myeloid cells, with a phenotypic and functional specialization and a high plasticity depending on the local cytokine milieu. Mph can be broadly divided into classically activated (Mph1) and alternatively activated macrophages (Mph2), with their cytokine production profile as an important functional difference [1]. Mph1 are characterized by production of pro-inflammatory cytokines like TNF-α and IL-12, whereas Mph2 produces large amounts of IL-10. In addition, Mph2 can be distinguished from Mph1 based on their expression of CD163 and CD206, although different expression levels have been reported depending on the Mph2 subtype [2-5]. For mouse, Mph2 specific markers, like FIZZ1 and Ym1, have been identified both *in vivo* and *in vitro* [6], but establishing specific phenotypic markers for human Mph2 is still an ongoing challenge. CD163 positive staining on resident tissue macrophages has been found dispersed throughout the body, indicating that *in vivo* Mph represent a Mph2 phenotype [7]. Placental Mph also display an Mph2-like phenotype, indicated by the expression of specific Mph2 markers, and their anti-inflammatory phenotype [8;9]. In addition, tumor-associated macrophages (TAMs) also have a phenotype and function similar to Mph2: high expression of CD206, poor antigen presentation, high IL-10, but low IL-12 production [10].
In vitro, Mph1 can be polarized by IFN-γ, LPS, and/or GM-CSF, thereby generating a pro-inflammatory phenotype. In addition, different Mph2 subsets have been described, depending on the different ways of generation, including culture in IL-4/IL-13, immune complexes, or IL-10/TGF-β, generating M2a, M2b, M2c respectively [4;11-14]. Recently, we and other have shown that also generation of Mph in the presence of M-CSF results in cells (Mph2) with

anti-inflammatory properties [4;13;15]. Since M-CSF is present constitutively in blood, this suggests that under homeostatic conditions macrophages will mostly acquire Mph2 properties [16].
One major characteristic which has been proposed to be different between Mph1 and Mph2 is their capacity to produce reactive oxygen species (ROS), which has been mostly linked to pro-inflammatory cells [3;12;17;18]. The importance of ROS in our immunity is demonstrated in patients with Chronic Granulomatous Disease (CGD) who have mutations in various components of the phagocytic NADPH oxidase (NOX2) complex, and suffer from recurrent infections. However, these patients are also characterized by chronic inflammation, increased autoimmunity and a hampered ability to control inflammation [19]. These latter findings have also been observed in mouse models with defects in NOX2 proteins and were shown to be macrophage dependent [20;21]. Recently, we showed that M-CSF differentiated Mph2 have a high ROS producing capacity, and were able to suppress T cell responses in a ROS-dependent manner [4;15].
In the current study we investigated whether there is a difference concerning the ROS producing capacity between various well defined human Mph2 subsets. Moreover, we studied the role of ROS in the Mph-T cell interaction. We show that Mph2 subsets display a differential capacity to produce ROS. However, the ROS inhibitor DPI inhibited the T cell stimulatory capacity and cytokine production by all Mph2 subsets, suggesting involvement of a ROS-dependent signaling pathway.

Materials and methods

Cell culture

Monocytes were isolated from buffy coats by CD14+ MACS-bead selection (Miltenyi Biotec) [22]. Monocytes were cultured for 7 days in 5 ng/ml M-CSF (R&D systems; Mph2), in 15 ng/ml IL-4 (Biosource; Mph2a), or in 15 ng/ml IL-10 (ITK; Mph2c). Cells were cultured in RPMI with 10% FCS and penicillin/streptomycin (RPMI++) in 6 wells plates with 1.5×10^6 cell per well; medium and cytokines were refreshed twice. Macrophages were harvested by gentle scraping after short trypsinization.

ELISA

Macrophages were harvested, replated and stimulated with 200 ng/ml LPS for 16 hours and subsequently supernatants were collected. In the experiments with diphenyleneiodonium (DPI) (Sigma) the cells were cultured for 48 hours in the presence of 200 ng/ml LPS or L-CD40L cells [23], thereafter supernatants were harvested. In other experiments cells were incubated with 50 U/ml Catalase (Sigma) for 48 hours hereafter supernatants were harvested. The IL-6, IL-10 (Sanquin) and IL-12p40 (Biolegend) production was assessed by ELISA according manufacturer's instructions.

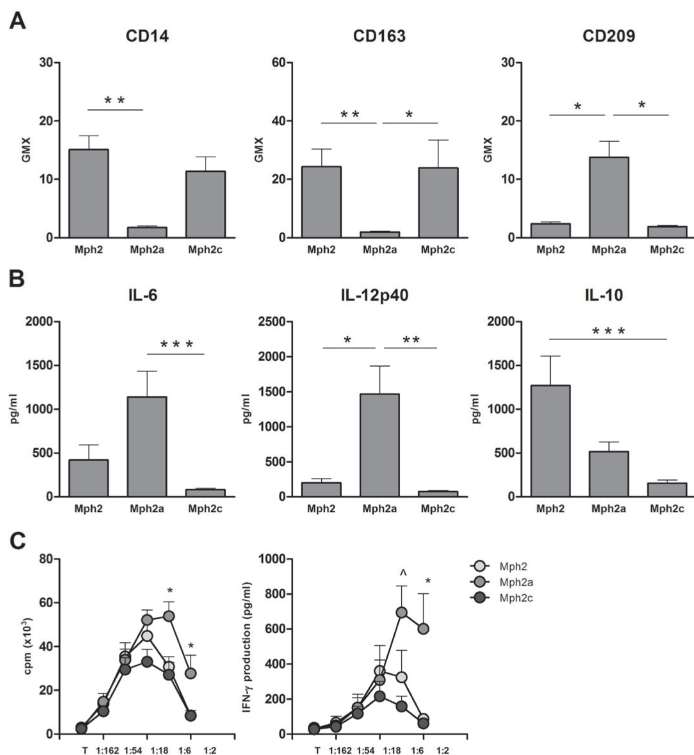

Figure 1: Mph2a are phenotypically and functionally distinct from Mph2 and Mph2c. (***A***) Expression levels of CD14 (left), CD163 (middle), and CD209 (right) for Mph2, Mph2a, and Mph2c as determined by flow cytometry. (***B***) IL-6 (left), IL-12p40 (middle), and IL-10 (right) production were determined for Mph2, Mph2a and Mph2c after 16 hours of LPS stimulation by ELISA. Mean and SEM are shown for 4-8 independent experiments. (***C***) Allogeneic T cells (150,000) were stimulated with respectively Mph2, Mph2a, or Mph2c in different ratios as depicted on the x-axis. Proliferation (left; * P<0.05 Mph2a vs Mph2 and Mph2c) and IFN-γ production (right; * P<0.05 Mph2a vs Mph2 and Mph2c, ^ P<0.05 Mph2a vs Mph2c) were determined after 5 days of coculture. Depicted are averages and SEM of 9-10 independent experiments. * P<0.05, ** P<0.01, *** P<0.001.

Flow cytometry

Cell surface molecule expression was assessed by flow cytometry using FACSCalibur and BD CellQuest software (BD Biosciences). ROS production was determined by staining the cells with 5 μM dihydrorhodamine123 (DHR123) for 10 min at 37°C in medium and stimulation with 300 ng/ml phorbol-12-myristate13-acetate (PMA) for 20 min at 37°C. In certain cases the cells were stimulated for 1 hour with DPI before measuring ROS production. Expression levels of DC-SIGN (R&D), CD14-PE , CD163, CD86 (all BD Biosciences), and HLA-DR (clone B8.11,2; ATCC) with secondary Ab GαM-PE (DAKO) were determined. Protein levels of p47*phox* and gp91*phox* were determined by intracellular staining. The cells were permeabilized, fixed (BD Biosciences), and stained with anti-p47*phox*, anti-gp91*phox* (Santa Cruz), or an isotype control (BD Biosciences) and detected with GαM-PE (DAKO). Cell death was determined by incubating the cells with propidium iodide (Invitrogen).

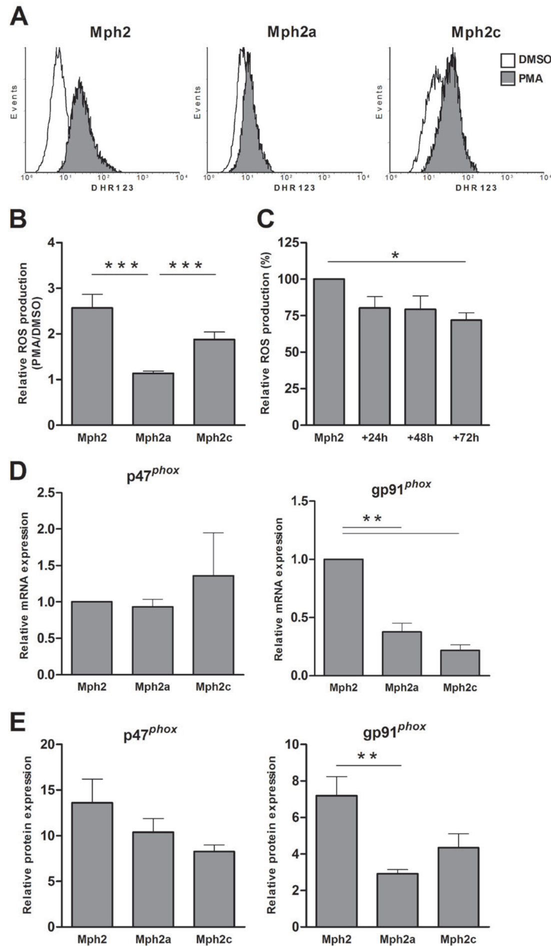

Figure 2: Mph2a have a decreased ROS producing capacity. (***A***) FACS histograms of DHR123 fluorescence after staining of Mph2 (left), Mph2a (middle) and Mph2c (right) to detect ROS production after PMA stimulation (filled histogram) compared to the DMSO control. (***B***) The ratio of ROS production as induced by PMA or in absence of stimulation (DMSO; vehicle control) was determined for the Mph2 subsets in paired samples (N=20). (***C***) Fully differentiated day 6 Mph2 were incubated with IL-4 for indicated time points and the ROS production relative to untreated Mph2 was determined (N=4). (***D***) mRNA levels for p47^{phox} (left) and gp91^{phox} (right) as determined by qPCR. In every experiment, mRNA expression normalized for GAPDH and determined for Mph2 was put on 1.0 and Mph2a and Mph2c expression was related to that value. Mean and SEM of 4-9 individual experiments are shown. (***E***) Protein levels for p47^{phox} (left) and gp91^{phox} (right) were determined by flow cytometry after intracellular staining. Mean with SEM are shown for 6 individual experiments. * P<0.05, ** P<0.01, *** P<0.001.

Amplex Red Assay

Hydrogen peroxide levels were determined using the Amplex Red assay. Mph2 subsets were incubated with 12.5 µM Amplex Red (Invitrogen) and 0.5 U/ml HRP (Sigma) for 20 min at 37°C. The reaction was measured for fluorescence intensity (excitation 530 nm, emission 590 nm) at a cytofluor (Perseptive Biosystems). Hydrogen peroxide levels determined in the presence of Cat were measured directly after adding Amplex Red and HRP.

T cell proliferation assay

150.000 total T cells were cocultured with different ratios of allogeneic Mph. In some experiments this was done in the presence of Catalase (50 U/ml) or DPI (0.02/0.3 μM). In other experiments T cells were activated with 0.25 μg/ml anti-CD3 and 0.5 μg/ml anti-CD28 (Sanquin) in the presence of different concentrations of Cat and DPI. After 5 days supernatants were harvested and IFN-γ (Biolegend) levels were determined by ELISA. T cell proliferation was measured by adding 0.5 μCi ^{3}H thymidine to the cocultures at day 5 for 16 hours.

L-CD40L cell activation assay

Mph were cultured in a 2:1 ratio with L-CD40L cells or its control L-Orient for 48 hours as previously described [23]. Different concentrations of DPI or diluent (DMSO) were added to the cultures and supernatants were harvested to detect marker expression and cytokine production.

mRNA isolation and Quantitative PCR

mRNA was isolated using a Qiagen kit. After making cDNA, quantitative PCRs were performed on a Bio-Rad Icycler machine using the following primers: p47^{phox} (Forward: CCTGACGAGACGGAAGAC; Reverse: GGGAAGTAGCCTGTGACG), gp91^{phox} (Forward: TAGTGGGAGCAGGGATTG; Reverse: TCAAAGGCATGTGTGTCC). The following GAPDH primers were used for normalization: Forward TTCCAGGAGCGAGATCCCT and reverse CACCCATGACGAACATGGG.

Statistics

Results of different independent experiments were averaged and subjected to statistical analyses. Significant differences were determined using either the Student's T-test, one-way or two-way ANOVA.

Results

Mph2a are phenotypically and functionally distinct from Mph2 and Mph2c

Different Mph2 subsets were generated by differentiating monocytes with M-CSF, IL-4 or IL-10 towards Mph2, Mph2a, and Mph2c, respectively. Phenotypic analysis showed that CD14 and CD163 were highly expressed by both Mph2 and Mph2c, whereas low CD209 (DC-SIGN) expression was observed (Figure 1A). The opposite pattern was observed for Mph2a, showing low CD14 and CD163, but high CD209 expression (Figure 1A). Functionally, upon LPS stimulation Mph2a had a higher IL-6 and IL-12p40 production compared to Mph2 and Mph2c (Figure 1B). High IL-10 production was observed for Mph2, while Mph2a and Mph2c produced only low amounts (Figure 1B).

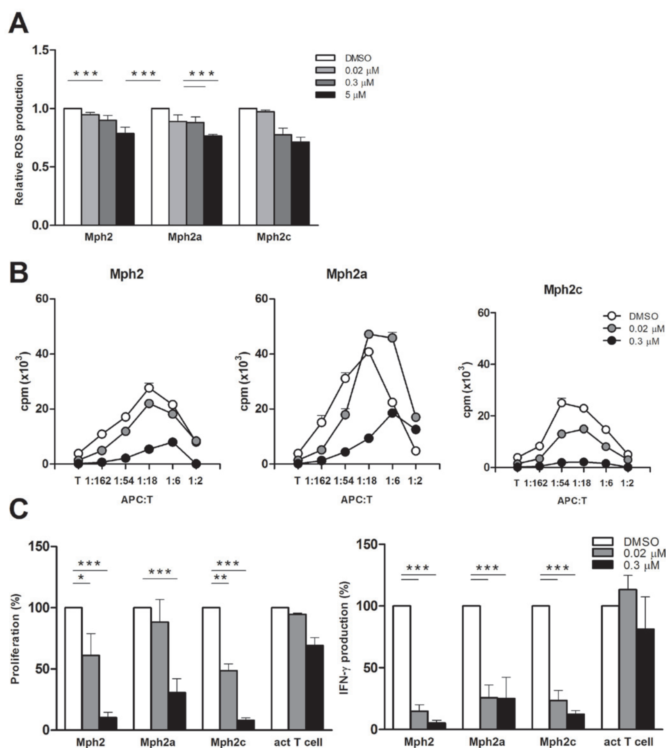

Figure 3: DPI decreases T cell proliferation in an MLR. (***A***) Flow cytometric analysis of the relative ROS producing capacity of Mph2, Mph2a, and Mph2c as induced by PMA stimulation in the presence of increasing concentrations of DPI. Mean and SEM are depicted from 5 independent experiments. (***B***) Allogeneic T cells (150,000) were stimulated with increasing amounts of Mph2, Mph2a, or Mph2c respectively in the presence of 0.02 or 0.3 µM DPI or DMSO as indicated, and proliferation was measured at day 5 of culture. A representative experiment is shown (***C***) Relative proliferation (left) and IFN-γ production (right) (DMSO at 100) is shown for Mph2, Mph2a, Mph2c at a ratio of 1:18, and anti-CD3/28-activated T cells in the presence of DPI. Mean and SEM are shown for 5-6 individual experiments. * P<0.05, ** P<0.01, *** P<0.001.

Next we investigated the T cell stimulatory capacity of the different Mph2 subsets in an MLR. Mph2a showed a higher T cell stimulatory capacity, especially at higher Mph:T cell ratios, with optimal activation at a 1:6 ratio (Figure 1C). A more pronounced, but similar result was observed for IFN-γ production. Thus Mph2a is both phenotypically and functionally different from Mph2 and Mph2c, showing a higher pro-inflammatory cytokine production and stronger T cell stimulatory capacity.

Mph2a have a decreased ROS producing capacity

Although anti-inflammatory Mph2 are usually described as non-ROS producing cell [3;17], we recently showed that ROS can be produced by Mph2 and has profound immuneregulatory effects [4;15]. Therefore, we investigated the ROS producing capacities of the different type 2 Mph as characterized above. In paired samples, upon PMA stimulation, Mph2a showed a significantly lower

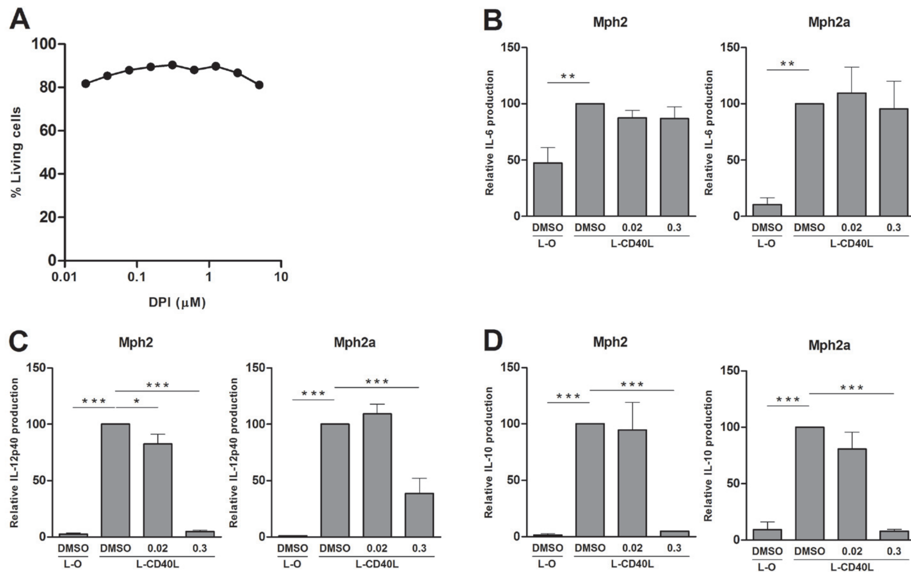

Figure 4: DPI affects IL-12p40 and IL-10 production of the Mph2 subsets. (***A***) Percentage living Mph2 in the presence of different concentrations of DPI as determined with PI staining by flow cytometry. A representative experiment out of 3 is shown. (***B***) Relative IL-6 production of Mph2 (left) and Mph2a (right) activated with L-CD40L cells (L-Orient as control) (L-CD40L DMSO set at 100) in the presence of DPI for 48 hours as determined by ELISA (L-CD40L DMSO, average production Mph2 241 pg/ml; Mph2a 315 pg/ml). (***C***) Relative IL-12p40 production of Mph2 (left) and Mph2a (right) (L-CD40L DMSO, average production Mph2 2846 pg/ml; Mph2a 4088 pg/ml). (***D***) Relative IL-10 Mph2 (left) and Mph2a (right) (L-CD40L DMSO, average production Mph2 609 pg/ml; Mph2a 501 pg/ml). Mean and SEM are shown for 5-6 independent experiments. * P<0.05, ** P<0.01, *** P<0.001.

ROS producing capacity compared to Mph2 and Mph2c (Figure 2A,B). We have shown before that monocyte-derived dendritic cells, differentiated with GM-CSF and IL-4, have low ROS producing capacity [15], whereas monocytes themselves have a high ROS producing capacity (data not shown). Therefore we investigated whether exposure to IL-4 directly affects the ROS producing capacity. When IL-4 was added to fully differentiated Mph2, we observed a time-dependent decrease in ROS producing capacity (Figure 2C).
Since Mph2 derived-ROS is produced by the NOX2 complex [15], we investigated the expression of two important proteins of the NOX2 complex as a possible explanation for the difference in ROS producing capacity. Both mRNA and protein expression of p47^{phox} was similar between Mph2 subsets (Figure 2D,E). In contrast, the mRNA levels of gp91^{phox} were lower in Mph2a and Mph2c compared to Mph2 (Figure 2D). Moreover, Mph2a also has diminished gp91^{phox} protein expression compared to Mph2 (Figure 2E). We conclude that the IL-4-generated Mph2a have a lower NOX2-mediated ROS production.

DPI decreases T cell proliferation in an MLR

In view of the immunosuppressive role of ROS, we hypothesized that the presence of ROS in Mph2 and Mph2c might contribute to their decreased T

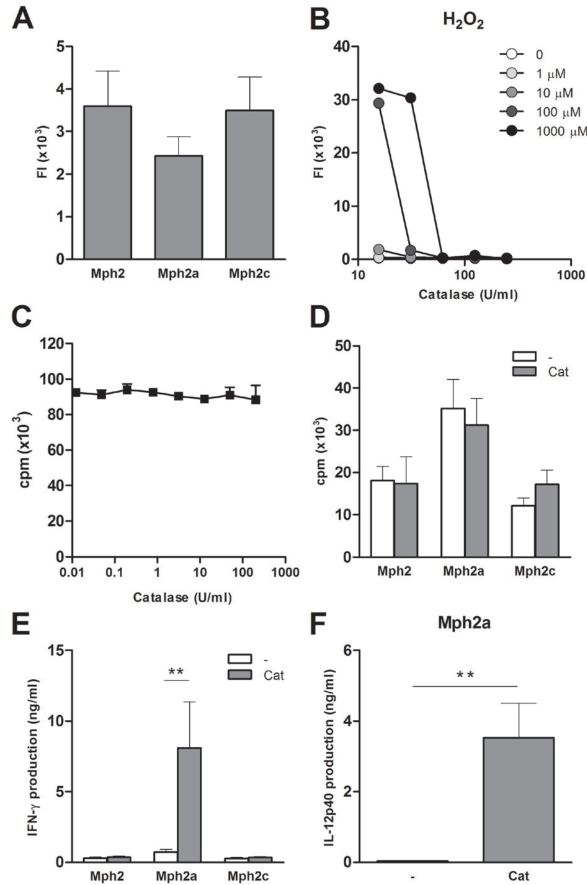

Figure 5: Catalase has no effect on T cell proliferation in an MLR. (***A***) Extracellular hydrogen peroxide levels measured as fluorescence intensity (FI) of Mph2, Mph2a, and Mph2c as determined by AmplexRed assay. (***B***) FI of different concentrations of hydrogen peroxide in the presence of different concentrations of Cat as indicated. (***C***) Allogeneic T cells (150,000) were stimulated with anti-CD3/28 in the presence of different concentrations of Cat. Proliferation was determined after 5 days of culture. A representative experiment depicting averages and SEM of triplicates is shown. Allogeneic T cells (150,000) were stimulated at a ratio of 1:6 with respectively Mph2, Mph2a, or Mph2c in the presence of 50 U/ml Cat. (***D***) Proliferation and (***E***) IFN-γ production are depicted. (***F***) IL-12p40 production of Mph2a after 48 hours incubation in the presence of 50 U/ml Cat as determined by ELISA. In all cases mean and SEM are shown of 5-7 independent experiments. * $P<0.05$, ** $P<0.01$.

cell stimulatory capacity (Figure 1C). To investigate this we used the commonly used ROS inhibitor diphenyleneiodonium (DPI), which interacts with the C-terminal flavin-binding domain of gp91^{phox} [24]. DPI inhibited the ROS production of Mph2, Mph2a, and Mph2c (Figure 3A). At high concentrations DPI inhibited proliferation and IFN-γ production by anti-CD3/CD28-activated T cells (data not shown). Therefore we used low concentrations of DPI (0.02 μM and 0.3 μM) to investigate the role of ROS in the T cell stimulatory capacity within the MLR. For all three Mph subsets, DPI dose-dependently inhibited the proliferation of T cells over a wide range of T cell:APC ratio's (Figure 3B). When

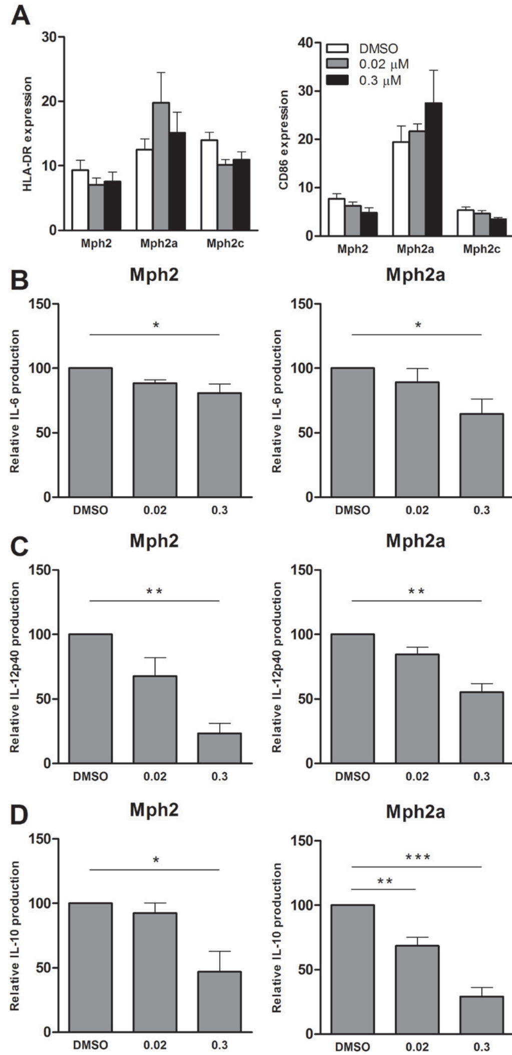

Figure S1: (***A***) HLA-DR (left) and CD86 (right) expression after 48 hours incubation with DPI for Mph2, Mph2a, and Mph2c. (***B***) Relative IL-6 production of Mph2 (left; average production of 1048 pg/ml) and Mph2a (right; average production of 1045 pg/ml) stimulated with LPS (DMSO set at 100) in the presence of DPI for 48 hours as determined by ELISA. (***C***) Relative IL-12p40 production of Mph2 (left; average production of 1010 pg/ml) and Mph2a (right; average production of 3351 pg/ml). (***D***) Relative IL-10 production of Mph2 (left; average production of 741 pg/ml) and Mph2a (right; average production of 442 pg/ml). In all cases mean and SEM are shown for 3-5 individual experiments. * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

quantifying the data, it appeared that Mph2a were less sensitive for inhibition, and higher concentrations were needed to reach a significant inhibition (Figure 3C). Similarly in all cases also the IFN-γ production was strongly inhibited by DPI. Under these conditions, proliferation and IFN-γ production by T cells activated by CD3/CD28 were not affected. These data show that, opposite to our hypothesis, ROS production by macrophages is essential for the T cell

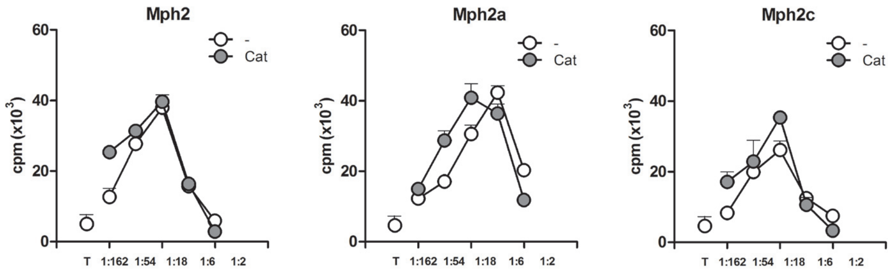

Figure S2: Allogeneic T cells (150,000) were stimulated with respectively Mph2 (left), Mph2a (middle), or Mph2c (right) in the presence of 50 U/ml Cat in different ratios as depicted on the x-axis. A representative experiment is shown. Depicted are averages and SEM of triplicates within this experiment.

stimulatory capacity, and cannot explain the reduced T cell stimulation by Mph2 and Mph2c.

DPI affects the cytokine production of the Mph2 subsets

We next explored the potential mechanisms how NOX2 might be involved in the T cell stimulatory capacity. DPI up to a concentration of 5 μM, showed no effect on the viability of Mphs (Figure 4A). The stimulation of T cells by APCs requires MHC class II and co-stimulatory molecule activation, as well as cytokine production. However, HLA-DR and CD86 expression on the Mph2 subsets were not affected by DPI (Supplementary Figure 1A).
Therefore we investigated the cytokine production of Mph2 subsets activated by CD40L to mimic the CD40-CD40L signal between APC-T cell as it occurs during the MLR reaction. IL-6 production by Mph2 and Mph2a was increased upon stimulation with L-CD40L cells, but no effect of DPI was observed (Figure 4B). In contrast, the strongly increased production of IL-12p40 (Figure 4C) and IL-10 (Figure 4D), was dose-dependently inhibited by DPI. In LPS-stimulated Mph2 and Mph2a, the IL-6, IL-12p40, and IL-10 production was suppressed by DPI (Supplementary Figure 1B,C,D). Similar trends were observed with the cytokine production of Mph2c (data not shown).

Catalase has no effect on T cell proliferation in an MLR

Although we identified an important role for ROS in Mphs for their T cell stimulatory capacity, this does not clarify the molecular mechanism. Hydrogen peroxide, a product of ROS generation is a known for its role as second messenger, its relatively long half-life and its ability to move through membranes [25;26]. Using the Amplex Red assay, which detects extracellular hydrogen peroxide, similar basal levels of extracellular hydrogen peroxide were observed for Mph2, Mph2a, and Mph2c (Figure 5A). The high signal in Mph2a indicates that apart from NOX2, also other sources produce ROS resulting in hydrogen peroxide. Catalase (Cat), a specific hydrogen peroxide-scavenger, is able to efficiently break down high concentrations of hydrogen peroxide (Figure 5B).

Neither the proliferation of anti-CD3/CD28-activated T cells (Figure 5C), nor the T cell proliferation induced by Mph2, Mph2a, or Mph2c (Figure 5D, Supplementary Figure 2) was affected by the addition of 50 U/ml of Cat. In addition, no effect on the IFN-γ production induced by Mph2 or Mph2c was observed (Figure 5E). However, when using Mph2a as T cell activator, addition of Cat strongly increased the IFN-γ production (Figure 5E). In line with this, when tested directly on Mph2a, Cat did also increase the IL-12p40 production (Figure 5F). Thus whereas hydrogen peroxide seems not involved in T cell proliferation, it does affect the quality of the T cell response induced by Mph2a.

Discussion

In this study we showed a difference in ROS producing capacity among human Mph2 subsets; Mph2 and Mph2c have a higher ROS producing capacity compared to Mph2a. In addition, we observed decreased T cell proliferation with DPI for all Mph2 subsets, indicating that the ROS producing capacity of Mph2 and Mph2c is not directly associated with their reduced T cell stimulatory capacity. This decreased T cell proliferation could be explained by decreased IL-10 and IL-12p40 production in the presence of DPI. These data suggests a ROS-dependent mechanism of T cell activation via the cytokine production.
In vitro, monocytes can be differentiated in functionally different Mph2 subsets. We found that Mph2 and Mph2c, differentiated by M-CSF and IL-10 respectively, are both phenotypically and functionally similar. The expression of markers was in agreement with another study demonstrating similarity between M-CSF and IL-10 polarized Mph, but a difference with IL-4 [2]. Traditionally, the ROS producing capacity has been associated with pro-inflammatory type-1 Mph, but less with anti-inflammatory Mph2. However, we showed before that the M-CSF differentiated Mph2 are capable of producing ROS [4;15]. Therefore we investigated the ROS-producing capacity of different Mph2 subsets. Importantly, Mph2a, generated in the presence of IL-4, produce lower levels of ROS after PMA stimulation compared with Mph2 and Mph2c. This is in line with the observation that also monocyte-derived dendritic cells, generated in the presence of IL-4 and GM-CSF lack the ROS producing capacity [15]. We showed that Mph2a have lower gp91^{phox} protein expression, an effect previously shown in IL-4-treated porcine and mouse Mph [27;28]. In addition we showed that IL-4 directly affects the ROS producing capacity, also in fully polarized Mph2 cells. Finally also in patients treated with IL-4 *in vivo*, a reduced capacity of monocytes to produce superoxide was observed [29]. Therefore, IL-4 signaling seems a key factor in myeloid cells to regulate their ROS producing capacity.
T cell proliferation was not affected in the presence of Cat, although the IFN-γ production was increased with Mph2a-stimulated T cells, indicating that hydrogen peroxide decreases the T cell activation capacity of Mph2a. This might be explained by the increase in IL-12p40 levels of Mph2a when Cat is present. In agreement, hydrogen peroxide inhibited the IL-12p40 induction in

murine Mph [30].
In contrast, DPI did affect the T cell activation, as shown by the suppressed proliferation and IFN-γ production in an MLR. There appears to be a discrepancy between the concentrations of DPI required to inhibit ROS production by the Mph and the T cell suppression by DPI. However, we did observe a trend with decreased ROS production by the Mph also with low concentrations of DPI. It should be noted that the measurement of ROS is a very short term experiment, whereas T cell proliferation concerns a 5 day assay. Attempt to monitor generation of ROS over longer periods was hampered by an increase of background staining using the ROS indicator DHR (data not shown). Moreover, DPI is not exclusive for the inhibition of NOX2, and is also able to inhibit nitric oxide synthase, xanthine oxidase or cytochrome P450 reductase [31]. This might explain the observation that Mph2a are significantly affected by DPI, even though they are very low in NOX2 expression. DPI has been shown to decrease the proliferation of different cell types both *in vitro* as *in vivo* [32-34]. In addition, T cell proliferation was decreased in response to antigen presentation in the presence of ROS inhibitors including DPI, whereas no effect was observed with Cat [35]. ROS are thus pivotal for T cell activation. This was also shown by experiments with an anti-oxidant, where decreased proliferation and IFN-γ production was observed, whereas a deficient ROS scavenger increased proliferation [36-38].
We showed that DPI diminished the IL-10 and IL-12p40 production of the Mph2 subsets, but showed no effect on IL-6 production. Thus although we observed a ROS-dependent component for the cytokine production, not all cytokines have the same dependence with regard to their production. The pro-inflammatory IL-12p40 is a known T cell activator, whereas the anti-inflammatory IL-10 diminishes the production of inflammatory mediators and favors Th2 T cell development [39;40]. DPI had no effect on the pro-inflammatory cytokine production, including IL-12, with LPS-stimulated dendritic cells [41]. However, mouse Mph stimulated with LPS did show decreased pro- and anti-inflammatory cytokine production with DPI [42], which we observed as well. Interestingly, in CGD patients it has been shown that ROS negatively regulates cytokine production during infection, indicating deregulated cytokine production due to dysfunctional NOX2 [43;44].

Conclusions

In conclusion, we here showed that the ROS producing capacity is different for different Mph2 subsets; Mph2 and Mph2c have a higher ROS producing capacity compared to Mph2a, which is most likely related to a reduced gp91^{phox} protein expression. The difference in T cell stimulatory capacity of Mph2 subsets is not directly linked with their differential ROS producing capacity. In contrast, DPI inhibited T cell proliferation, possibly through decreasing the cytokine production of all Mph2 subsets, indicating a ROS-dependent mechanism of T cell activation.

Acknowledgements

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Chapter 5

The NOX2-mediated ROS producing capacity of recipient cells is associated with reduced T cell infiltrate in an experimental model of chronic renal allograft inflammation

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Abstract

We previously showed that anti-inflammatory Mph (Mph2) can both *in vitro* and *in vivo* induce regulatory T cells (Tregs) in a reactive oxygen species (ROS)-dependent fashion. As influx of Mph is an important characteristic of chronic inflammatory responses, we investigated the impact of NOX2-mediated ROS production by recipient cells in an experimental model of chronic allograft inflammation. We used a kidney transplantation (Tx) model with Lewis (Lew) rats as donor and congenic DA.$Ncf1^{DA/DA}$ (low ROS) and DA.$Ncf1^{E3/E3}$ (normal ROS) rats as recipients. At day 7 the contralateral kidney was removed, and the animals were sacrificed four weeks after Tx. Renal function and injury was monitored in serum and urine and the composition of the infiltrate was analyzed by immunohistochemistry. Four weeks after Tx, large leukocyte clusters were observed in the allograft, in which signs of ROS production could be demonstrated. These clusters showed no difference regarding composition of myeloid cells or the number of FoxP3 positive cells. However, T cell infiltrate was significantly reduced in the DA.$Ncf1^{E3/E3}$ recipients having normal ROS production. Therefore, this study suggests a regulatory effect of ROS on T cell infiltration, but no effect on other inflammatory cells in the allograft.

Introduction

Loss of renal function will ultimately result in the need for a renal transplantation. Despite significant improvement, especially in the early post-transplantation period, the occurrence of allograft rejection remains a problem, which negatively affects the function and survival of transplanted organs. Allograft rejection is characterized by the influx of recipient immune and inflammatory cells in the transplanted organ [1;2]. Although initial attention focused on T cell infiltrates, it has become clear that also other cells, including B cells, macrophages, dendritic cells are part of this infiltrate. However, not all infiltrating cells actively contribute to the rejection process, and there is accumulating evidence that some of these cells have regulatory functions [3]. Macrophages appear in different subsets like the pro-inflammatory Mph (Mph1) which maintain inflammation, and the anti-inflammatory Mph (Mph2) that will clear up cell debris and repair the tissue [4]. Regulatory T cells (Tregs) can also be part of the infiltrate, and have been considered a protective biomarker of graft outcome [5-7]. Therefore, it is of great importance to characterize cellular infiltrates and the local inflammatory conditions, since these will contribute to the composition of the infiltrate within the graft.
The inflammatory response at time of rejection is characterized by the presence of reactive oxygen species (ROS), which are generated amongst others by the Mph [8;9]. Next to oxidative stress, also a role for reactive oxygen species (ROS) in immune regulation has been observed [10;11]. Recently we showed that ROS produced by type 2 Mph contribute to the generation of Tregs [12].

Mph from chronic granulomatous disease (CGD) patients, who have a deficient ROS production due to mutations in the phagocytic NADPH oxidase (NOX2) complex, were shown to be less efficient in the induction of Tregs. Considering that CGD patients are characterized by chronic inflammation, these findings point towards a role for ROS in dampening inflammation. In line with this, rats and mice with a decreased ROS production have increased T cell activation and more severe arthritis, which, in the mouse, was shown to be dependent on the ROS producing capacity of Mph [13;14].
Here we studied the impact of NOX2-mediated ROS production by recipient cells on the inflammatory response in the transplanted kidney. For this we used the model of Lewis-to-DA kidney transplantation, resulting in a model of chronic inflammation. As recipients we used the congenic rat strains DA.*Ncf1*$^{DA/DA}$ and DA.*Ncf1*$^{E3/E3}$genotypes, which only differ in their phagocytic NOX2-derived ROS-producing capacity. In this way, we were able to investigate the role of ROS produced by infiltrating inflammatory cells, in chronically inflamed kidneys. When sacrificed at 4 weeks, we observed a decreased T cell infiltration in grafts derived from recipients with a normal ROS production (DA.*Ncf1*$^{E3/E3}$), compared with the congenic DA.*Ncf1*$^{DA/DA}$ recipients.

Objective

To study the impact of NOX2-mediated ROS production by infiltrating cells on the inflammatory response in the transplanted kidney.

Materials and Methods

Animals

Littermates (DA.*Ncf1*$^{DA/DA}$ and DA.*Ncf1*$^{E3/E3}$) were obtained by intercrossing F1 animals from a DA (Harlan, Horst, The Netherlands) x DA.*Ncf1*$^{E3/E3}$ cross (DA.*Ncf1*$^{E3/E3}$ founders originating from Medical Inflammation Research, Karolinska Institute, Stockholm, Sweden) [15]. Lewis rats were purchased from Harlan (Horst, The Netherlands). The animals were housed in IVC cages and had free access to water and standard rat chow. Animal care and experimentation were performed in accordance with the Dutch law and the local committee of animal experiments of the Leiden University Medical Center.

Kidney transplantation model

Lewis rats were used as donors and DA.*Ncf1*$^{E3/E3}$ (N=5) and DA.*Ncf1*$^{DA/DA}$ (N=5) rats (190–350 g) were used as recipients. Kidney transplantations were performed under isoflurane anesthesia and 0.02 mg/kg buprenophine (Temgesic®, Schering-Plough). The left kidney from the donor was perfused using cold ringer (Fresenius Kabi) with 400 U/ml Heparin (LEO, Pharma BV) and kept on ice. The left kidney from the recipient was removed and the donor kidney was transplanted in the abdominal cavity from the recipient. The donor renal artery and vein were anastomosed end-to-site to respectively the

recipient aorta and vena cava using running sutures. The donor urether was anastomosed end-to-end to the urether of the recipient using lose stitches. Postoperatively, 10 mg/kg of cyclosporine (Sandimmune®, Novartis) was given daily s.c. and provided up till day 7. Seven days after Tx the contralateral kidney was removed. Blood samples were collected at several time points after Tx by tail vein puncture. The rats were twice a week placed in metabolic cages to collect urine samples. The animals were sacrificed four weeks after Tx. Creatinine and urea in serum samples were measured using standard autoanalyzer methods by our hospital diagnostic services. In addition, rocket immuno-electrophoresis (protocol modified from [16]) was used to quantify albumin levels in urine.

Immunohistochemistry

Snap frozen rat kidney sections (4 μm) were air dried and acetone fixed. Sections were stained for OX-42 (anti-CD11b/c, kindly provided by Dr. P. Kuppen, LUMC, Leiden)), ED-1 (anti-CD68; kindly provided by Dr. C.D. Dijkstra, VU, Amsterdam), ED-2 (anti-CD163), R73 (anti-TCR; both kindly provided by Dr. E. de Heer, LUMC, Leiden), and CD45 (BD Biosciences), followed by horseradish peroxidase (HRP)-conjugated goat anti-mouse (Jackson). Goat anti-rat KIM-1 (TIM-1; R&D) staining was assessed by HRP-conjugated rabbit anti-goat (DAKO) as secondary antibody. In addition, the sections were incubated with Tyramide-fluorescein isothiocyanate (FITC). FoxP3 (eBioscience) staining was followed by secondary rabbit anti-fluorescein HRP antibody (Abcam). All stainings were visualized by 3,3'-Diaminobenzidine tetrahydrochloride hydrate (DAB, Sigma), followed by nuclear counterstaining with Mayer's hematoxylin (Merck). Quantification of immunohistochemistry was performed by assessing 10 consecutive fields on each section. Using image J software, the positive area in each image (expressed in area fraction) was quantified, except for the FoxP3 staining where analysis was performed by counting the number of positive cells.

Immunofluorescence

Snap frozen rat kidney sections (10 μm) were air dried. Sections were stained with 10 μM dihydroethidium (DHE; Invitrogen) for 30 min at 37°C in the dark. After washing the sections were incubated with 1.6 μM Hoechst (Invitrogen) for 1 min. Quantification was performed by assessing 15 consecutive fields on each section. The positivity of the staining was semi-quantified by assigning 1 (weak), 2 (positive), or 3 (strong) to each field. The average of all fields was takes as measurement of positivity per section.

Statistical analysis

All data were presented as mean ± standard error of the mean (SEM) and subjected to statistical analysis with one- or two-way ANOVA and the Mann-Whitney U-test using GraphPad Prism software. A value of $p<0.05$ was considered statistically significant.

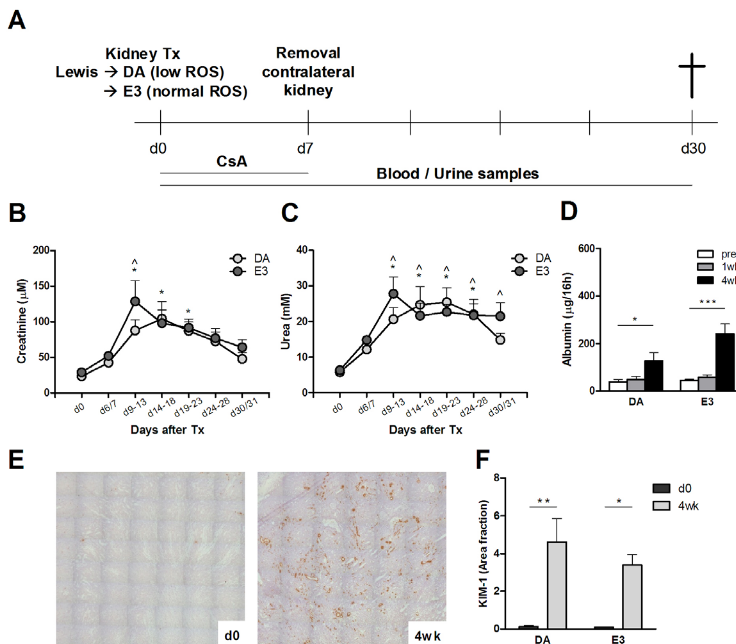

Figure 1: The ROS producing capacity of recipients does not affect the injury of chronically inflamed transplanted kidneys. (***A***) Experimental model with donor Lewis kidney transplanted into either DA.*Ncf1*$^{DA/DA}$ (DA) or DA.*Ncf1*$^{E3/E3}$ (E3) rats. Creatinine (***B***) and urea (***C***) levels measured in serum from DA rats (N=5) and E3 rats (N=5) at different time points after Tx. * $p<0.05$ compared to d0 for DA, ^ $p<0.05$ compared to d0 for E3. (***D***) Albumin levels measured in urine and correlated with urine out-put before, at one week and four weeks after Tx. (***E***) KIM-1 staining in kidney tissue of control kidney (d0) and four weeks after Tx (4wk). (***F***) Analysis of KIM-1 staining in cortex at d0 and 4wk. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Results

Dysfunctional and damaged kidney four weeks after Tx

In an experimental rat model we transplanted Lewis kidneys into either a DA.*Ncf1*$^{DA/DA}$ (low ROS) or DA.*Ncf1*$^{E3/E3}$ (normal ROS) recipient (Fig. 1A). To assess renal function, we measured serum creatinine and urea levels and observed that both slightly increased at day 6/7 after Tx, but showed a steep increase following removal of the remaining native kidney (Fig. 1 B,C). Until sacrifice at week 4, a descending trend was observed with creatinine, but urea levels remained significantly elevated compared with the pretransplant situation. For both parameters, there was no difference between DA.*Ncf1*$^{DA/DA}$ and DA.*Ncf1*$^{E3/E3}$ recipients.

Albumin levels in urine were measured as a sign of injury and were significantly increased four weeks after Tx, but no difference was observed between DA.*Ncf1*$^{DA/DA}$ and DA.*Ncf1*$^{E3/E3}$ recipients (Fig. 1D). It should be noted that the albuminuria in this model is low compared to other strain combinations, where 8-10 mg/24h have been measured [17;18]. In addition, damage to the kidney

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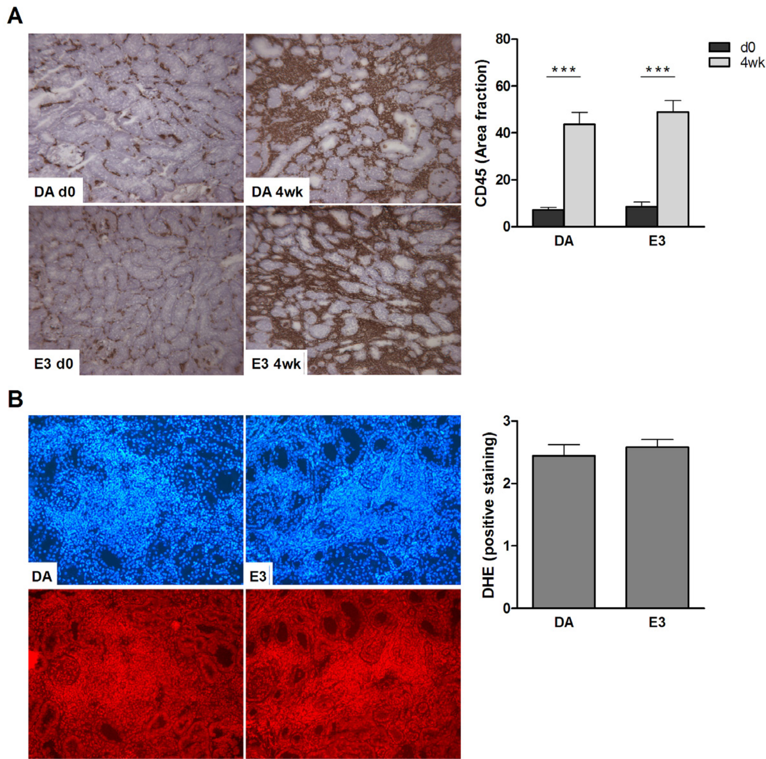

Figure 2: Chronically inflamed kidneys are characterized by leukocyte clusters and presence of ROS. (***A***) Representative picture from CD45 staining for both a DA and E3 rat at d0 and 4wk (left). Digital image analysis of CD45 staining in of the cortex at d0 and 4wk (right). (***B***) DHE (upper) and Hoechst (lower) staining of kidney tissue from a representative DA and E3 rat at 4wk (left), and semi-quantative analysis of DHE staining at 4wk (right).* $p<0.05$, **$p <0.01$, *** $p<0.001$.

four weeks after Tx was confirmed by strong expression of the tubular the injury marker KIM-1, but again no difference was observed between DA.*Ncf1*$^{DA/DA}$ and DA.*Ncf1*$^{E3/E3}$ recipients (Fig. 1E,F).

Leukocyte clusters and ROS present in infiltrate

We next investigated the number of infiltrating cells by staining with the leukocyte marker CD45. In the cortex of control kidneys, small numbers of CD45 positive cells were observed located in the peritubular space, most likely representing resident myeloid cells. In contrast, four weeks after Tx the cortex was characterized by a strong increase of CD45 positive cells, observed in large leukocyte clusters (Fig. 2A). Both in distribution and quantity, no difference was observed between DA.*Ncf1*$^{DA/DA}$ and DA.*Ncf1*$^{E3/E3}$ recipients.

To monitor the contribution of ROS in the graft inflammation, we stained the tissue with the ROS marker dihydroethidium (DHE). DHE staining was observed, and most prominently in the leukocyte clusters as indicated by the high cell density observed with Hoechst staining, although there was no difference

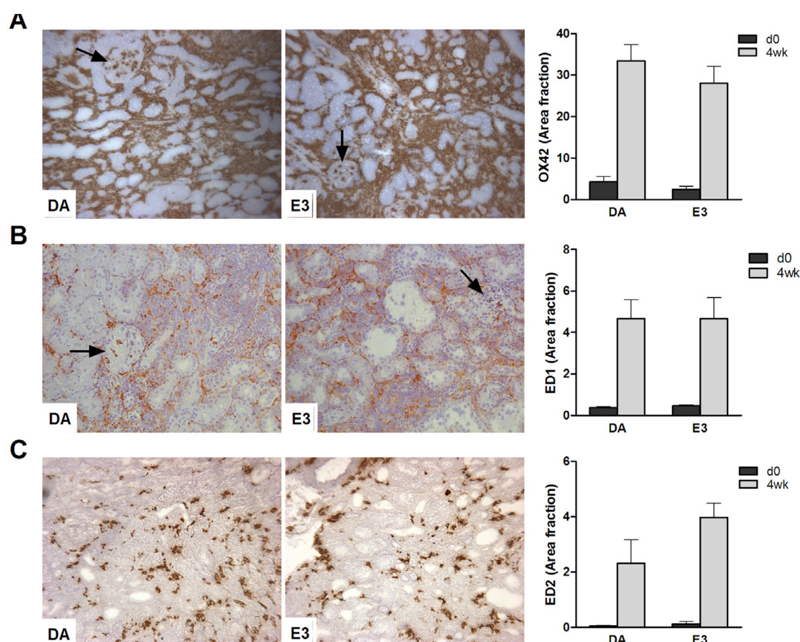

Figure 3: The ROS producing capacity of recipients does not affect the composition of myeloid cell populations. (***A***) OX42, (***B***) ED-1, (***C***) and ED-2 staining in the cortex at d0 and 4wk after Tx. On the left side a representative picture of the cortex at 4wk from both a DA and E3 rat, and quantification of positive area fraction on the tissue on the right side. Arrows indicate positive staining in glomeruli.

between the rats (Fig. 2B).

Similar composition of myeloid cell populations

As Mph have an important role in graft rejection [4] and are a major cell type expressing NOX2, we investigated the presence of myeloid cells in the kidney tissue. A high number of OX42 (CD11b/c) positive cells were seen distributed throughout the entire cortex (Fig. 3A). ED-1 (CD68) positive Mph were observed both in leukocyte clusters and the peritubular area, whereas ED-2 (CD163) positive Mph, most likely representing Mph2, were mostly located in the leukocyte clusters (Fig. 3B,C). However, the quantity of ED1 and ED2 positive cells was lower compared with the OX42 staining.

We also observed OX42 and ED1 positive cells in the glomeruli (indicated by arrows), which has been shown before [19]. No difference in DA.*Ncf1*$^{E3/E3}$ and DA.*Ncf1*$^{DA/DA}$ rats was shown regarding the number of myeloid cells, although a trend towards higher numbers of ED2 positive Mph was observed in recipients with normal ROS-producing cells.

Decreased T cell infiltrate with ROS-producing cells

T cells are an essential cell involved in graft rejection [2]. Therefore we stained the kidney with R73, a pan T cell marker specifically detecting the TCR. T cells

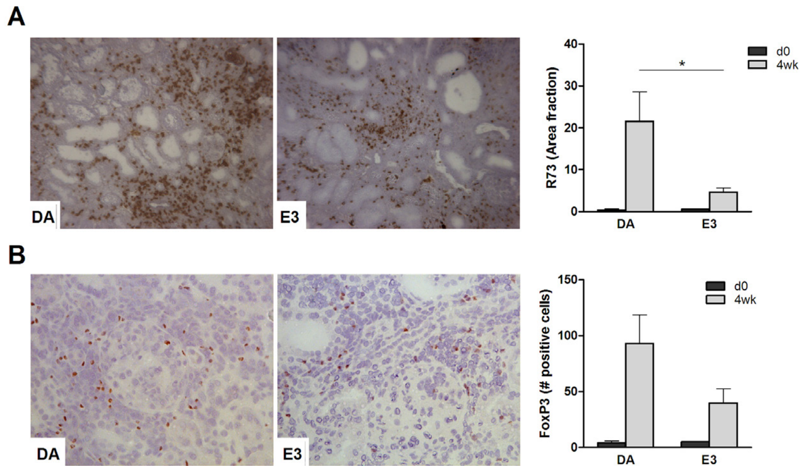

Figure 4: Decreased T cell infiltrate in recipients with a normal ROS-producing capacity. (***A***) A representative picture of the cortex 4wk after Tx (left) and analysis of R73 staining at d0 and 4wk (right). (***B***) FoxP3 positive cells observed in the cortex at d0 and 4wk after Tx. On the left side a representative picture of the cortex at 4wk from a DA and E3 rat, and quantification of the tissue on the right side. * $p<0.05$

were mostly observed in the leukocyte clusters throughout the cortex, but some positive cells were also located in the interstitium (Fig. 4A). The number of T cells was significantly lower in DA.*Ncf1*$^{E3/E3}$ rats compared to DA.*Ncf1*$^{DA/DA}$ rats (Fig. 4A).

Since the presence of Tregs can be beneficial for graft outcome [5], we investigated the presence of these cells in the kidney tissue. We observed FoxP3 staining especially in the leukocyte clusters in the cortex, with a trend towards lower numbers observed in DA.*Ncf1*$^{E3/E3}$ recipients compared to DA.*Ncf1*$^{DA/DA}$ rats (Fig. 4B). In view of the reduced T cell infiltrate, the relative amount of FoxP3 positive cells (ratio FoxP3: R73 cells) appeared higher in DA.*Ncf1*$^{E3/E3}$ rats (43 positive cells/ 5 % positive area fraction) compared to DA.*Ncf1*$^{DA/DA}$ rats (22 positive cells/ 5 % positive area fraction).

Discussion

In this study we investigated the contribution of NOX2-derived ROS in a model of chronic renal inflammation. Lewis rat kidneys were transplanted into recipients with either normal or reduced NOX2-derived ROS, making use of the congenic DA rat strain DA.*Ncf1*$^{E3/E3}$ and DA.*Ncf1*$^{DA/DA}$. Four weeks after Tx, the allografts were still dysfunctional and damage to the kidney tissue was observed. Moreover, large leukocyte clusters were observed in the kidney, characterized by CD45 and CD11b/c expression. For these parameters, there was no difference between DA.*Ncf1*$^{E3/E3}$ and DA.*Ncf1*$^{DA/DA}$ recipients. However, a decreased number of T cells infiltrated the Tx kidney when recipients had a

normal ROS producing capacity.
The transplant model using DA rats as donor and Lewis rats as recipient has been more widely described in the literature [17;20]. In this study we wanted to specifically address the contribution of ROS by infiltrating inflammatory cells. Therefore we used the congenic DA rats as recipients. We observed ROS staining in the kidney tissue, especially in the leukocyte clusters. Making use of DHE staining, we observed that also DA.*Ncf1*$^{DA/DA}$ rats still have ROS producing capacity [12;21], indicating that next to NOX2, also other sources of ROS are present. ROS produced during oxidative stress (high levels) has been implicated in chronic kidney diseases or kidney Tx in a negative way [22;23]. In contrast, it has been shown that ROS produced in the immunological synapse or produced in lower amounts can inhibit T cell activation and has been indicated in various signaling pathways as well [24-27].
Mph2 have been shown to induce Tregs via the production of ROS [12]. In the current experiments we found no difference in absolute numbers of FoxP3 positive cells in the cortex of transplanted kidneys between DA.*Ncf1*$^{DA/DA}$ and DA.*Ncf1*$^{E3/E3}$ recipients. However, the reduction in Tregs was not as prominent as the reduction in total T cells. Therefore it is tempting to speculate that in DA.*Ncf1*$^{E3/E3}$ recipients the relative amount of FoxP3 positive cells was higher, thereby tilting the balance towards more regulation. This relative increase may be important since it has been assumed that the ratio between effector T cells and Tregs is a main determinant in allograft outcome [28]. *In vitro*, Tregs can steer monocyte differentiation towards CD163 positive Mph [29], whereas *in vivo* Tregs expansion increased the number of CD163 positive Mph in the infiltrate and decreased pro-inflammatory cytokine production [30]. This further indicates that Tregs might exert an immune regulatory role on the infiltrating cells [31]. However, in the current study this altered balance did not affect any of the other inflammatory, injury or functional markers.
When investigating the renal tissue for different myeloid cells, we observed OX42 staining throughout the entire cortex located both in the leukocyte clusters and between the tubuli. There was considerably less ED1 and ED2 staining in the cortex compared with OX42. This might suggest that apart from Mph also granulocytes (which abundantly express OX42) were present in the kidney tissue four weeks after Tx. However, the presence of neutrophils at this late stage after Tx is unlikely, although a constant influx of these cells due to chronic inflammation is possible. An alternative explanation is that OX42 identifies another myeloid population not recognized by ED1 or ED2, or that the OX42 expression is higher and therefore more easily detected in immunohistochemistry. Similarly, the abundant expression of OX42 can explain why reduced amount of T cell infiltrate (as shown by R73 staining) was not reflected by a difference in CD45 staining.
ED1 is considered to be a general Mph marker, whereas ED2 is more specific for Mph2. Since, compared to ED1, there was less ED2 staining present in the infiltrate, it is likely that also Mph1 were present. This indicates that both pro-inflammatory mechanisms and tissue repair were simultaneously taking place at this stage after Tx. Since no difference was observed between DA.*Ncf1*$^{E3/E3}$

and DA.*Ncf1*$^{DA/DA}$ rats, this suggests that the ROS producing capacity of the recipient's immune cells is not involved in the active process of Mph recruitment and function after Tx. However a trend towards more Mph2 was observed in the grafts from DA.*Ncf1*$^{E3/E3}$ recipients. The presence of the diverse immune cells in the lymphoid clusters would allow interactions between these cells and therefore also allow for the possibility of ROS signaling within the graft. Nevertheless, diminished numbers of R73 positive T cells were observed in the cortex of the Lew kidney with DA.*Ncf1*$^{E3/E3}$ rats as recipients. By performing the renal transplantation and analyzing the tissue of this chronically inflamed allograft (with infiltrating macrophages having a difference in ROS producing capacity), we hypothesized to pick up differences in the 'local' inflammatory response (ie in the graft). However, we cannot exclude that part of the difference we ultimately found has its origin at other locations (like in the lymphoid organs). This will be the place where most T cell activation will be initiated, and also here signaling through ROS might take place. Since these molecules only act on a short distance it could be hypothesized that the immunological synapse could be important for this process. The reduced T cell infiltrate could potentially hint towards an immunosuppressive role of NOX2-derived ROS. In a collagen-induced arthritis model Mph-derived ROS was clearly shown to be beneficial in suppressing T cell responses [13]. It most likely that the subtle ROS producing capacity by Mph in our model was insufficient to overcome the high inflammatory state in the kidney after Tx, which only showed a decreased T cell infiltrate. Due to the nature of our model, we believe that the amount of ROS produced here by Mph was insufficient to have an impact on the inflammatory response in the allograft. We recently showed that inhibition of ROS can under some conditions also suppress T cell proliferation [32]. In that study we used a human *in vitro* model of monocyte-derived macrophages and used the inhibitor DPI for blocking ROS-mediated effects. Unfortunately, DPI is not specific for NOX2, and therefore this can explain the inhibitory effects of DPI on T cell activation. In the current study the difference is based on a genetic polymorphism in one of the NOX2 gene, and therefore can specifically address differences in NOX2-mediated ROS. A partially protective role of ROS under such conditions is in line with various auto-immune diseases where Mph-derived ROS has been shown to be essential for protection [13;14].
In conclusion, in the Lew-to-DA renal Tx model we observed leukocyte clusters four weeks after Tx. In these clusters the presence of myeloid cells and FoxP3 positive Tregs were observed, but no difference was shown between DA.*Ncf1*$^{E3/E3}$ and DA.*Ncf1*$^{DA/DA}$ rats. Although a decreased number of infiltrating T cells was shown in allografts of DA.*Ncf1*$^{E3/E3}$ recipients having a normal ROS-producing capacity, in this experimental model no effect was observed on other inflammatory cells or signs of injury.

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Chapter 6

General discussion and summary

Discussion

In recent years the view on the biological role of reactive oxygen species (ROS) has dramatically changed and a functional role for macrophage (Mph)-derived ROS in T cell activation and auto-immunity has been documented. Rats with a reduced ROS producing capacity showed an increased susceptibility to pristane-induced arthritis, which was mediated in a T cell-dependent fashion (1). In addition, mice expressing functional NOX2 protein p47^{phox} only in their Mph were protected against disease compared to mice with fully functional p47^{phox} in an arthritis model (2). Finally, T cell activation is inhibited by hydrogen peroxide and ROS have been implicated to play a role in the process of antigen presentation (3-5). Altogether, since especially Mph-derived ROS seems to play an important role, in the current thesis we aimed to explore this further by investigating different Mph subsets and the immune modulating potential of Mph-derived ROS. This chapter summarizes and discusses the contribution of the research described in this thesis in order to understand the role of ROS in the Mph-T cell interaction.

ROS producing capacity of macrophages

The NOX2-derived ROS producing capacity is different between human Mph subsets, showing a high ROS producing capacity with pro-inflammatory Mph1, whereas most literature suggested that the anti-inflammatory Mph2 has little to no ROS producing capacity (6,7). Since we were interested in Mph-derived ROS, gaining knowledge about the ROS producing potential of these different Mph subsets would be essential. Comparing GM-CSF differentiated Mph1 with M-CSF differentiated Mph2 showed opposite results, with little ROS producing capacity of Mph1 and high ROS production for Mph2 (**Chapter 3**).

Mph2 display a high plasticity and can undergo phenotypic and functional changes due to the surrounding milieu (7,8). We therefore wanted to investigate certain Mph2 subsets in more detail. The most commonly described Mph2 in literature are Mph2a, which are differentiated with either IL-4 or IL-13. In addition, Mph2c are a well-known Mph2 subset and are differentiated with IL-10 or glucocorticoids. When comparing the phenotype of three different Mph2 subsets, namely M-CSF differentiated Mph2, Mph2a, and Mph2c, it is striking that Mph2 and Mph2c demonstrate a similar phenotype, whereas Mph2a are clearly distinct (**Chapter 4** and Table 1). The IL-4 differentiated Mph2a drives Th2 immune responses, predominantly mediated via STAT6 signaling, whereas Mph2c requires STAT3 signaling, which could in part explain the difference (9,10). Thus although they are all named anti-inflammatory Mph, phenotypically and functionally these Mph2 subsets are different. We showed this *in vitro*, where we observed differences in marker expression and cytokine production, and this was also observed *in vivo* by others (11,12).

As we showed that M-CSF-differentiated Mph2 had a high NOX2-derived ROS producing capacity, which is in contrast with the general opinion, we wanted to compare this Mph2 with Mph2a and Mph2c with regard to their ROS producing

		Mph2 (M-CSF)	Mph2a (IL-4)	Mph2c (IL-10)
markers	CD14	+	-	+
	CD163	+	-	+
	CD209	-	+	-
	HLA-DR	+	+	+
	CD86	+	++	+
cytokines	IL-6	+	++	-
	IL-12p40	-	+	-
	TNF-α	-	+	-
	IL-10	++	+	+
other	ROS producing capacity	++	-	+
	T cell stimulatory capacity	++	+++	+

Table 1: Characteristics of Mph2, Mph2a, and Mph2c regarding markers, cytokines, ROS producing capacity and T cell stimulatory capacity.

capacity (**Chapter 4**). Mph2a do not produce NOX2-derived ROS upon PMA stimulation, which is indeed in agreement with literature. In contrast Mph2c did show ROS-producing capacity, clearly demonstrating the functional diversity of diverse Mph2 subsets. Thus in addition of different expression of cell surface markers or cytokine production, the ROS producing capacity is also different among Mph2, Mph2a, and Mph2c. This indicates that ROS will most likely have different functions within the Mph subsets, related to the various effects observed on the immune system, and that subsets should be well described and characterized before results can be extrapolated.

Effect of immunosuppressive drugs on ROS

Immunosuppressive drugs (IS drugs) are commonly given to prevent T cell activation and graft rejection. When considering the side-effects of these drugs, developing an alternative therapy is essential. Mph2 have the potential to be used as cellular therapy, since they may be instrumental in diminishing T cell responses in autoimmunity or preventing allograft rejection in transplantation. Currently, cellular therapy with Mph has already been performed. Two patients required low-dose immunosuppressive therapy to preserve their grafts from rejection, after they received a transfusion with regulatory Mph a week prior to their kidney transplantation (13). In protocols where regulatory Mph will be used as cellular therapy, this will most likely be in combination with the current therapies therefore the effects observed can be potentially of significance. However, the IS drugs may affect the immunosuppressive function of the Mph2 administered and specifically the ROS producing capacity of Mph may be effected. Therefore we investigated in **Chapter 3** the effect of IS drugs on the ROS-producing capacity of Mph2. Interestingly, we observed that IS drugs did not affect the ROS-producing capacity of already differentiated

Mph, which is important when considering Mph2 for cell therapy. The ROS-producing capacity of Mph2 was augmented by a number of these drugs when present during differentiation, indicating that the drugs only influence the NOX2 complex as long as monocytes are differentiating into mature Mph2. Dexamethasone, which is experimentally one of the strongest and most widely used corticosteroid, was one of the IS drugs shown to increase the ROS-producing capacity of Mph2. In line with this, it was shown that dexamethasone could upregulate NOX2 proteins and *in vivo* increased ROS production was demonstrated (14,15). In addition, dendritic cells cultured in dexamethasone were frozen in an immature state associated with lower levels of T cell activation (16). Dendritic cells were shown to have a very low ROS production *in vitro*, but with the addition of dexamethasone, the ROS producing capabilities of dendritic cells were strongly increased (**Chapter 3**). In summary, in **Chapter 3** we describe an additional anti-inflammatory mechanism of dexamethasone, since dexamethasone amplified the down-regulation of T cell responses via the production of ROS in Mph2.

ROS in T cell activation

A role for ROS in activating T cells was previously shown in a rat model of pristane-induced arthritis (1). Suppressed T cell activation by ROS was also demonstrated for granulocyte-derived hydrogen peroxide and through the effect of catalase on lymphocytes (4,5). Therefore we wanted to investigate the role of human Mph-derived ROS on T cell activation (**Chapter 4**). Interestingly, differences in the T cell-stimulatory capacity by Mph2, Mph2a, and Mph2c were observed, which was inversely correlated with their ROS-producing capacity. Therefore we hypothesized that the ROS-producing capacity of Mph2 could be functionally important with regards to T cell activation. However, in contrast to this hypothesis, T cell activation by Mph2a did demonstrate ROS dependency, as T cell activation by Mph2a was inhibited by the ROS inhibitor DPI, even when we could not demonstrate any NOX2-derived ROS by Mph2a upon stimulation. This indicates that apart from ROS derived from NOX2, ROS are also being produced elsewhere in the Mph, although Mph rely primarily on NOX2 for ROS production (17). Since DPI not only inhibits NOX2, but also the other NOX, nitric oxide synthase, xanthine oxidase or cytochrome P450 reductase, this suggests involvement of ROS from above mentioned sources in T cell activation (18). It was recently shown that mitochondrial ROS are required for T cell activation and the subsequent IL-2 induction (19). The effect of ROS on T cell activation was independent on the ROS producing capacity of the Mph2 subsets observed in our studies.

To unravel this effect we searched for possible mechanisms how Mph-derived ROS affects T cell function (**Chapter 4**). We focused on the signals between Mph and T cell that allows for T cell activation. These signals involve the MHC class 2 molecules, co-stimulatory molecules and cytokines. By means of DPI we investigated the MHC class 2 molecule HLA-DR and the co-stimulatory molecule CD86 expression on Mph2, Mph2a, and Mph2c and observed no effect on the expression of these markers, indicating that there is no ROS dependency of

Gene	Ratio	Gene summary
IFIT3	0.61	Interferon-induced protein with tetratricopeptide repeats 3
IL1RN	0.68	Interleukin 1 receptor antagonist
IFIT1	0.73	Interferon-induced protein with tetratricopeptide repeats 1
IFIT2	0.74	Interferon-induced protein with tetratricopeptide repeats 2
TNFRSF4	0.76	Tumor necrosis factor receptor superfamily, member 4
FOS	1.84	FBJ murine osteosarcoma viral oncogene homolog
DUSP1	1.69	Dual specificity phosphatase 1
JUN	1.66	Jun proto-oncogene
KLF9	1.56	Kruppel-like factor 9
CD69	1.49	Early T-cell activation antigen p60

Table 2: Genes changed in expression when exposed to hydrogen peroxide with the top 5 most downregulated genes and top 5 most upregulated genes. Ratio: CD4+ T cells exposed to 4 hours of hydrogen peroxide / CD4+ T cells without hydrogen peroxide.

these specific signals. Therefore we investigated the cytokine production of Mph2 subsets. The Mph2 subsets were activated by CD40L in order to mimic the CD40-CD40L signal between APC-T cell. When testing directly on the three different Mph2 subsets, DPI dose-dependently decreased the IL-10 and IL-12p40 production of CD40L-stimulated Mph2 subsets. However, the IL-6 production was not affected, which is interesting due to its pro-inflammatory nature. Interestingly, when stimulated with LPS, DPI suppressed the cytokine production in all three Mph2 subsets. In mouse Mph decreased IL-10, IL-6 and TNF-α production with DPI has been observed as well (20). Thus the decreased T cell proliferation in the presence of DPI could be linked to alterations in cytokine production, as clearly shown for IL-10 and IL-12p40 production. However, a more detailed analysis of the complete cytokine production profile, as well as information on other signaling pathways, will be required to fully explain the mechanism of ROS-dependent T cell activation.

ROS-dependent Treg induction

We observed a high ROS producing capacity in Mph2, but in contrast with literature no ROS production upon PMA stimulation was shown in GM-CSF-differentiated Mph1 (**Chapter 3**). We hypothesized that if Mph-derived ROS prevent T cell mediated immune responses, it could do so by inducing regulatory T cells (Tregs). It was already shown that anti-inflammatory Mph were capable of inducing potent Tregs, whereas in that setting the same was not observed with pro-inflammatory Mph (21). Induction of Tregs in a ROS-dependent manner could be demonstrated in an *in vivo* DTH model and an expansion in the number of Tregs after increasing the ROS producing capacity with dexamethasone. This confirmed that Mph-derived ROS can play a role in the induction of Tregs (Chapters 2 and 3). In addition, NOX2-derived ROS was shown to be involved in suppression of CD4 positive effector cells by Tregs (22). These data indicate diminished pro-inflammatory activities and thus less inflammation, showing that ROS affect the immune regulatory capacity.
Regulatory immune cells promote tolerance and enhance graft survival *in vivo*

(23), which may include both natural and inducible Tregs. Natural Tregs are derived from the thymus, whereas inducible Tregs are derived from naïve T cell precursors in the periphery and upregulate FoxP3 expression (24). In grafts their number is limited and consequently when acute rejection takes place they will not be able to prevent rejection. However, the allograft itself has been shown to be able to induce or expand Tregs (25,26). Tregs affect immune cells in different ways. For example, they produce IL-10 or express cytotoxic T lymphocyte antigen 4 (CTLA4), causing decreased APCs activity, which will diminish the activity of effector immune cells and decreases the chance of graft rejection. Inhibition of CTLA4 or IL-10 *in vivo* indeed showed impaired Treg-mediated regulation in transplantation (27,28). These data show the importance of the presence of Tregs in an allograft. Based on our experiments, we believe that there may be a link between ROS and Treg induction *in vivo*. In **Chapter 5** we investigated the impact of NOX2-mediated ROS production by recipient cells in an experimental model of chronic allograft inflammation. We used the model of Lewis-to-DA kidney transplantation. As recipients we used the congenic rat strains DA.*Ncf1*$^{DA/DA}$ and DA.*Ncf1*$^{E3/E3}$genotypes, which only differ in their phagocytic NOX2-derived ROS-producing capacity. DA rats are more prone to develop rheumatoid arthritis compared with other rat strains. A naturally occurring polymorphism of *Ncf1*, component of the NADPH oxidase complex, was found to regulate the severity of arthritis in rats (29). This polymorphism caused reduced ROS production and promoted the activation of arthritogenic T cells. Thus the congenic DA.*Ncf1*$^{DA/DA}$ rats have a reduced NOX2-mediated ROS production compared to the DA.*Ncf1*$^{E3/E3}$ rats. Large leukocyte clusters were observed in the allograft four weeks after transplantation. In addition, signs of ROS production was also demonstrated. The leukocyte clusters showed no difference regarding composition of myeloid cells or the number of FoxP3 positive Treg cells. However, T cell infiltrate was significantly reduced in the DA.*Ncf1*$^{E3/E3}$ recipients having normal ROS production. As a consequence, this means that there are relatively more Tregs in the infiltrate. This could suggests a regulatory effect of ROS on T cell infiltration, but no effect on other inflammatory cells in the allograft.

Molecular mechanisms of ROS-mediated effects

NOX2-derived ROS from Mph can be produced intracellular, into the phagosome, or extracellular, at the plasma membrane (17). The intracellular produced ROS are associated with the killing of pathogens and inflammatory signaling, extracellular ROS are considered to be second messengers (17). We observed that NOX2-derived ROS from Mph affects T cells by inducing Tregs and are able to activate T cells in a ROS-dependent manner (Chapters 2 and 4). Signaling by ROS depends on the location, time, concentration and duration of the ROS being produced, contributing to homeostasis and physiological cell activation (30). There are several potential ways how ROS could work; 1) ROS could work intracellular in the Mph, thereby interfering with the signaling pathways determining the T cell stimulatory capacity, 2) ROS could be a molecule delivered at the contact between Mph and T cell causing a

Gene	Ratio	Gene summary
IFIT1	0.61	Interferon-induced protein with tetratricopeptide repeats 1
IFIT2	0.67	interferon-induced protein with tetratricopeptide repeats 2
CCL5	0.74	Chemokine (C-C motif) ligand 5
IFIT3	0.74	Interferon-induced protein with tetratricopeptide repeats 3
TNFRSF4	0.83	Tumor necrosis factor receptor superfamily, member 4
JUN	1.95	Jun proto-oncogene
ZMIZ1	1.76	Zinc finger, MIZ-type containing 1
FOS	1.61	FBJ murine osteosarcoma viral oncogene homolog
MAPK13	1.56	Mitogen-activated protein kinase 13
HMOX1	1.49	Heme oxygenase (decycling) 1

Table 3: Most differently expressed genes when exposed to hydrogen peroxide with top 5 most downregulated genes and top 5 most upregulated genes. Ratio: αCD3/αCD28 activated CD4+ T cells exposed to 4 hours of hydrogen peroxide / αCD3/αCD28 activated CD4+ T cells without hydrogen peroxide.

local change in the redox state of the cell surface molecules important in T cell activation, 3) ROS might end up in the T cells and there actively interfere with T cell signal transduction.
We investigated the effect of ROS on T cells at the level of gene and protein expression. Hydrogen peroxide was used as the ROS to conduct these experiments. We tried to establish potential mechanisms on how Mph-derived ROS could affect T cells by searching for genes and proteins that directly or indirectly could have an effect on T cell activation.

Molecular mechanisms of ROS-mediated effects on genetic level

In order to investigate gene expression we performed gene expression analysis on CD4+ T cells using microarrays (in collaboration with M Eikmans, Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden, The Netherlands). We hypothesized that we would find genes involved in transcriptional regulation of T cell activation and signaling pathways, including cell surface markers and cytokines, able to activate and differentiate the T cells. We studied both unstimulated and αCD3/αCD28-stimulated T cells. First we examined whether our method was valid by comparing genes from unstimulated T cells with αCD3/αCD28-stimulated T cells. Increased expression of well-known T cell activation genes including ICOS, CD69, IL1B, and NFKB1 was observed, confirming T cell activation by αCD3/αCD28 (data not shown). Hydrogen peroxide-induced gene expression has already been investigated in among others the human HeLa cell line (31), and even though the focus in these studies was on oxidative stress, genes in involved in apoptosis, cell cycle regulation, cell-cell communication, signal transduction, and transcriptional regulation were differentially regulated upon treatment (32).
In the microarray we investigated the effect of 4 hours incubation with hydrogen peroxide on CD4+ T cells and Table 2 summarizes the top 5 genes that changed in expression compared to the controls. Genes with decreased expression included type 1 IFN signaling genes IFIT1, IFIT2 and

IFIT3 which upon transcription, inhibit various cellular and viral processes like cell proliferation, cell migration and viral replication (33). Anti-inflammatory therapy negatively regulates the transcription of the IFIT genes (33), as we observed as well, indicating that ROS signaling affects these genes in an anti-inflammatory manner. IL1RN encodes the protein IL-1RA which inhibits the activity of the pro-inflammatory cytokines IL-1α and IL-1β. TNFRS4 (CD134, OX40) encodes a stimulatory receptor on T cells (34). The TNF receptor family is vital for regulation of T cell activation, differentiation and survival. Members of the TNFR family regulate TCR-independent NF-κB activation. Genes with increased expression due to hydrogen peroxide included the genes JUN and FOS encoding proteins that dimerize forming the transcription factor complex AP-1. DUPS1 (MKP-1) dephosphorylates and inactivates MAPKs and can attenuate LPS-induced IL-6, IL-10, and TNF-α expression (35). In addition, the gene KLF9 encodes a member of the Krüppel-like family of transcription factors and CD69 is a T cell activation marker. The genes which are down- and upregulated by ROS indicate an increase of inflammatory events and activation of the T cells, and although the downregulation of TNFRS4 is not in agreement with this, these data indicate that ROS plays a role in TCR-dependent activation of T cells.
We investigated also whether ROS would change the gene expression of αCD3/αCD28-activated T cells when simultaneously exposed to ROS. Activated cells could have a changed thiol group formation compared with unstimulated cells, therefore the effect of ROS on already activated cells might be different. Incubation with hydrogen peroxide showed several genes with changed expression (Table 3). We again observed decreased expression of IFIT1, IFIT2, IFIT3, and TNFRS4. In addition, expression of CCL5 was reduced. CCL5 is a chemoattractant secreted by T cells upon activation. Genes with increased expression included JUN and FOS. ZMIZ1 encodes a member of the protein inhibitor of activated STAT family of proteins (36). MAPK13 is involved in a variety of cellular processes, whereas HMOX1 has antioxidant, anti-inflammatory, and anti-apoptotic properties. The effect of ROS on the gene expression on activated T cells is inconsistent showing genes that indicate activation (IFIT genes, JUN, FOS, and MAPK13), but also inhibition (TNFRS4, CCL5, ZMIZ1, and HMOX1). These data indicate the complexity regarding the effect of ROS on T cells and will therefore need more investigation in order to beginning to understand ROS signaling pathways.

Molecular mechanisms of ROS-mediated effects at the protein level

As an alternative mechanistic explanation, we investigated the effect of ROS at the protein level, since there is evidence for a functional role for cysteine modifications in response to redox signals. For example, it has been shown that ROS can affect cell surface thiols of the proteins integrin α-4, CD4 receptor and the virus envelope protein gp120 (37,38). However, the identity and significance of these alterations in the protein thiols are still largely unknown. For this purpose proteomics were performed by a newly developed method

of Isotope-coded affinity tag (ICAT) labeling put into a mass spectrometry (in collaboration with PA van Veelen and GMC Janssen, Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden, The Netherlands). In order to test this method and trying to validate it with known literature, we used Jurkat T cells and hydrogen peroxide as a simplified model for Mph and T cells.

Proteomics was performed using ICAT labeled Jurkat cells which were incubated for a short period of time with hydrogen peroxide. We observed and identified several membrane and cytoplasmic proteins that upon hydrogen peroxide treatment changed their thiol groups. However, although the technical part of the CAT appeared to work fine, interpretation of the data was still difficult. For example, the period in which hydrogen peroxide could potentially change thiol groups of proteins was limited. In addition, when the cells were rested after incubation with hydrogen peroxide they would change back to their original state. Therefore, the technique and procedures need to be further optimized to identify ROS-sensitive proteins involved in T cell activation. However in the future this redox-proteomics will allow for a better understanding on how ROS may contribute to signaling.

Concluding remarks

Anti-inflammatory Mph have a high plasticity. Both *in vivo* and *in vitro* different types of Mph2 have been observed, all presenting an overlapping phenotype, but each having specific characteristics. Classifying Mph2 is difficult, especially since no specific markers for human Mph2 have yet been identified, as is the case for mouse Mph2. Currently, there are a number of markers being used, but these are all based on the distinction between Mph1 and Mph2. The expression of these specific Mph2 markers is diverse among different Mph2 subsets. Therefore it will be more important to perform functional characterization of the different Mph2 subsets. As a consequence molecules involved in these diverse functions could ultimately serve as specific markers.

At present, the full mechanism by which Mph-derived ROS affect T cell activation and signaling is unknown. We observed that Mph2 activate T cells in a ROS-dependent manner but also that the suppression of activated T cells by Mph2 was ROS-dependent. We made a first step in trying to identify proteins and genes which are redox-sensitive. We decided to make use of inhibitors and focus on the signals between Mph and T cell that involve the activation of T cells. We observed that some cytokines were affected by DPI: the IL-12p40 and IL-10 production were suppressed by DPI, whereas no effect was observed with IL-6, suggesting that ROS is specifically involved in the regulation of some cytokines that may exert its effect on T cells. Affected cytokine production in the absence of ROS was also observed by others (39). These results can be a new start point for further research in unraveling the mechanism, but how ROS affects T cell activation will need further investigations. In summary, T cells are activated by Mph-derived ROS and regulation of the cytokine production by the APC is a potential mechanism how ROS can affect T cell functions.

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6

Appendix

Nederlandse samenvatting

De afgelopen jaren is het inzicht in de biologische rol van zuurstofradicalen (‘reactive oxygen species’, ROS) dramatisch veranderd. Over het algemeen werd er gedacht dat ROS alleen maar schadelijk waren, maar intussen is duidelijk geworden dat ROS ook functioneren als belangrijke fysiologische regulatoren van intracellulaire signaaltransductie routes. ROS spelen een rol in antigeen presentatie en er is aangetoond dat waterstofperoxide, een belangrijke vorm van ROS, T cel activatie kan remmen. Ten slotte is aangetoond dat DA ratten met een verminderde ROS productie gevoeliger waren voor artritis, en dat ook dit gepaard ging met ene veranderde T cel activatie. De reden voor deze verminderde ROS productie kwam door een polymorfisme in het *Ncf1* gen. Dit *Ncf1* gen codeert voor het eiwit p47^{phox}, wat een van de belangrijkste eiwitten is van het NADPH oxidase complex (NOX2). Dit NOX2 complex produceert de ROS superoxide. Muizen die een functioneel NOX2 eiwit p47^{phox} exclusief in hun macrofagen tot expressie konden brengen, waren beschermd tegen artritis. ROS afkomstig van macrofagen lijken dus een belangrijke rol te spelen en daarom wilden wij dit onderwerp in dit proefschrift verder bestuderen door verschillende macrofaag-subsets en het immuunmodulerende potentieel van ROS geproduceerd door macrofagen te onderzoeken.

Hoofdstuk 1 geeft algemene achtergrondinformatie met betrekking tot macrofagen. Daarnaast wordt er een overzicht van de verschillende soorten macrofagen gegeven. Ook wordt de activatie van macrofagen en de rol van macrofagen tijdens inflammatie besproken. Verder wordt er achtergrondinformatie over ROS gegeven. Ook wordt het NOX2 complex beschreven en de gevolgen van mutaties in dit complex. Ten slotte wordt in dit overzicht dieper ingegaan op de potentieel immuunmodulerende rol van ROS.

In **Hoofdstuk 2** onderzochten we de ROS-producerende capaciteit van humane macrofagen. De anti-inflammatoire macrofagen (gedifferentieerd met M-CSF; Mph2) hebben een hoge ROS productie na PMA stimulatie, terwijl dendritische cellen (gedifferentieerd met GM-CSF en IL-4) hoegenaamd geen ROS produceren. In een ‘mixed lymphocyte reaction’ (MLR) waarin T cellen daarnaast geactiveerd werden met anti-CD3/anti-CD28, stimuleerden dendritische cellen de T cel proliferatie, terwijl Mph2 de T cel proliferatie juist onderdrukten. Deze remming bleek ROS-afhankelijk te zijn, want na toevoeging van de NOX2-remmer apocynin werd de remming opgeheven. De macrofagen induceerden regulatoire T cellen (CD4+CD25+FoxP3+) nadat CD4+CD25- T cellen aan deze cellen waren blootgesteld. Na toevoeging van apocynin bleek deze inductie ROS-afhankelijk te zijn. Dit konden we bevestigen met Mph2 afkomstig van patiënten met ‘chronic granulomatous disease’ (CGD) die een mutatie in NOX2 hebben, waarbij we in afwezigheid van ROS productie opnieuw verminderde inductie van regulatoire T cellen en meer T cel activatie

observeerden. Verder bestudeerden we de rol van ROS *in vivo* door de T cel activatie te bestuderen in een ratmodel met de 'delayed-type hypersensitivity' (DTH)-respons als uitleessysteem. Hieruit bleek dat het primen van naïeve Lewis ratten met Mph2 van DA ratten met een normale ROS-producerende capaciteit leidde tot een sterkere inductie van regulatoire T cel in vergelijking met het primen met Mph2 van DA ratten met een lage ROS productie. Bovendien was de T cel activatie lager met normaal ROS-producerende Mph2 gezien de verminderde DTH-respons in vergelijking met laag ROS-producerende Mph2. Deze data tonen aan dat ROS geproduceerd door Mph2 een rol spelen in de inductie van regulatoire T cellen.

In **Hoofdstuk 3** hebben we onderzocht of medicijnen die het immuunsysteem onderdrukken een negatief effect kunnen hebben op de ROS productie van macrofagen. Dit zou namelijk een nadelig effect kunnen hebben op de mogelijkheid om macrofagen als therapie te gebruiken in combinatie met de huidige therapieën. We vergeleken hierbij de pro-inflammatoire macrofaag (gedifferentieerd met GM-CSF; Mph1) met de anti-inflammatoire Mph2. Terwijl Mph2 een hoge ROS productie hebben na PMA stimulatie, vertoonden Mph1 juist een lage ROS-producerende capaciteit. Verschillende immuunsuppressiva (cyclosporine, tacrolimus, dexamethason, rapamycine, en mycofenolaat mofetil) werden aan de Mph1 en Mph2 toegevoegd. Wanneer de medicijnen werden toegevoegd op dag 6 van kweek, het moment dat de macrofagen gedifferentieerd zijn, was er geen effect op de ROS-producerende capaciteit aantoonbaar. Echter, de medicijnen hadden wel een effect op de ROS productie wanneer toegediend tijdens het differentiëren van monocyt-naar-macrofaag (gedurende de eerste 6 dagen). De medicijnen cyclosporine, tacrolimus, dexamethason en mycofenolaat mofetil verhoogden de capaciteit van Mph2 om ROS te produceren, terwijl rapamycine een remmend effect had. Hoewel de verschillende medicijnen geen effect hadden op de ROS-producerende capaciteit van Mph1, die om te beginnen al laag was, verhoogde dexamethason wel de ROS productie van dendritische cellen. Ook zorgde dexamethason voor een toename in de expressie van p47^{phox} op zowel mRNA als eiwit niveau. Functioneel versterkte dexamethason behandeling van Mph2 de suppressie van de productie van IFN-gamma en IL-4 door T cellen, hoewel er geen effect te zien was op de proliferatie van T cellen. Ten slotte zorgden dexamethason-injecties *in vivo* voor toename van ROS productie en tot verhoogde inductie van regulatoire T cellen, met name in de DA ratten met normaal ROS-producerende capaciteit. Hiermee tonen we een nieuw anti-inflammatoir mechanisme van dexamethason aan.

In **Hoofdstuk 4** onderzochten we de productie van ROS in verschillende Mph2 subsets en bestudeerden de rol van ROS geproduceerd door Mph2 op T cel activatie. Monocyten kunnen op diverse manieren gedifferentieerd worden tot Mph2. Deze Mph2 hebben overeenkomende kenmerken, maar zijn fenotypisch en functioneel toch verschillend van elkaar. In dit hoofdstuk hebben wij gekeken naar Mph2 gedifferentieerd met respectievelijk M-CSF (Mph2), IL-4

(Mph2a) en IL-10 (Mph2c). Mph2 en Mph2c lijken veel op elkaar: hoge CD14 en CD163 expressie, lage DC-SIGN expressie, lage productie van de pro-inflammatoire cytokines IL-6 en IL-12p40, terwijl Mph2a het tegenovergestelde laat zien. Ook functioneel zit er een verschil tussen de drie verschillende macrofagen: Mph2a is de meest potente T cel activator, terwijl Mph2c de slechtste is. De ROS-producerende capaciteit van Mph2 is het hoogst, Mph2c heeft een gemiddelde ROS producerende capaciteit, terwijl Mph2a nauwelijks in staat zijn om ROS te produceren. Om te onderzoeken of er een omgekeerde correlatie was tussen de ROS-producerende capaciteit en de T cel stimulatoire capaciteit, hebben we gebruik gemaakt van de NOX2-remmer DPI. Interessant genoeg zagen we dat DPI de T cel proliferatie en IFN-gamma productie remde. Verder onderzoek toonde aan dat het effect van DPI in de MLR niet op het niveau van de T cel plaats vond, maar juist bij de macrofagen. De interactie tussen de macrofaag en T cel, bepalend voor de activatie van de T cel, vindt plaats op verschillende niveaus waaronder MHC moleculen, co-stimulatoire moleculen en cytokine productie. DPI behandeling van Mph had geen effect op de expressie van MHC class 2 molecuul HLA-DR en ook geen effect op het co-stimulatoire molecuul CD86. Er was echter wel een remmend effect op de productie van de cytokines IL-12p40 en IL-10, maar niet IL-6, wanneer de Mph gestimuleerd werden met CD40L. DPI onderdrukte ook de IL-12p40 en IL-10 productie van Mph2, Mph2a, en Mph2c na LPS stimulatie. Deze remming van de cytokine productie door Mph kan bijdragen aan de verminderde capaciteit om T cellen te activeren. Dus we zien dat ROS een associatie heeft met de cytokine productie, maar meer experimenten zijn nodig om het volledige mechanisme te kunnen ontrafelen.

In **Hoofdstuk 5** hebben we de consequenties onderzocht van NOX2-gemedieerde ROS productie in een experimenteel model van chronische ontsteking in een transplantaat. Hiervoor hebben we een niertransplantatiemodel gebruikt met als donor Lewis ratten en als ontvanger DA.*Ncf1*$^{DA/DA}$ en DA.*Ncf1*$^{E3/E3}$ ratten, die alleen verschillen in de NOX2-producerence capaciteit van hun fagocyten. We waren hierdoor in staat het effect te onderzoeken van ROS op de inflammatoire respons in de getransplanteerde nier. Een week na de transplantatie van de nier werd de contralaterale nier verwijderd, waarna de ratten op dag 30 werden opgeofferd. We zagen dat 4 weken na transplantatie de creatinine en ureum waardes verhoogd waren ten opzichte van de normaalwaardes. Ook waren de albumine waardes in de urine significant verhoogd. Dit bevestigde dat we naar een model keken met een chronisch nierfunctie verlies. Schade in de getransplanteerde nier werd inderdaad bevestigd door op weefsel niveau een verhoogde mate van aankleuring van de schademarker KIM-1 aan te tonen. Daarnaast werd het weefsel gekarakteriseerd door een zeer sterke influx van ontstekingscellen, aangetoond met de leukocytenmarker CD45. Voor al deze parameters was er geen verschil aantoonbaar tussen de ontvanger stammen met een verschillende ROS producerende capaciteit.
Om een beter inzicht te krijgen hebben we ook gekeken naar verschillende

typen infiltrerende cellen. Verschillende subsets van myeloide cellen waren aantoonbaar (aangetoond met antilichamen tegen OX42, ED1, ED2), maar de hoeveelheid aankleuring was niet verschillend. Er was ook geen significant verschil in de hoeveelheid regulatoire T cellen (aangetoond met een antilichaam tegen FoxP3). Wel was er een significant verschil in de T cel aankleuring (R73), waarbij er minder T cellen aanwezig waren in de DA.*Ncf1*$^{E3/E3}$ ratten in vergelijking met DA.*Ncf1*$^{DA/DA}$ ratten. Deze data suggereren een onderdrukkend effect van ROS op T cel infiltratie, terwijl er geen effect op de andere immuuncellen in het transplantaat werd gezien.

Tenslotte wordt in **Hoofdstuk 6** een overzicht gegeven van de studies beschreven in dit proefschrift waarin we verschillende facetten hebben bestudeerd hoe ROS een rol zouden kunnen spelen in de regulatie van een immuunrespons. Daarnaast bespreken we aanvullende gegevens over potentiele moleculaire mechanismen waarmee ROS zowel op eiwit- als op gen niveau hun effecten zouden kunnen bewerkstelligen. We hebben duidelijk aangetoond dat ROS-producerende Mph2 in staat zijn regulatoire T cellen te induceren, en identificeerden een ROS-gemedieerd effect op T cel infiltratie in een niertransplantatiemodel. Naast het feit dat ROS als boodschapper tussen antigeen presenterende celen en T cellen kan fungeren, toonden we ook duidelijk aan dat ROS een belangrijke plaats hebben in de functionele capaciteiten van verschillende Mph subsets. Naast de bestaande gegevens over de belangrijke plaats van ROS in oxidatieve stress en andere pro-inflammatoire processen, hebben de huidige studies nieuwe inzichten opgeleverd over de 'andere kant' van ROS en de betrokkenheid bij immuunregulerende processen. Het verder ontrafelen van deze complexe, en soms tegenstrijdige processen, zal een uitdaging zijn voor de komende periode.

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Curriculum vitae

De schrijver van het proefschrift, Marina Kraaij, werd geboren op 28 december 1984 te Hillegom. In 2003 behaalde zij het VWO diploma aan het Fioretti college te Lisse. In datzelfde jaar begon zij met de studie Biomedische Wetenschappen aan de Universiteit Leiden. Tijdens deze studie verrichtte zij een Bachelor onderzoeksstage op de afdeling Infectieziekten van het LUMC te Leiden onder supervisie van Dr. P.H. Nibbering. Tijdens deze stage deed zij onderzoek naar de antimicrobiele en immuunmodulerende activiteiten van genetische varianten van humaan lactoferrine. De eerste Master onderzoeksstage tijdens deze opleiding verrichtte zij op de afdeling Pathologie van het LUMC te Leiden onder supervisie van Dr. N.F. de Miranda. Tijdens deze stage werd onderzoek gedaan naar de activatie van hypoxia in darmtumoren. De afsluitende Master onderzoeksstage tijdens deze opleiding verrichtte zij op de afdeling Cellular Medicine in de Musculoskeletal Research Group van de Newcastle University te Newcastle onder supervisie van Prof. Dr. J.M. van Laar. Tijdens deze stage werd onderzoek gedaan naar de rol van B-cellen in systemische sclerosis. De studie Biomedische Wetenschappen werd afgesloten in 2008. Aansluitend hierop begon zij in september 2008 aan een Nierstichting-gefinancierd promotieonderzoek getiteld 'Inducing T cell tolerance via macrophage-derived radicals: a radical way to prevent transplant rejection' op de afdeling Nierziekten van het LUMC onder de supervisie van Dr. K.A. Gelderman en Prof. Dr. C. van Kooten. De resultaten van het promotieonderzoek zijn beschreven in dit proefschrift. In 2013 verrichtte zij postdoctoraal onderzoek op de afdeling Inwendige Geneeskunde, sectie Immunologie en Transplantatie, van het Erasmus MC te Rotterdam waar onderzoek werd gedaan naar de rol van monocyten in transplantatie in de onderzoeksgroep van Dr. A.T. Rowshani. Vanaf 2014 verricht zij postdoctoraal onderzoek op de afdeling Infectieziekten en Immunologie, sectie Moleculaire Afweer, op de faculteit Diergeneeskunde van de universiteit Utrecht te Utrecht met als onderwerp de immuunmodulerende mechanismes van de kip host defense peptide cathelicidin-2 in de onderzoeksgroep van Prof. Dr. H.P. Haagsman.

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