ROS-producing macrophages in immune modulation

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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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