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.

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.

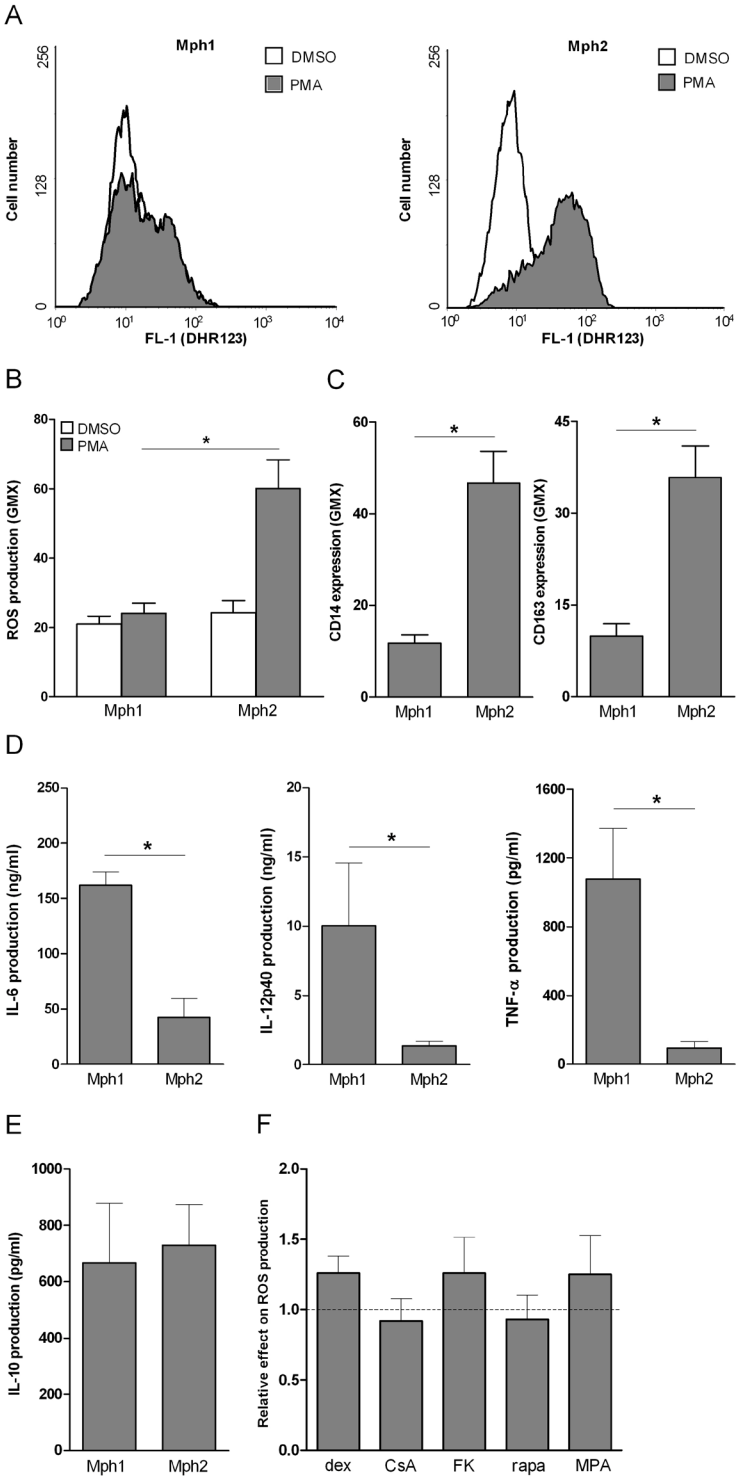
Statistics

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
Cell number
256
128
0
10^0
10^1
10^2
10^3
10^4
FL-1 (DHR123)
Mph2
DMSO
PMA
Cell number
256
128
0
10^0
10^1
10^2
10^3
10^4
FL-1 (DHR123)
B
DMSO
PMA
*
ROS production (GMX)
80
60
40
20
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Mph1
Mph2
C
*
CD14 expression (GMX)
60
40
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Mph1
Mph2
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CD163 expression (GMX)
45
30
15
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IL-6 production (ng/ml)
250
200
150
100
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Mph1
Mph2
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IL-12p40 production (ng/ml)
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Mph1
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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*$^{E3/E3}$

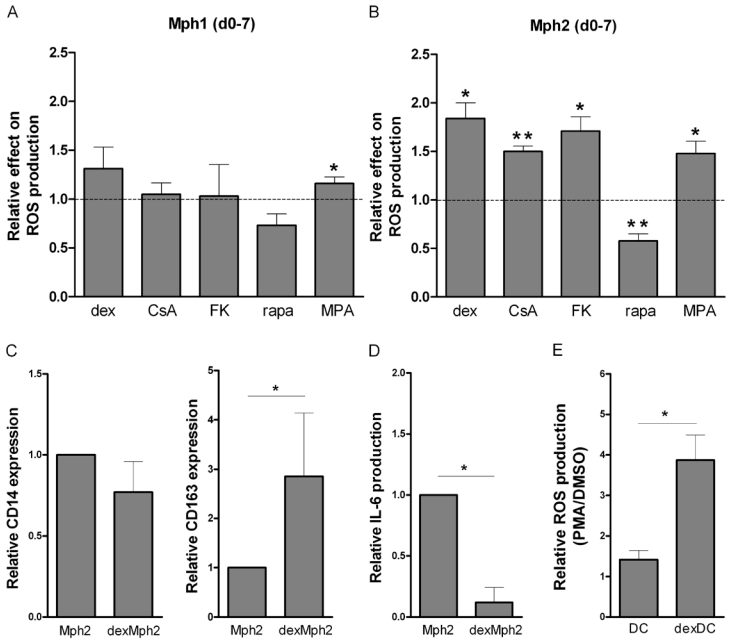

Figure 2: Dex increases ROS producing capacity. (***A***, ***B***) The effects of different immunosuppressive drugs (dex, CsA, FK, rapa, and MPA) during Mph differentiation were tested on ROS producing capacity. ROS production after PMA stimulation for both Mph1 (A) and Mph2 (B) is shown. Figures A and 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.

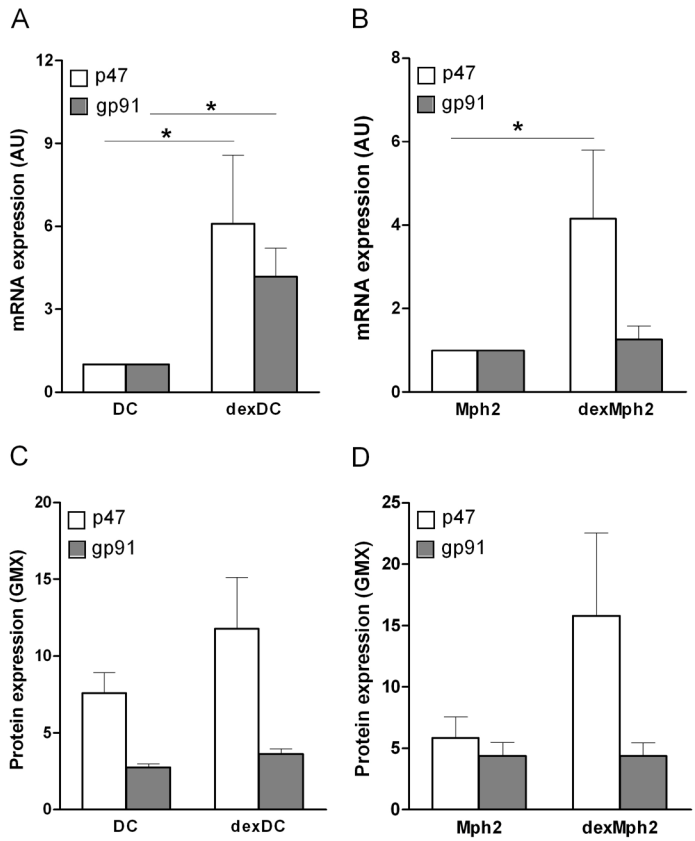

Figure 3: Dexamethasone increases the expression of components of the NOX2 complex. (***A***, ***B***) mRNA levels for p47^{phox} and gp91^{phox} as determined by qPCR. In every experiment, normalized mRNA expression for DC and Mph2 was put on 1.0 and dexDC/dexMph2 expression was related to that value. (***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

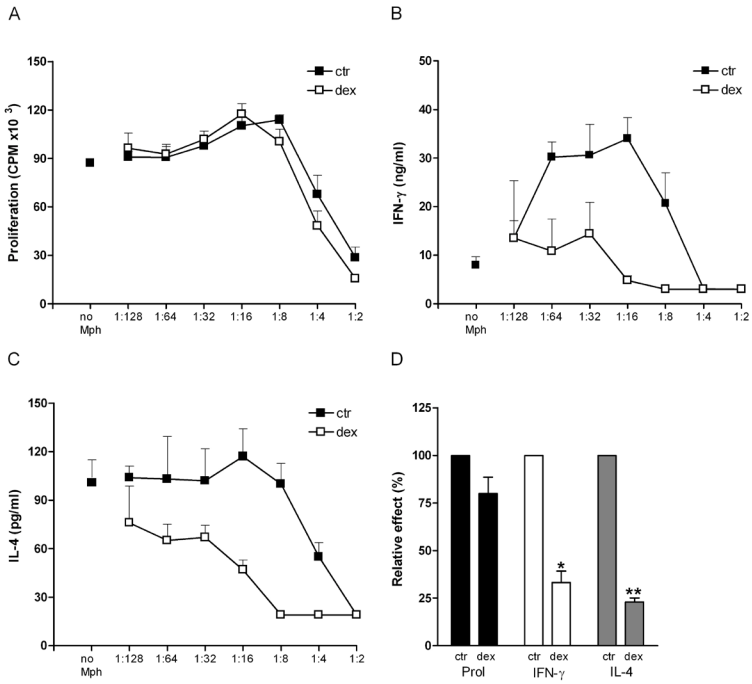

Figure 4: Mph2 differentiated in dex show decreased cytokine response. (***A***, ***B***, ***C***, ***D***) Allogeneic T cells (150,000) were stimulated with anti-CD3/28 Ab in combination with Mph2 differentiated in presence of dex or no drugs, and added in a dose-dependent fashion in ratios as depicted 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

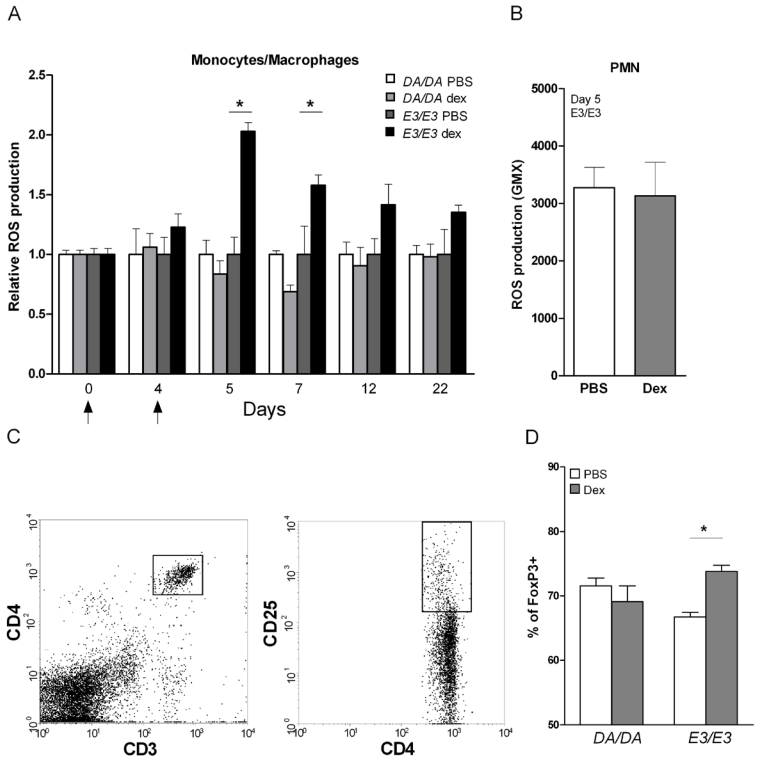

Figure 5: Dex increases ROS producing capacity and ROS dependent Treg induction *in vivo*. (***A***) DA.*Ncf1*$^{DA/DA}$ (DA/DA) rats that express alleles leading to low ROS producing capacity and DA.*Ncf1*$^{E3/E3}$ (E3/E3) rats that express alleles conferring normal levels of ROS production were injected 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.

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