ROS-producing macrophages 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

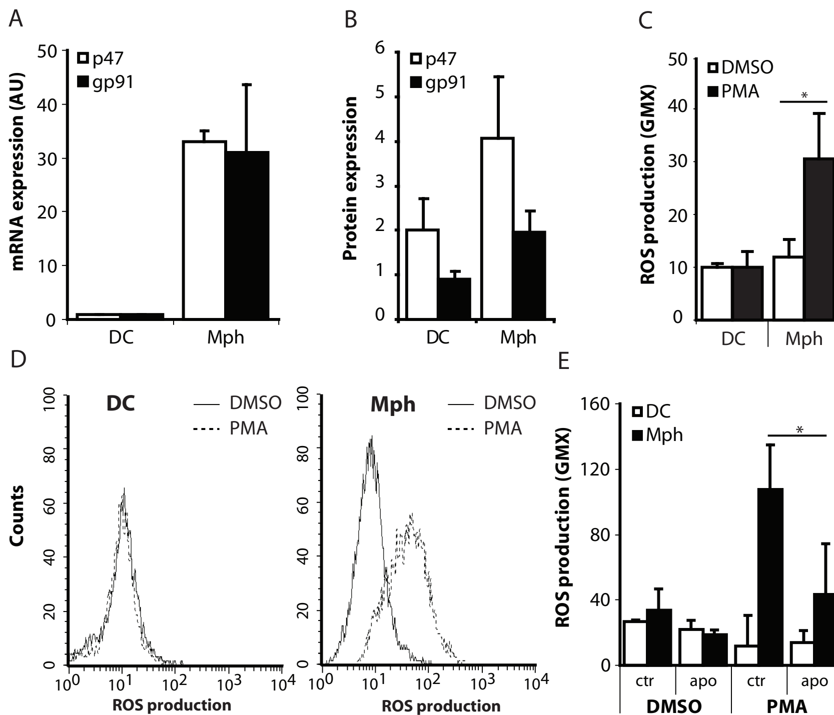

Figure 1: Mph produce ROS. (***A***) mRNA and (***B***) protein expression levels of the two most important NADPH oxidase complex components, p47*phox* and gp91*phox*, determined in DC and Mph by RT-PCR and flowcytometry, respectively. In (A) mRNA expression levels were corrected for GAPDH expression 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

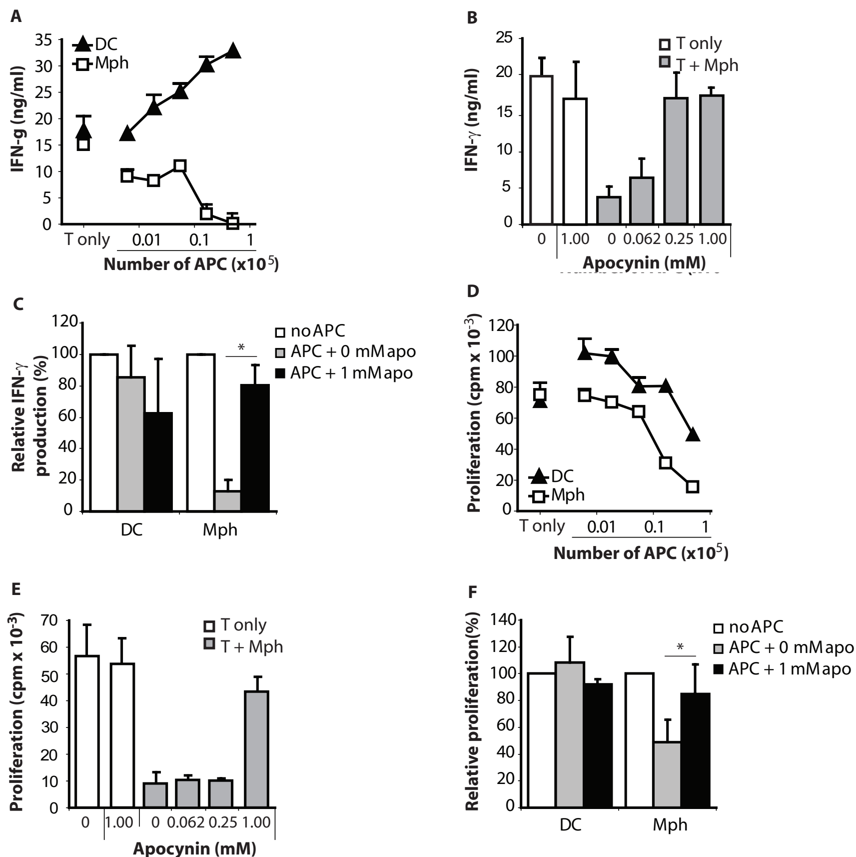

Figure 2: Mph suppress T cell activation in a ROS-dependent fashion. (***A***, ***D***) T cells (150.000) were activated with anti-CD3/28 Ab and Mph or DC were added in increasing numbers (x-axes). After 5 days of coculture IFN-γ production (A) and proliferation (D) were determined by ELISA and 3H thymidine 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).

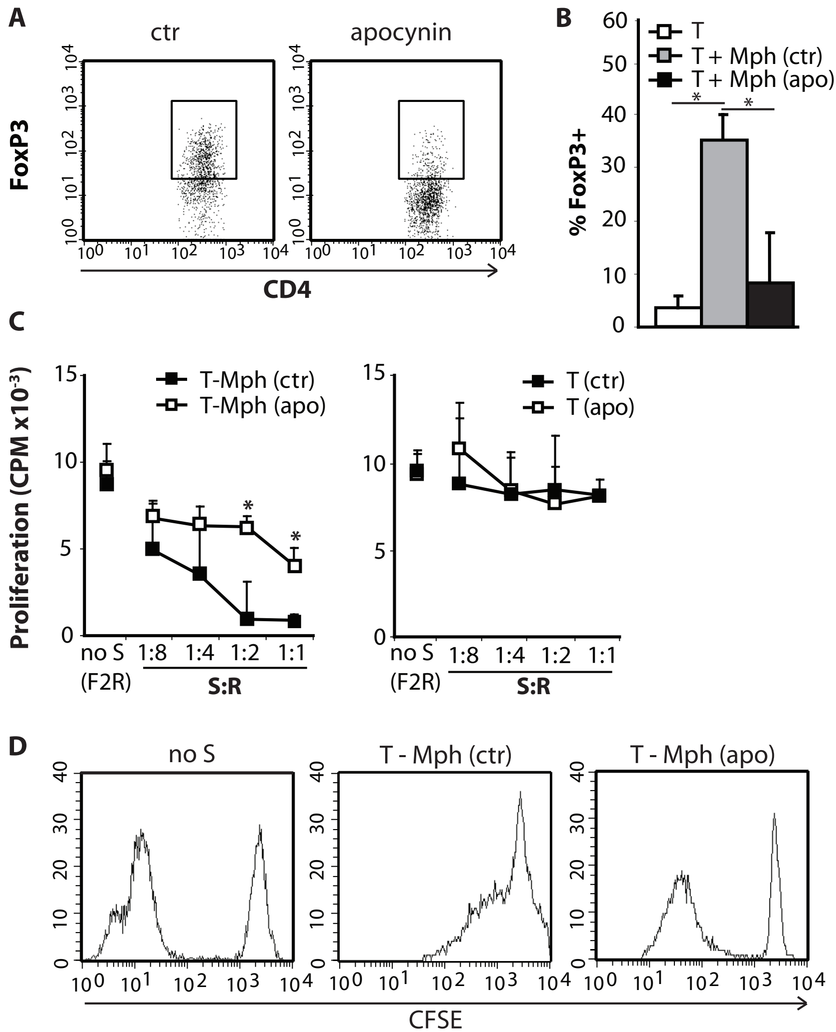

Figure 3: Mph induce Tregs via ROS. (***A***) Expression of FoxP3 within CD3⁺CD4⁺CD25⁺ population of T cells primed with Mph in absence (left) or presence (right) of apocynin. (***B***) Priming of CD4⁺CD25⁻ T cells with Mph in the presence or absence of apocynin for 7 days, followed by the analysis of 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,

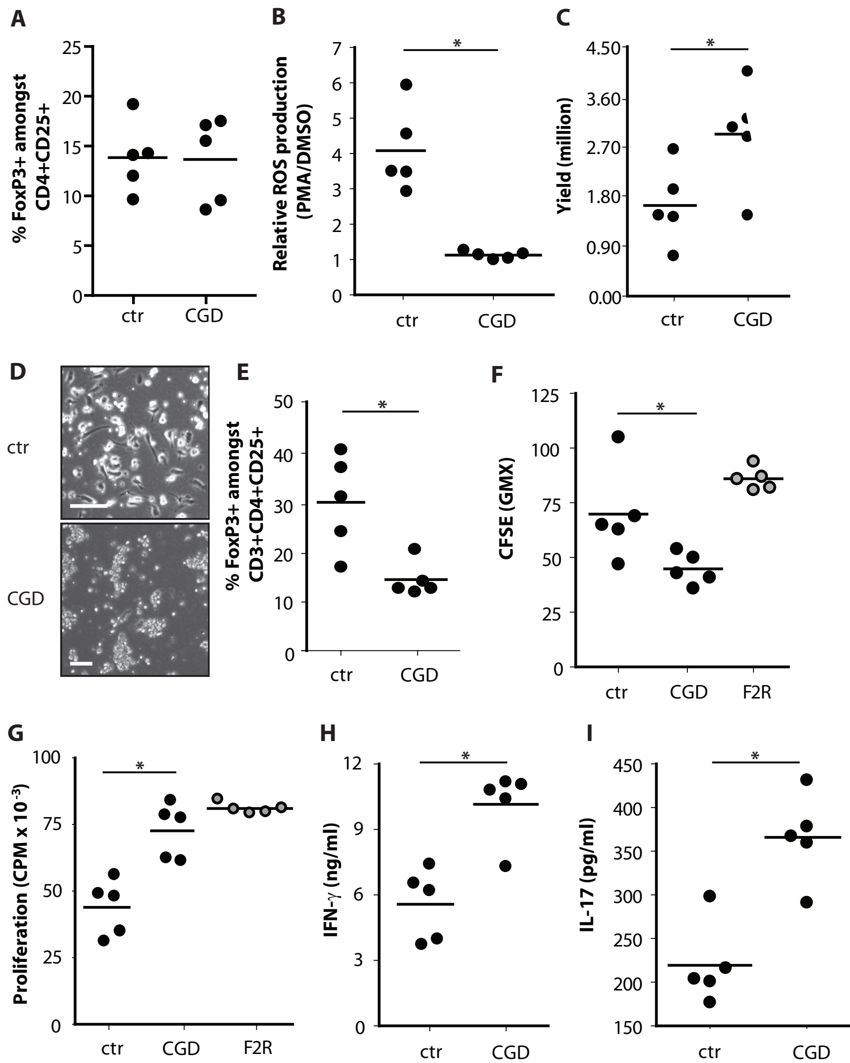

Figure 4: CGD macrophages induce less Tregs. (***A***) The % of FoxP3⁺ cells amongst CD4⁺CD25⁺ in peripheral blood of CGD patients and healthy controls. (***B***) ROS production by Mph differentiated from healthy control or CGD monocytes, as determined by flow cytometry after DHR123 staining. The ratio 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, ^3H 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. * = $p<0.05$

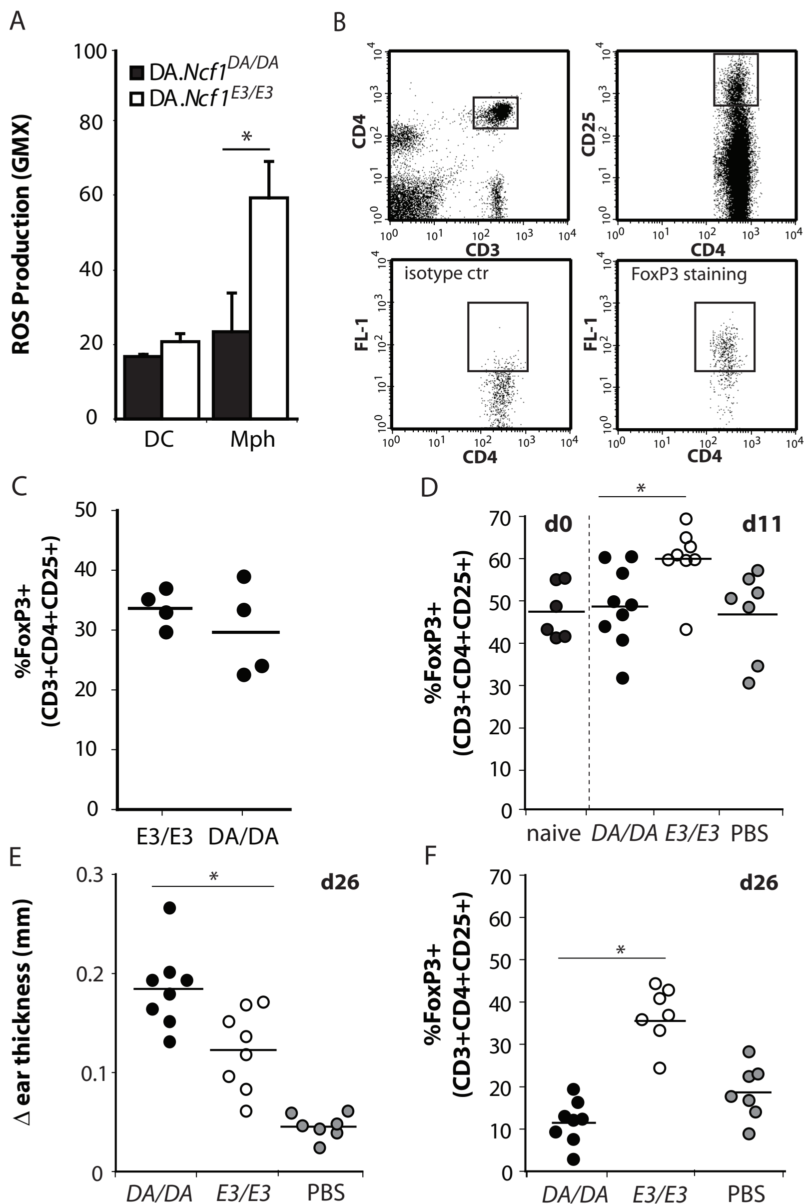

Figure 5: Mph suppress DTH reponses in vivo in a ROS-dependent fashion. (***A***) Rat BM cells were cultured with rat GM-CSF and IL-4 or human M-CSF to obtain DC and Mph, respectively. ROS production by PMA-stimulated DC and Mph generated from DA.*Ncf1*$^{DA/DA}$ or congenic *Ncf1*-wildtype DA.*Ncf1*$^{E3/E3}$ 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*.

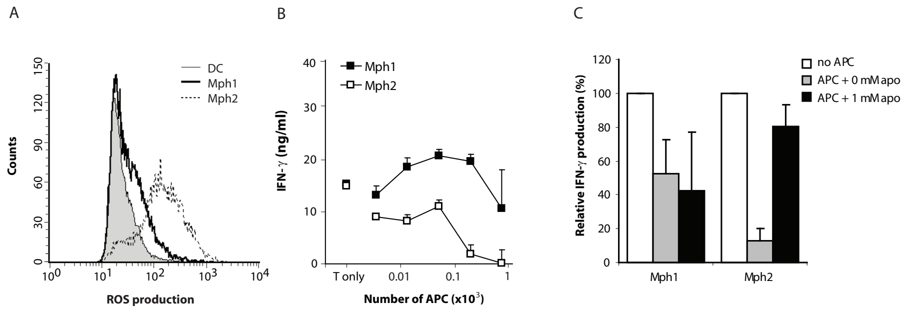

Figure S1: Anti-inflammatory Mph2 but not pro-inflammatory Mph1 suppress T cell activation in a ROS dependent fashion. (***A***) Proinflammatory Mph (Mph1) produce lower levels of ROS as compared to anti-inflammatory Mph2 as measured by DHR123 fluorescence by flow cytometry after stimulation with PMA. (***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

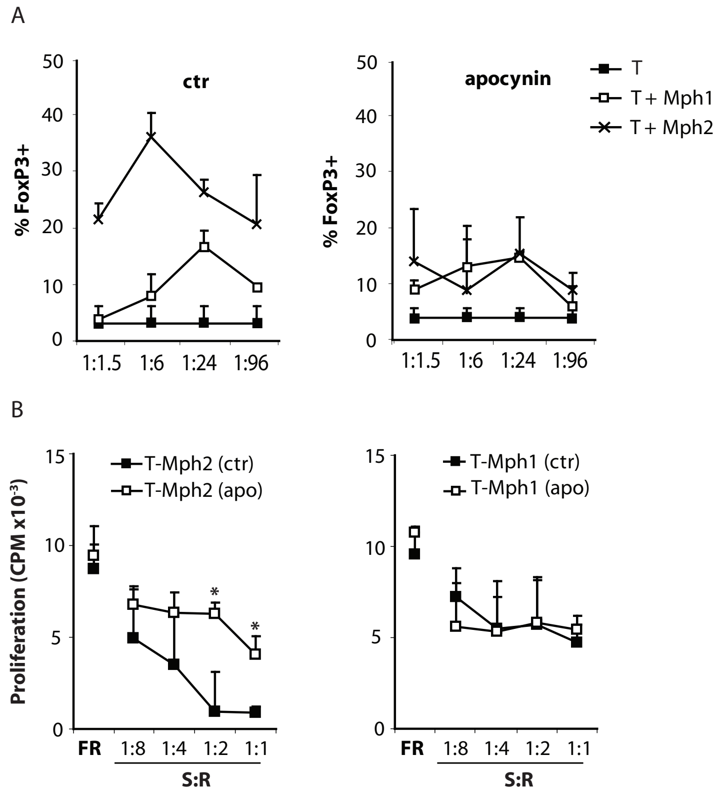

Figure S2: Mph2 but not Mph1 induce Treg in a ROS dependent fashion. (***A***) Priming of $CD4^+CD25^-$ T cells with anti-inflammatory Mph2 but not with pro-inflammatory Mph1 induces FoxP3 expression by these T cells. (***B***) T cells primed with Mph2 but not when primed with Mph1 suppress proliferation 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).

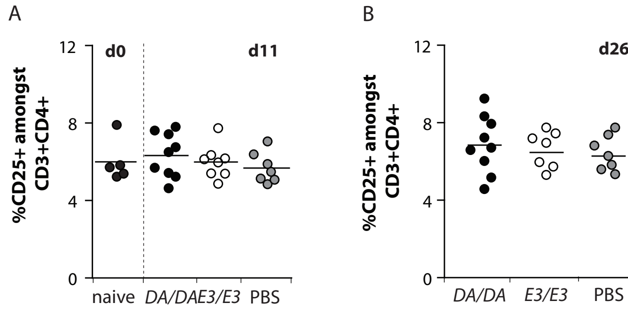

Figure S3: The number of activated T cells during DTH remains similar between groups. (***A***, ***B***) The percentage of CD25+ cells amongst CD3+CD4+ cells 11 days (A) or 26 days (B) after priming the rats with DA.Ncf1DA/DA Mph, DA.Ncf1E3/E3 Mph or PBS is comparable between groups.

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