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Chapter 4

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

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Abstract

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

Introduction

Macrophages (Mph) represent a heterogeneous group of myeloid cells, with a phenotypic and functional specialization and a high plasticity depending on the local cytokine milieu. Mph can be broadly divided into classically activated (Mph1) and alternatively activated macrophages (Mph2), with their cytokine production profile as an important functional difference [1]. Mph1 are characterized by production of pro-inflammatory cytokines like TNF-α and IL-12, whereas Mph2 produces large amounts of IL-10. In addition, Mph2 can be distinguished from Mph1 based on their expression of CD163 and CD206, although different expression levels have been reported depending on the Mph2 subtype [2-5]. For mouse, Mph2 specific markers, like FIZZ1 and Ym1, have been identified both *in vivo* and *in vitro* [6], but establishing specific phenotypic markers for human Mph2 is still an ongoing challenge. CD163 positive staining on resident tissue macrophages has been found dispersed throughout the body, indicating that *in vivo* Mph represent a Mph2 phenotype [7]. Placental Mph also display an Mph2-like phenotype, indicated by the expression of specific Mph2 markers, and their anti-inflammatory phenotype [8;9]. In addition, tumor-associated macrophages (TAMs) also have a phenotype and function similar to Mph2: high expression of CD206, poor antigen presentation, high IL-10, but low IL-12 production [10].

In vitro, Mph1 can be polarized by IFN-γ, LPS, and/or GM-CSF, thereby generating a pro-inflammatory phenotype. In addition, different Mph2 subsets have been described, depending on the different ways of generation, including culture in IL-4/IL-13, immune complexes, or IL-10/TGF-β, generating M2a, M2b, M2c respectively [4;11-14]. Recently, we and other have shown that also generation of Mph in the presence of M-CSF results in cells (Mph2) with

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

Materials and methods

Cell culture

Monocytes were isolated from buffy coats by CD14+ MACS-bead selection (Miltenyi Biotec) [22]. Monocytes were cultured for 7 days in 5 ng/ml M-CSF (R&D systems; Mph2), in 15 ng/ml IL-4 (Biosource; Mph2a), or in 15 ng/ml IL-10 (ITK; Mph2c). Cells were cultured in RPMI with 10% FCS and penicillin/streptomycin (RPMI++) in 6 wells plates with $1.5x10^6$ cell per well; medium and cytokines were refreshed twice. Macrophages were harvested by gentle scraping after short trypsinization.

ELISA

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

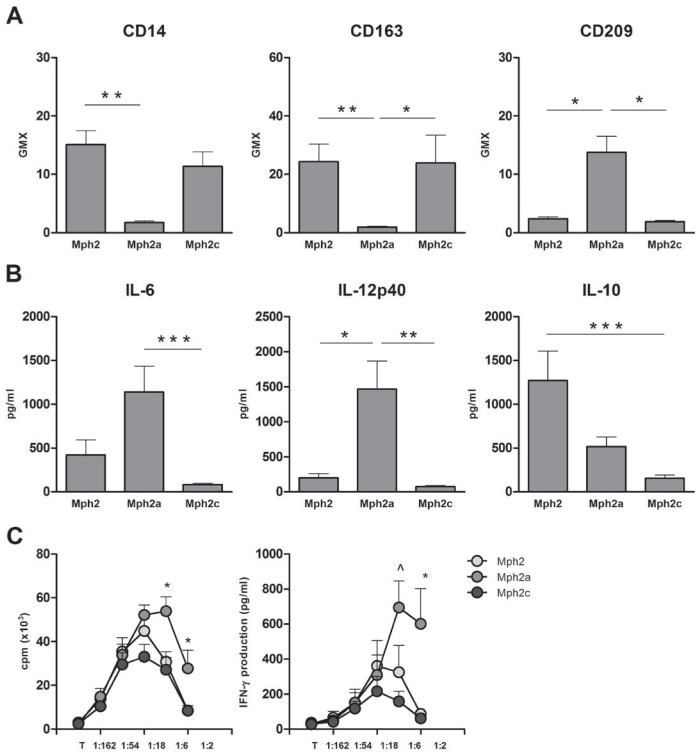

Figure 1: Mph2a are phenotypically and functionally distinct from Mph2 and Mph2c. (***A***) Expression levels of CD14 (left), CD163 (middle), and CD209 (right) for Mph2, Mph2a, and Mph2c as determined by flow cytometry. (***B***) IL-6 (left), IL-12p40 (middle), and IL-10 (right) production were determined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

Flow cytometry

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

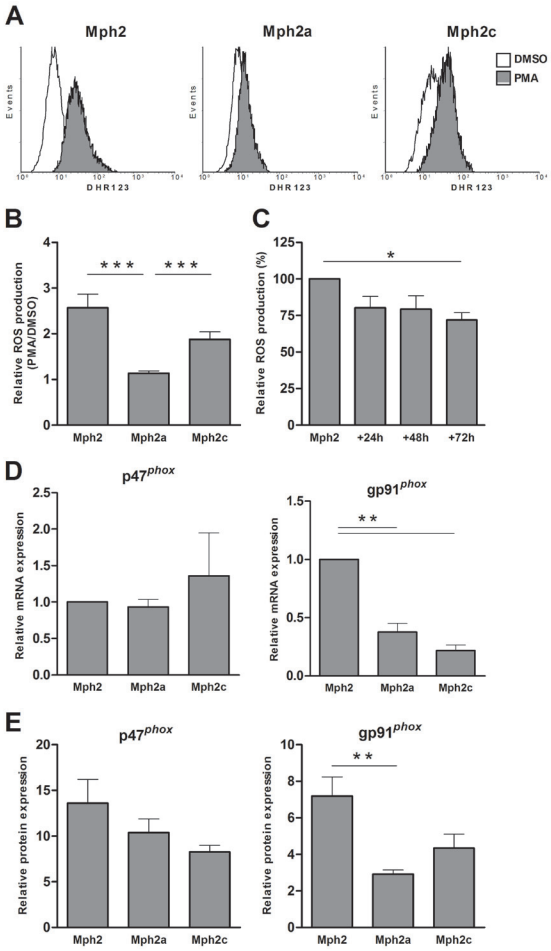

Figure 2: Mph2a have a decreased ROS producing capacity. (***A***) FACS histograms of DHR123 fluorescence after staining of Mph2 (left), Mph2a (middle) and Mph2c (right) to detect ROS production after PMA stimulation (filled histogram) compared to the DMSO control. (***B***) The ratio of ROS production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

Amplex Red Assay

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

T cell proliferation assay

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

L-CD40L cell activation assay

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

mRNA isolation and Quantitative PCR

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

Statistics

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

Results

Mph2a are phenotypically and functionally distinct from Mph2 and Mph2c

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

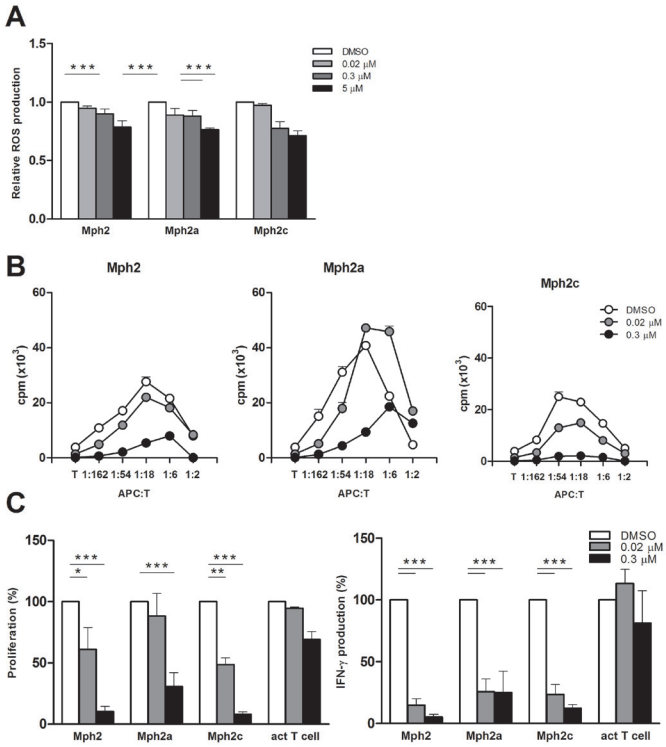

Figure 3: DPI decreases T cell proliferation in an MLR. (***A***) Flow cytometric analysis of the relative ROS producing capacity of Mph2, Mph2a, and Mph2c as induced by PMA stimulation in the presence of increasing concentrations of DPI. Mean and SEM are depicted from 5 independent experiments. (***B***)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

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

Mph2a have a decreased ROS producing capacity

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

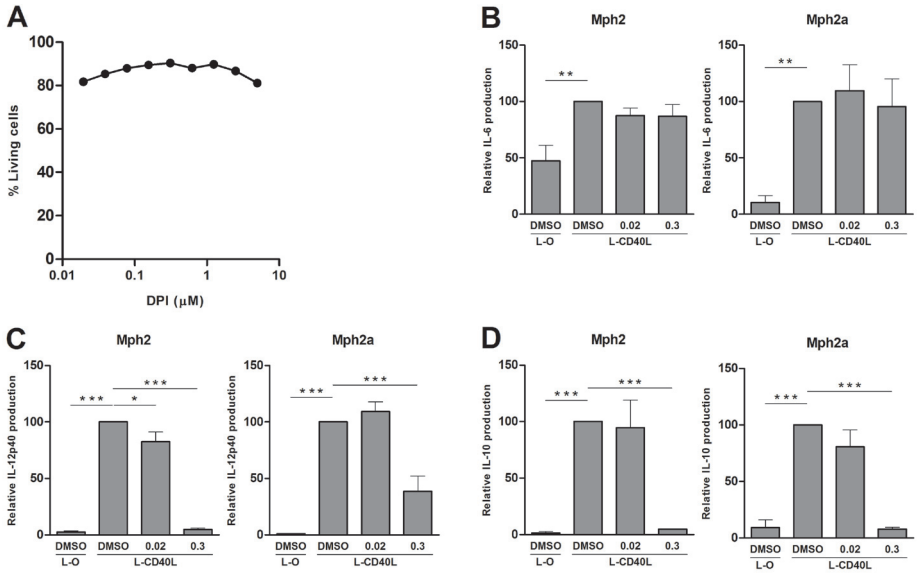

Figure 4: DPI affects IL-12p40 and IL-10 production of the Mph2 subsets. (***A***) Percentage living Mph2 in the presence of different concentrations of DPI as determined with PI staining by flow cytometry. A representative experiment out of 3 is shown. (***B***) Relative IL-6 production of Mph2 (left)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

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

DPI decreases T cell proliferation in an MLR

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

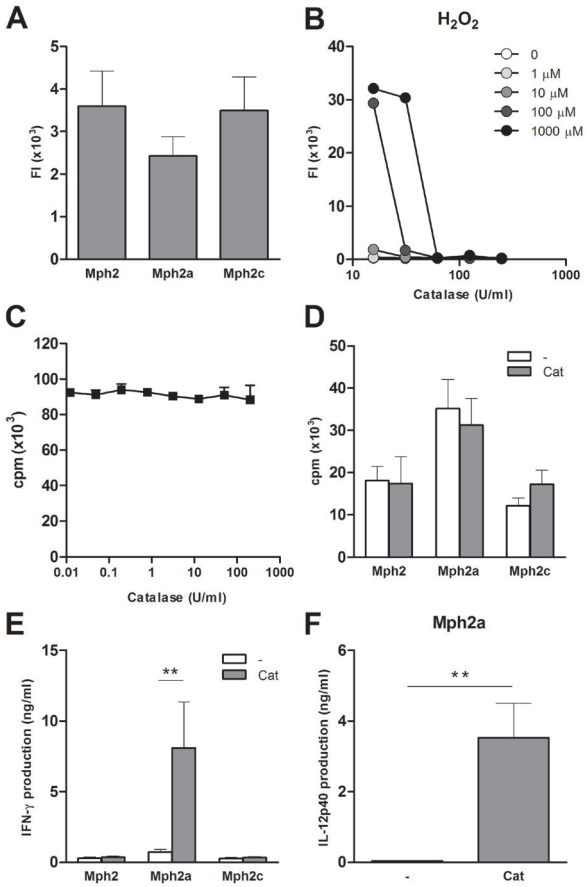

Figure 5: Catalase has no effect on T cell proliferation in an MLR. (***A***) Extracellular hydrogen peroxide levels measured as fluorescence intensity (FI) of Mph2, Mph2a, and Mph2c as determined by AmplexRed assay. (***B***) FI of different concentrations of hydrogen peroxide in the presence of different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

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

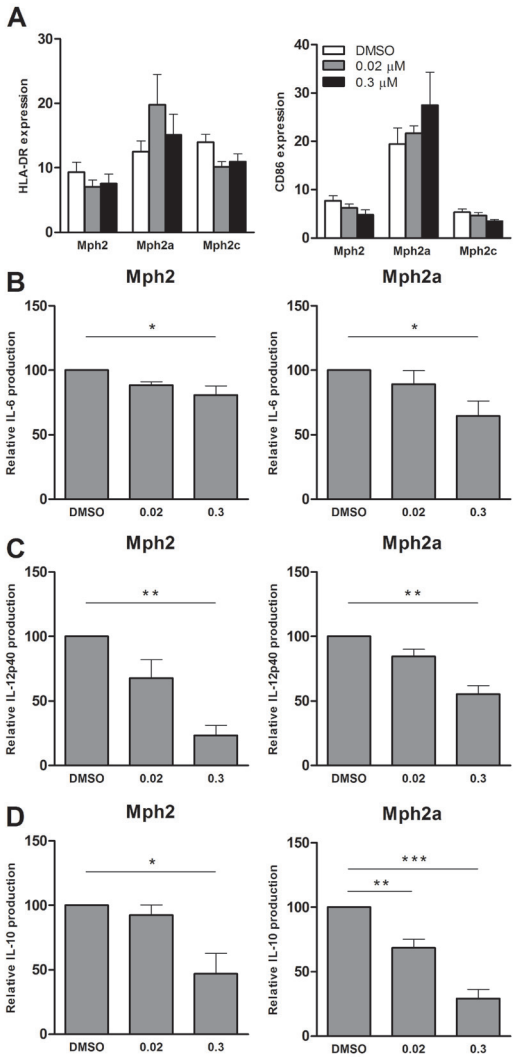

Figure S1: (***A***) HLA-DR (left) and CD86 (right) expression after 48 hours incubation with DPI for Mph2, Mph2a, and Mph2c. (***B***) Relative IL-6 production of Mph2 (left; average production of 1048 pg/ml) and Mph2a (right; average production of 1045 pg/ml) stimulated with LPS (DMSO set at 100) in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

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

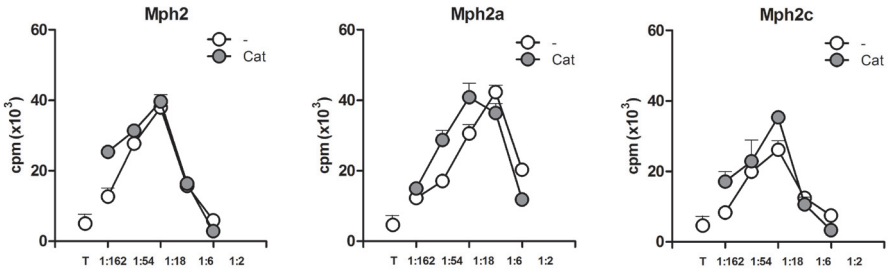

Figure S2: Allogeneic T cells (150,000) were stimulated with respectively Mph2 (left), Mph2a (middle), or Mph2c (right) in the presence of 50 U/ml Cat in different ratios as depicted on the x-axis. A representative experiment is shown. Depicted are averages and SEM of triplicates within this experiment.

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

DPI affects the cytokine production of the Mph2 subsets

We next explored the potential mechanisms how NOX2 might be involved in the T cell stimulatory capacity. DPI up to a concentration of 5 μM, showed no effect on the viability of Mphs (Figure 4A). The stimulation of T cells by APCs requires MHC class II and co-stimulatory molecule activation, as well as cytokine production. However, HLA-DR and CD86 expression on the Mph2 subsets were not affected by DPI (Supplementary Figure 1A).

Therefore we investigated the cytokine production of Mph2 subsets activated by CD40L to mimic the CD40-CD40L signal between APC-T cell as it occurs during the MLR reaction. IL-6 production by Mph2 and Mph2a was increased upon stimulation with L-CD40L cells, but no effect of DPI was observed (Figure 4B). In contrast, the strongly increased production of IL-12p40 (Figure 4C) and IL-10 (Figure 4D), was dose-dependently inhibited by DPI. In LPS-stimulated Mph2 and Mph2a, the IL-6, IL-12p40, and IL-10 production was suppressed by DPI (Supplementary Figure 1B,C,D). Similar trends were observed with the cytokine production of Mph2c (data not shown).

Catalase has no effect on T cell proliferation in an MLR

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

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

Discussion

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

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

Conclusions

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

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