Research Article

The NADPH Oxidase Nox4 Controls Macrophage Polarization in an NFκB-Dependent Manner

V. Helfinger,1 K. Palfi,1 A. Weigert2, and K. Schröder1

1Institute for Cardiovascular Physiology, Goethe-University, Frankfurt, Germany
2Institute for Biochemistry I, Goethe-University, Frankfurt, Germany

Correspondence should be addressed to K. Schröder; schroeder@vrc.uni-frankfurt.de

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The family of NADPH oxidases represents an important source of reactive oxygen species (ROS) within the cell. Nox4 is a special member of this family as it constitutively produces H2O2 and its loss promotes inflammation. A major cellular component of inflammation is the macrophage population, which can be divided into several subpopulations depending on their phenotype, with proinflammatory M(LPS+IFNγ) and wound-healing M(IL4+IL13) macrophages being extremes of the functional spectrum. Whether Nox4 is expressed in macrophages is discussed controversially. Here, we show that macrophages besides a high level of Nox2 indeed express Nox4. As Nox4 contributes to differentiation of many cells, we hypothesize that Nox4 plays a role in determining the polarization and the phenotype of macrophages. In bone marrow-derived monocytes, ex vivo treatment with LPS/IFNγ or IL4/IL13 results in polarization of the cells into M(LPS+IFNγ) or M(IL4+IL13) macrophages, respectively. In this ex vivo setting, Nox4 deficiency reduces M(IL4+IL13) polarization and forces M(LPS+IFNγ). Nox4−/− M(LPS+IFNγ)-polarized macrophages express more Nox2 and produce more superoxide anions than wild type M(LPS+IFNγ)-polarized macrophages. Mechanistically, Nox4 deficiency reduces STAT6 activation and promotes NFκB activity, with the latter being responsible for the higher level of Nox2 in Nox4-deficient M(LPS+IFNγ)-polarized macrophages. According to those findings, in vivo, in a murine inflammation-driven fibrosarcoma model, Nox4 deficiency forces the expression of proinflammatory genes and cytokines, accompanied by an increase in the number of proinflammatory Ly6C+ macrophages in the tumors. Collectively, the data obtained in this study suggest an anti-inflammatory role for Nox4 in macrophages. Nox4 deficiency results in less M(IL4+IL13) polarization and suppression of NFκB activity in monocytes.

1. Introduction

Reactive oxygen species (ROS) regulate a variety of complex cellular processes including angiogenesis, inflammation, differentiation, and proliferation. The family of NADPH oxidases (Nox) consists of 7 members with tissue- and cell type-specific expression profiles. The main function of all family members is a controlled ROS production [1]. Importantly, the NADPH oxidases differ in the type of ROS produced. While Nox2 upon activation produces \( \cdot \mathrm{O}_2^- \), Nox4 is constitutively active and predominantly produces H2O2 [2, 3].

Inflammation and wound healing are processes that strongly depend on the function of macrophages. Macrophages are quite heterogeneous and represent a group of diversely polarized cells from the same monocyte origin [4]. The nomenclature of polarized macrophages has been changed recently. In particular, the M1 and M2 phenotypes have now been replaced by M(LPS+IFNγ) and M(IL4+IL13), respectively, according to the stimulation by cytokines forcing in vitro polarization to one or the other phenotype [5]. We followed this new nomenclature throughout the manuscript.

Nox2 and its product \( \cdot \mathrm{O}_2^- \) promote an M(LPS+IFNγ) phenotype with phagocytic activity and proinflammatory properties [6, 7]. In contrast, in tissue remodeling and wound healing, M(IL4+IL13) polarization of macrophages is characterized by both reduced Nox2 activity and reduced superoxide anion production [8]. H2O2 is a second messenger that enforces the polarization of monocytes to the M(IL4+IL13) phenotype (despite a lower Nox2-dependent
ROS production observed in other studies [9]). Although, there is evidence that Nox4 is expressed in macrophages [10], this is rather inconsistent throughout the literature, leading to the conclusion that Nox4 expression is dynamic over the course of a macrophage life. Nox4 is a major determinant of differentiation of a number of cells, including adipocytes [11] and osteoclasts [12]. Therefore, we hypothesize that Nox4 plays a role in macrophage polarization. With the aid of an in vivo model of tumorigenesis, as well as isolated murine bone marrow and human blood monocytes, we analyzed the contribution of Nox4 in macrophage polarization.

2. Material and Methods

2.1. Material. The following chemicals were used: 3-methylcholanthrene (MCA), NaCl, NH₄Cl, NaHCO₃, Hank’s BSS without Ca²⁺ and Mg²⁺, Trypsin-EDTA solution (T3924) and LPS from Sigma-Aldrich (Munich, Germany), Dulbecco’s PBS (Gibco, Life Technologies, Carlsbad, CA, USA), Hank’s buffer, Sybr Green from Bio-Rad (California, USA), Tris (Carl Roth) NFxB inhibitor #sc-3060 from Santa Cruz (Texas, USA), and GKT 137928 from Genkyotex (Switzerland). IL4, IL13, and IFNγ were purchased from PeproTech (NJ, USA). The following antibodies were used: anti-β-actin (AC-15) from Sigma-Aldrich (Munich, Germany), pSTAT6, STAT6, pSTAT1, and STAT1 from Cell Signaling (Danvers, MA, USA), and p65, β-tubulin, and topoisomerase from Santa Cruz (Texas, USA). YM1 was from Chemicon-Millipore (Darmstadt, Germany), and YY1 was from Bethyl Laboratories (Texas, USA).

2.2. Animals and Animal Procedures. All animal experiments were approved by the local governmental authorities (approval numbers: F28/27 and F28/46) and were performed in accordance with the animal protection guidelines. C57BL/6j and Nox2/- mice were purchased from Jackson Laboratories (Bar Harbor, Maine). Nox4/- mice were generated by targeted deletion of the translation initiation site and of exons 1 and 2 of the Nox4 gene [13] and backcrossed into C57BL/6j for more than 10 generations. Nox1y/- mice, kindly provided by Karl-Heinz Krause and previously characterized, were used for the same experiments [14]. Mice were housed in a specific pathogen-free facility with 12/12 hours day and night cycle and free access to water and chow. All experiments were performed with male mice at the age of 10-12 weeks. To induce fibrosarcomas, the chemical carcinogen MCA was injected subcutaneously into the right flank of the mice. In response to this, tumors were formed within the next three to four months. Once the tumors reached a diameter of 1.5 cm (around 100 days), mice were sacrificed by isoflurane anesthesia and subsequent decapitation. Subsequently, the tumor tissue was processed for biochemical analysis.

2.3. Cell Culture. Cell populations were isolated using the tumor dissociation kit for the mouse and the gentleMACS Dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany), following the manufacturer instructions. Briefly, tumor tissue was homogenized enzymatically, erythrocytes were lysed, and only fibrosarcoma cells were cultured whereas the rest of the cell suspension was only used for FACS analysis. Murine monocytes were isolated from bone marrow by flushing the bones with PBS containing 1% of PenStrep. Cells were filtered (Falcon; #340605, BD) and centrifuged, and erythrocytes were lysed. Erythrocyte depletion buffer contained 155 mM NH₄Cl, 10 mM NaHCO₃, and 100 mM EDTA in double distilled water, pH = 7.4. For isolation of human monocytes, whole blood samples were centrifuged (400 x g for 30 minutes) on a Ficoll gradient (Ricoll separation solution #L6115, Millipore) without brake. In order to force macrophage development, human peripheral blood mononuclear cells (PBMCs) and murine bone marrow-derived monocytes were cultured in Dulbecco’s modified Eagle’s medium (DMEM+glutaMAX) (Gibco, Life Technologies, Carlsbad, CA, USA), supplemented with 10% fetal calf serum (FCS), 1% penicillin (50 U/ml), and streptomycin (50 μg/ml), as well as 20% conditioned medium of L929 cells (contains M-CSF) for one week. Media were changed every 4 days. Before polarization, medium was exchanged to an unsupplemented DMEM/FCS. Polarization to M(LPS+IFNγ) was induced by 1 μg/ml LPS and 100 U/ml IFNγ; and M(IL4+IL13) polarization by IL4 and IL13 100 ng/ml each. After 4 hours, cells were used for nuclear extraction, Western Blot, PCR, or ROS measurements.

2.4. mRNA Isolation and RT-qPCR. Total mRNA from frozen homogenized tissue and isolated cells was obtained with an RNA-Mini-kit (Bio&Sell, Feucht, Germany) according to the manufacturers’ protocol. Random hexamer primers (Promega, Madison, WI, USA) and SuperScript III Reverse Transcriptase (Invitrogen, Darmstadt, Germany) were used for cDNA synthesis. Semi-quantitative real-time PCR was performed with the Mx3500P qPCR cycler (Agilent Technologies, Santa Clara, CA, USA) using the PCR Sybr Green qPCR Mix with ROX (Bio&Sell, Feucht, Germany) and appropriate primers. Relative expression of target genes was normalized to eukaryotic translation elongation factor 2 (EF2), analyzed by the delta-delta-ct method. Primer sequences are listed in Table 1.

2.5. Protein and Western Blot Analysis. For protein isolation, cells were lysed in a buffer containing 20 mM Tris/cl pH 7.5, 150 mM NaCl, 10 mM NaPPγ, 20 mM NaF, 1% Triton, 10 mM okadaic acid (OA), 2 mM orthovanadate (OV), protein inhibitor mix (PIM), and 40 μg/ml phenylmethylsulfonylfluorid (PMSF). Separation of nucleus and cytosol was achieved by lysing the cells in hypotonic buffer (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1% Nonidet, 10 mM DTT, protein inhibitor mix (PIM), and 40 μg/ml phenylmethylsulfonylfluorid (PMSF)). Cells were centrifuged at 17000 g, and the supernatant was collected as the cytosolic fraction. The pellet was further lysed with a hypertonic buffer (20 mM HEPES pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM DTT, protein inhibitor mix (PIM), and 40 μg/ml phenylmethylsulfonylfluorid (PMSF)). After centrifugation at 17000 g, the supernatant contained most soluble nuclear proteins, while membranes, organelles, and DNA were collected in the pellet. Protein
content was determined with the Bradford assay [15]. Samples were boiled in reducing the Laemmli sample buffer and were subjected to SDS-PAGE followed by Western Blotting. After incubation with first antibodies, membranes were analyzed with an infrared-based detection system, using fluorescent dye-conjugated secondary antibodies from LI-COR Biosciences.

2.6. Electrophoretic Mobility Shift Assay. The electrophoretic mobility shift assay (EMSA) was performed according to the manufacturer protocol (LI-COR). Shortly, cells were lysed, and nuclear extract was gained as described above. 5 μg nuclear extract (14 μl including water and sample) was incubated with 2 μl 10x binding buffer (100 mM Tris, 500 mM KCl, and 10 mM DTT; pH 7.5), 1 μl poly(dI·dC) (1 μg/μl in 10 mM Tris and 1 mM EDTA; pH 7.5), 2 μl 25 mM DTT/2.5% Tween® 20 (all components of the Odyssey® EMSA Buffer Kit #829-07910), and 1 μl IRDye® NFκB Oligonucleotide for 30 min in the dark. After that, 10x Orange loading buffer was added, and the total mixture was loaded onto a 4% native polyacrylamide gel. Detection was performed with an Odyssey® Infrared Imaging System at 700 nm.

2.7. ROS Measurements with Chemiluminescence. After polarization, macrophages were dissociated from the plate with Ca²⁺-free EDTA/EGTA (Versene). ROS levels were assessed in intact cells with either L-012 (200 μmol/l) or luminol (100 μmol/l)/horseradish peroxidase (HRP at 1 U/ml) in a Berthold 6-channel luminometer (LB9505, Berthold, Wildbad, Germany). All measurements were performed in the HEPES-Tyrode (HT) buffer containing (in mmol/l) 137 NaCl, 2.7 KCl, 0.5 MgCl₂, 1.8 CaCl₂, 5 glucose, 0.36 NaH₂PO₄, and 10 HEPES. Substances added during the experiment were used as follows: PMA 100 nM, DPI 10 μM, L-NAME 300 μM, PEG-catalase 250 U/ml, and PEG-SOD 50 U/ml.

2.8. Flow Cytometry. Tumor tissue was lysed with the aid of the tumor dissociation kit, mouse (Miltenyi) according to the manufacturer protocol. 3∗10⁶ cells were used for flow cytometry. Cells were pelleted by centrifugation at 500 g and resuspended in 100 μl PBS+0.5% BSA. CD16/32 blocking antibody was added to the cells for 15 minutes subsequently followed by a 15-minute incubation with the prepared mastermix of all antibodies indicated in Table 2. After staining, FACS flow was added; cells were centrifuged and resuspended in FACS flow for measurement. Samples were acquired with a LSRII/Fortessa flow cytometer (BD Biosciences) and analyzed using FlowJo software Vx (Treestar).

2.9. Statistics. All values are displayed as mean ± SEM. Statistical analysis was performed by ANOVA followed by LSD post hoc testing or by the t test if appropriate. Densitometry was performed with the aid of the Odyssey software. A p value of less than 0.05 was considered statistically significant.

3. Results

3.1. Nox4 Deficiency Promotes Inflammation in Murine Fibrosarcomas. In a murine fibrosarcoma model, the absence of Nox4 forces tumor growth [16]. Simultaneously, mRNA abundance of proinflammatory cytokines such as IL1β and TNFα and other markers of inflammation, such as ICAM-1, was elevated (Figure 1(a)). Accordingly, IL1β and TNFα

| Gene    | Forward (5’ to 3’) | Reverse (5’ to 3’) |
|---------|--------------------|--------------------|
| m TNFα  | CGGACTAGTGCTCTCCA   | CTCCAGCTGGAGACTCTCCCAG |
| m IL1β  | GACCTCCAGGATGAGCATGAG | GGTGGTGTGCGCTTTTCATTAC |
| m ICAM-1| TGACCTGGAGGTGCAACT  | GCGCTAGTGGGCTGCCCCG |
| m iNOS  | TGAAGAAACCCCTGTGCT | TTCTGTGCTGTCCCAGTGA |
| m YM1   | CGTAAATTGGTGGCCCTCTAG | TTCATACACCAACCTCACTT |
| m FIZZ1 | GCACTCGTGTGCTTATCTC | AGAAGCAGGGATATGGGCA |
| m ARG1  | GACAGGGCTCCTTTACAGAGA | CTTGGAGAGAGAGGCTT |
| m Nox2  | GTGACCACTATGAGAGGGA  | TTCGAAAGCGTCTTGAAGCC |
| m Nox1  | CGCTCCCAGCAGAGGGTGATTACCAAAGG | GGAGTACCCCAATCCCCTGCCCACCA |
| m Nox4  | TGCTGGGCGCTAAGTGTGT | AGGGACCTCTTGACCTCG |
| h Nox1  | TTCACAATCTCCAGGATGGTAGTGGTCT | GACCTGTCAGTCAGTGGCCCCTGTGTC |
| h Nox4  | CGACGTAGCTGGGCTGCCAACAGAAG | GTTAGCATAGGAATAGCACCACCACCACATGCAG |
| h iNOS  | GACCTGGGACCCCGCACACT | AGGTATGTCGACGGCTGGA |
| h TNFα  | TGAGAAGGGTGGACCGCTC  | TCTCCACAGGGAATGTATC |
| h IL1β  | CGTACAGCTAGCTGAAGCTGC | CACCATCTGTTGCTCATACT |
| h ARG1  | TTCCTAAAGGGAGACGCCAG | AGCACCTGGCTATCTCC |
| h MRC1  | GGAATGAGTGAAGCCACGTG | CTGTCGCCCCAGTATCCATC |
| h TGM2  | TTCAGGTTACAACCTAGGGCTGTC | TATTCGACTACCCACCTGAG |

Table 1: Primer sequences used.
were elevated, when measured with an ELISA or a cytometric bead assay, respectively. In contrast, the anti-inflammatory cytokine IL10 was strongly reduced in tumors of Nox4-deficient mice (Figure 1(b)). These data point towards a more severe inflammation in tumors of Nox4−/− mice. However, the total number of immune cells per tissue was similar in wild type and Nox4−/− mice (Supplemental Figure 1) as measured by flow cytometry. Therefore, we analyzed the number of proinflammatory macrophages, identified by high expression of the surface marker Ly6C [17], and found a substantial increase in Ly6C+ monocytes in the tumors of Nox4−/− mice (Figure 1(c)). When we further analyzed the tumor tissue for pro- and anti-inflammatory markers, we observed a trend towards more inflammation, together with lower expression of markers typical for M(IL4+IL13)-polarized macrophages (Figures 3(a) and 3(b)). Surprisingly, the absence of Nox4 further increased ROS formation in M(LPS+IFNγ)-polarized macrophages (Figures 3(a) and 3(b)). A major source of ROS in M(LPS+IFNγ)-polarized macrophages is Nox2, whose expression was elevated in Nox4-deficient macrophages (Figure 1(b)). These data point towards a specific increase of proinflammatory macrophages in the absence of Nox4, favoring the polarization of macrophages towards a proinflammatory phenotype, which was further investigated.

3.2. Loss of Nox4 Promotes M(LPS+IFNγ) Polarization of Macrophages. Human and murine macrophages were generated and analyzed for the expression of individual NADPH oxidases. As expected, Nox2 expression was the highest in both macrophage populations, followed by Nox4 and Nox1 (Supplemental Figure 3). In order to analyze if Nox4 influences macrophage polarization, we isolated monocytes from bone marrow of wild type and Nox4-deficient mice, challenged them (with M-CSF) to become macrophages, and eventually polarized them to either M(LPS+IFNγ) or M(IL4+IL13) phenotype. Nox4 knockout promoted the expression of M(LPS+IFNγ) macrophage markers including TNFα and IL1β (Figure 2(a)), whereas typical M(IL4+IL13) markers were significantly downregulated (Figure 2(b)). This effect was mediated by H2O2, the product of Nox4: external H2O2 or increased intracellular H2O2 formation via PMA-activated induction of Nox2 and conversion of the resulting -O2− into H2O2 by SOD induced M(IL4+IL13) polarization. Depletion of H2O2 by catalase forces the expression of M(LPS+IFNγ) markers, both without any further treatment with cytokines (Supplemental Figure 4). Exemplary verification of the PCR results on the protein level revealed the same effect for the M(IL4+IL13) marker Ym1 (Figure 2(c)). STAT6 is one of the main transcription factors involved in the expression of M(IL4+IL13) markers. In line with the decreased level of M(IL4+IL13) markers in Nox4−/− cells, phosphorylation of STAT6 was attenuated (Figure 2(d)). In order to analyze whether or not the effects seen are specific for Nox4, macrophage polarization was determined in Nox2- and Nox1-deficient macrophages as well. In contrast to Nox4−/− macrophages, loss of Nox2 induced a small but significant reduction in M(LPS+IFNγ) polarization with no effect on M(IL4+IL13) polarization or STAT6 phosphorylation (Supplemental Figure 5). Knockout of Nox1 had no effect on macrophage polarization, compared to wild type littermates (Supplemental Figure 6).

3.3. Formation of Reactive Oxygen Species upon M(LPS+IFNγ) Polarization Is Elevated in the Absence of Nox4. Several publications indicate that polarization of macrophages is dependent on ROS production and simultaneously forces ROS formation [18]. Polarization of macrophages towards the proinflammatory M(LPS+IFNγ) phenotype resulted in an increase in superoxide anion as well as in hydrogen peroxide production compared to M(IL4+IL13)-polarized macrophages (Figures 3(a) and 3(b)). Surprisingly, the absence of Nox4 further increased ROS formation in M(LPS+IFNγ)-polarized macrophages (Figures 3(a) and 3(b)). A major source of ROS in M(LPS+IFNγ)-polarized macrophages is Nox2, whose expression was elevated in Nox4-deficient M(LPS+IFNγ)-polarized macrophages (Figure 3(c)). Accordingly, when measuring -O2− in a more specific way with the aid of L-012 in intact cells, we found that both LPS and IFNγ separately increase the level of -O2− production in macrophages as well as the combination of both (Figure 3(d)). Knockout of Nox2 in macrophages completely abolished L-012 detectable -O2− formation (Figure 3(e)). In conclusion, the increase in Nox2 expression, which predominantly produces -O2− over H2O2, indicates that Nox2 is the major source of ROS in M(LPS+IFNγ)-polarized macrophages.

3.4. Nox4 Mediates the Proinflammatory Macrophage Polarization via Activation of NFκB. Inflammation is often associated with an increased activity of NFκB [19]. Indeed, TNFα and IL1β as well as ICAM-1 and Nox2 are target genes of NFκB. Therefore, we analyzed the potential role of Nox4 in NFκB activation in the course of macrophage polarization. M(LPS+IFNγ) polarization was accompanied by an increased translocation of p65 from the cytosol to the nucleus in the Nox4-deficient macrophages when compared to wild type cells (Figures 4(a) and 4(b)). However, nuclear translocation alone is not sufficient as the indicator of a transcription factor activity. In order to test for both, NFκB nuclear translocation and DNA binding activity, an electro mobility shift assay (EMSA) was utilized. Activity of NFκB was enhanced in M(LPS+IFNγ) macrophages in the absence...
of Nox4 (Figure 4(c)). In M(IL4+IL13)-polarized macrophages, no such effect of Nox4 was observed.

3.5. Activated NFκB Promotes Nox2 Expression in the Absence of Nox4. NFκB is one of the transcription factors that control Nox2 expression. We therefore hypothesized that elevated activation of NFκB in the absence of Nox4 promotes Nox2 expression during macrophage M(LPS+IFNγ) polarization. The upregulation of Nox2 however is not accompanied by an elevated expression of its cytosolic subunits or antioxidative enzymes such as SOD1 or 3 in wild type vs. Nox4-/- cells (Supplemental Figure 7). Treatment of the cells with an NFκB inhibitor prevented the increase in p65 nuclear translocation (Supplemental Figure 8), and Nox2 expression was reduced in Nox4-/- macrophages to the level similar to that of the wild type, when cells were pretreated with the NFκB inhibitor prior to M(LPS+IFNγ) polarization (Figure 4(d)). NFκB acts in concert with other transcription factors to regulate the expression of Nox2 [20]. One of which is the redox-sensitive zinc-finger transcription factor Yin Yang 1 (YY1), which directly controls the activity of NFκB [21]. As such, YY1 represents a potential target of Nox4-derived ROS, which is upstream of NFκB and controls Nox2 expression. A significant increase in the YY1 protein level was observed in M(LPS+IFNγ)-polarized Nox4-/- macrophages; which was not the case for M(IL4+IL13)-polarized macrophages (Supplemental Figure 9A). Inhibition of Nox4 with GKT137928 in Nox2-deficient macrophages results in a small but significant increase in LPS and IFNγ-induced M(LPS+IFNγ) polarization. Under basal conditions, treatment with GKT only increased iNOS, compared to DMSO-treated samples. Those results indicate that inhibition of Nox4 favors M(LPS+IFNγ) polarization even in the absence of Nox2 (Supplemental Figure 10). The interpretation of this result could be that NFκB even in the absence of Nox2 promotes M(LPS+IFNγ) polarization in macrophages. Although many studies provide evidence for the involvement of NFκB in macrophage polarization, the exact role of NFκB and its effects besides induction of Nox2 are unclear. Therefore, investigation of how NFκB triggers M(LPS+IFNγ) polarization in the absence of Nox2 would be worth a second study. Another transcription factor involved in M(LPS+IFNγ) polarization is STAT1 [22]. We therefore checked for a potential effect of Nox4 on phosphorylation of STAT1 in M(LPS+IFNγ) polarization without observing any effect of Nox4 (Supplemental Figure 9B). Thus, Nox4 appears to selectively regulate the activity of NFκB and potentially YY1. In conclusion, the absence of Nox4 promotes Nox2 expression and subsequently M(LPS+IFNγ) polarization of macrophages.
3.6. Pharmacological Inhibition of Nox4 Promotes M(LPS+IFNγ) Polarization of Human Macrophages. In order to determine whether our findings in a mouse can be translated to human cells, human macrophages generated from peripheral blood of healthy donors were analyzed. Inhibition of Nox4 was achieved by treatment of the cells with the Nox1/Nox4 inhibitor GKT137928. Upon treatment of the macrophages with GKT137928, an increased M(LPS+IFNγ) polarization was observed. This was accompanied by a decrease in M(IL4+IL13) polarization (Figure 5). As shown above in the murine system, knockout of Nox1 has no influence on macrophage polarization. Therefore, we assume that usage of the inhibitor will mainly affect Nox4-mediated signaling in the process of polarization. We conclude that the findings in the murine system also apply to the human system.

4. Discussion

Macrophages are a heterogeneous population of cells. Generally, they can be categorized into two discrete subsets as either classically activated M1 or alternatively activated M2. The specific M(LPS+IFNγ) markers IL1β, TNFα, and iNOS (a) and specific M(IL4+IL13) markers arginase 1, YM1, and FIZZ1 (b) were quantified by RT-qPCR after stimulation with cytokines polarizing the bone marrow-derived macrophages from WT and Nox4-/- mice to M(LPS+IFNγ) or M(IL4+IL13) phenotype. Protein expression of the M(IL4+IL13) marker YM1 (c) and the ratio of pSTAT6 to STAT6 (d) as determined by Western Blot; *p < 0.05 WT vs. Nox4-/- and #p < 0.05 WT/Nox4-/- M(LPS+IFNγ) vs. WT/Nox4-/- M(IL4+IL13), n = 5-6.

Figure 2: Nox4 knockout leads to a decreased M(IL4+IL13) polarization of macrophages. The specific M(LPS+IFNγ) markers IL1β, TNFα, and iNOS (a) and specific M(IL4+IL13) markers arginase 1, YM1, and FIZZ1 (b) were quantified by RT-qPCR after stimulation with cytokines polarizing the bone marrow-derived macrophages from WT and Nox4-/- mice to M(LPS+IFNγ) or M(IL4+IL13) phenotype. Protein expression of the M(IL4+IL13) marker YM1 (c) and the ratio of pSTAT6 to STAT6 (d) as determined by Western Blot; *p < 0.05 WT vs. Nox4-/- and #p < 0.05 WT/Nox4-/- M(LPS+IFNγ) vs. WT/Nox4-/- M(IL4+IL13), n = 5-6.
M2 macrophages, herein referred to as M(LPS+IFNγ) or M(IL4+IL13). In this context, M(LPS+IFNγ) macrophages represent proinflammatory “killers,” while M(IL4+IL13) macrophages serve as “builders” in inflammatory wound repair. This polarization of the macrophage population results from interactions with other cells or molecules within WT Nox4-/-. The figures show ROS measurements revealing increased ROS production in Nox4-deficient cells due to an increase in Nox2. Superoxide anion production measured with L-012 (a) and hydrogen peroxide levels measured with luminol and HRP (b) in polarized macrophages of wild type and Nox4 knockout mice. (c) RT-qPCR for Nox2 mRNA expression in polarized macrophages of WT and Nox4-deficient animals; *p < 0.05 (n = 3–8). (d) Superoxide anion production, as measured with L-012 in WT macrophages with or without LPS (10 µg/ml) and IFNγ (100 U/ml) directly after stimulation or 4 hours after addition. (e) Superoxide anion production in polarized WT and Nox2-deficient macrophages; *p < 0.05 WT vs. Nox4-/- or treated vs. CTL and #p < 0.05 WT/Nox4-/- M(LPS+IFNγ) vs. WT/Nox4-/- M(IL4+IL13) (n = 3–5).

**Figure 3**: ROS measurements reveal increased ROS production in Nox4-deficient cells due to an increase in Nox2. Superoxide anion production measured with L-012 (a) and hydrogen peroxide levels measured with luminol and HRP (b) in polarized macrophages of wild type and Nox4 knockout mice. (c) RT-qPCR for Nox2 mRNA expression in polarized macrophages of WT and Nox4-deficient animals; *p < 0.05 (n = 3–8). (d) Superoxide anion production, as measured with L-012 in WT macrophages with or without LPS (10 µg/ml) and IFNγ (100 U/ml) directly after stimulation or 4 hours after addition. (e) Superoxide anion production in polarized WT and Nox2-deficient macrophages; *p < 0.05 WT vs. Nox4-/- or treated vs. CTL and #p < 0.05 WT/Nox4-/- M(LPS+IFNγ) vs. WT/Nox4-/- M(IL4+IL13) (n = 3–5).
In previous work, we found that knock-out of the NADPH oxidase Nox4 enhances inflammation and tumorigenesis [16, 24]. In an angiotensin II-induced model of vascular dysfunction, loss of Nox4 promoted the expression of the proinflammatory cytokines IL6 and IL1β [12]. The present study underlines the protective potential of Nox4 in inflammation, as it promotes M(IL4+IL13) polarization of macrophages. Our results were confirmed in a very recent study in a myocardial infarction model, where overexpression of Nox4 promoted M(IL4+IL13) polarization of cardiac macrophages and protects from postinfarction remodeling [25].

The balance between activation of STAT1 and STAT3/STAT6 plays a crucial role in macrophage polarization: a predominance of STAT1 activation promotes M(LPS+IFNγ), while STAT3/STAT6 activation increases M(IL4+IL13) macrophage polarization [26]. In fact, STAT6 is the most important transcription factor regulating M(IL4+IL13) polarization of macrophages [27], and phosphorylation of STAT6 can be regulated by redox-sensitive phosphatases [28]. Therefore, it is likely that Nox4-derived H2O2 at least contributes to STAT6 phosphorylation and thereby to M(IL4+IL13) polarization. Importantly, STAT6 suppresses NFκB activation via Klf4. Here, we provide evidence that Nox4 deficiency prevents STAT6 phosphorylation and supports NFκB activation. NFκB has been shown to promote M(LPS+IFNγ) polarization of phagocytes [29]. Besides regulation by STAT6/Klf4, the activity of NFκB is redox sensitive and potentially regulated by NADPH oxidases [30]. Both, increased NFκB and reduced phosphorylation of STAT6,

Figure 4: Increased NFκB activation in M(LPS+IFNγ)-polarized macrophages of Nox4-/- is responsible for elevated Nox2 expression. Translocation of p65 was analyzed by Western Blot in the cytosol (a) and nuclear fraction (b) of M(LPS+IFNγ)- and M(IL4+IL13)-polarized macrophages of WT and Nox4-/- mice. (c) Electrophoretic mobility shift assay for NFκB was performed in M(LPS+IFNγ)-polarized macrophages of WT and Nox4-/- animals. The left bar graph shows quantification, and the right bar graph representative shift. (d) Nox2 mRNA expression was quantified by RT-qPCR after M(LPS+IFNγ) polarization with and without an NFκB inhibitor (30 ng/ml, 1 h pretreatment before polarization); *p < 0.05 WT vs. Nox4-/- and #p < 0.05 CTL vs. NFκB inhibitor (n = 3-8). TOPO: topoisomerase I.
Therefore, our data can be supported by a superoxide anion to hydrogen peroxide. Consequently, less H$_2$O$_2$ in isolated murine (A) and human (B) macrophages, and Nox4-/- mice were analyzed for cell composition with FACS using specific antibodies for cells indicated. The table contains the different T cell populations in cells/g tumor tissue, no statistical differences ($n = 6-10$).
and polarization markers ARG1, FIZZ1, and YM1 were quantified with RT-qPCR; \( \cdot p < 0.05 (n = 6) \). (B) WT macrophages were treated with basal medium or LPS and IFN\( \gamma \) or PEG-catalase (500 U/ml) for 4 h to polarize, followed by subsequent analysis of polarization markers TNF\( \alpha \), IL1\( \beta \), and iNOS; \( \cdot p < 0.05 (n = 3) \). Supplemental Figure 5: Nox2 knockdown decreases M(LPS+IFN\( \gamma \)) polarization of macrophages. The specific M(LPS+IFN\( \gamma \)) markers IL1\( \beta \), TNF\( \alpha \), and iNOS (A) and specific M(IL4+IL13) markers arginase 1, YM1, and FIZZ1 (B) were quantified by RT-qPCR after stimulation with cytokines polarizing the bone marrow-derived macrophages from Nox2KO/C57Bl6J mice to M(LPS+IFN\( \gamma \)) or M2(IL4+IL13) phenotype. Protein expression of the M(IL4+IL13) marker YM1 (C) and the ratio of pSTAT6 to STAT6 as determined by Western Blot; \( \cdot p < 0.05 \) and \( \# p < 0.05 \) WT/Nox2y/- M(LPS+IFN\( \gamma \)) vs. WT/Nox2y/- M(IL4+IL13) (n = 4 – 8). Supplemental Figure 6: deficiency in Nox1 does not affect polarization of macrophages. The specific M(LPS+IFN\( \gamma \)) markers IL1\( \beta \), TNF\( \alpha \), and iNOS (A) and specific M(IL4+IL13) markers arginase 1, YM1, and FIZZ1 (B) were quantified by RT-qPCR after stimulation with cytokines polarizing the bone marrow-derived macrophages from Nox1KO/C57Bl6J mice to M(LPS+IFN\( \gamma \)) or M2(IL4+IL13) phenotype. Protein expression of the M(IL4+IL13) marker YM1 (C) and the ratio of pSTAT6 to STAT6 (D) as determined by Western Blot; \( \cdot p < 0.05 \) and \( \# p < 0.05 \) WT/Nox1y/- M(LPS+IFN\( \gamma \)) vs. WT/Nox1y/- M(IL4+IL13) (n = 3 – 6). Supplemental Figure 7: SOD and Nox2 cytosolic subunit expressions in WT and Nox4-deficient macrophages. SOD1 (A) and SOD3 (B) expressions were analyzed in WT and Nox4-/- M(LPS+IFN\( \gamma \))- and M(IL4+IL13)-polarized macrophages by RT-qPCR. Expressions of cytosolic Nox2 subunits (C: p40phox, D: p47phox, and E: p67phox) and Nox1 (F) were analyzed in WT and Nox4-/- M(LPS+IFN\( \gamma \))- and M(IL4+IL13)-polarized macrophages using RT-qPCR; \( \cdot p < 0.05 \) WT/Nox4-/- M(LPS+IFN\( \gamma \)) vs. WT/Nox4-/- M(IL4+IL13) (n = 5–8). Supplemental Figure 8: NFkB inhibition prevents p65 translocation into the nucleus in M(LPS+IFN\( \gamma \))-polarized macrophages of Nox4-/-; P65 expression in cytosol (A) and nucleus (B) was assessed with Western Blot after M(LPS+IFN\( \gamma \)) polarization with and without treatment of NFkB inhibitor (30 ng/ml, 1 h pretreatment before M(LPS+IFN\( \gamma \)) polarization); \( \cdot p < 0.05 \) WT vs. Nox4-/- and \# p < 0.05 CTL vs. NFkB inhibitor (n = 3–8). TOPO: topoisomerase I. Supplemental Figure 9: YY1 is increased in Nox4-deficient macrophages after M(LPS+IFN\( \gamma \)) polarization. (A) YY1 expression was determined by Western Blot after polarization of WT and Nox4-/- macrophages. (B) Phosphorylation of pSTAT1 and total STAT1 quantified by Western Blot in M(LPS+IFN\( \gamma \))- and M(IL4+IL13)-polarized macrophages of WT and Nox4-deficient animals; \( \cdot p < 0.05 \) WT vs. Nox4-/- and \# p < 0.05 WT/Nox4-/- M(LPS+IFN\( \gamma \)) vs. WT/Nox4-/- M(IL4+IL13) (n = 3–5). Supplemental Figure 10: inhibition of Nox4 in Nox2-deficient macrophages elevates M(LPS+IFN\( \gamma \)) polarization in M(LPS+IFN\( \gamma \))-polarized macrophages. Nox2-deficient macrophages were treated with Nox4 inhibitor GKT (10 \( \mu \)M) 2 h prior to cell polarization to M(LPS+IFN\( \gamma \)) or only control medium (CTRL). M(LPS+IFN\( \gamma \)) markers TNF\( \alpha \), IL1\( \beta \), and iNOS were evaluated using RT-qPCR; \( \cdot p < 0.05 \) DMSO vs. GKT and \# p < 0.05 DMSO/GKT CTRL vs. DMSO/GKT M(LPS+IFN\( \gamma \)) (n = 3). (Supplementary Materials)

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