NADPH oxidases are essential for macrophage differentiation

Qing Xu†, Swati Choksi†, Jianhui Qu*, Jonathan Jang*, Moran Choe*, Botond Banfi†, John F. Engelhardt† and Zheng-gang Liu*

*Center for Cancer Research, National Cancer Institute, National Institutes of Health, 37 Convent Drive, Bethesda, MD 20892; †Department of Anatomy and Cell Biology, University of Iowa, Room 1-111 Bowen Science Building, 51 Newton Road, Iowa City, Iowa 52242-1109

Running Title: NOX1 and NOX2 in macrophage differentiation

# To whom correspondence should be addressed: Zheng-gang Liu, Center for Cancer Research, NCI, NIH, Bldg37, Rm1130, 37 Convent Dr., Bethesda, MD 20892, USA Tel: 301-435-6351; Fax: 301-402-1997; E-mail: zgliu@helix.nih.gov

† These authors contributed equally to this work.

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Abstract

NADPH oxidases (NOXs) are involved in inflammation, angiogenesis, tumor growth and osteoclast differentiation. However, the role of NOX1 and NOX2 in macrophage differentiation and tumor progression is still elusive. Here, we report that NOX1 and NOX2 are critical for the differentiation of monocytes to macrophages, the polarization of M2-type, but not M1-type, macrophages and the occurrence of tumor associated macrophages (TAMs). We found that the deletion of both NOX1 and NOX2 led to the dramatic decrease of ROS production in macrophages and resulted in an impaired efficiency in monocyte to macrophage differentiation and M2-type macrophages polarization. We further showed that NOX1 and NOX2 were critical for the activation of MAP kinases JNK and ERK during macrophage differentiation and that the deficiency of JNK and ERK activation was responsible for the failure of monocyte to macrophage differentiation and in turn affecting M2 macrophage polarization. Furthermore, we demonstrated that the decrease in M2 macrophages and TAMs, concomitant with the reduction of cytokines and chemokines secretion, contributed to the delay in wound healing and the inhibition of tumor growth and metastasis in NOX1/2 double knockout mice compared to WT mice. Collectively, these data provide direct evidence that NOX1 and NOX2 deficiency impairs macrophage differentiation and the occurrence of M2-type TAMs during tumor development.

Introduction

The NADPH oxidases (NOXs) are enzymes specifically dedicated to the production of reactive oxygen species (ROS) and their activation leads to the generation of superoxide (O2–). To date, seven NOX isoforms NOX1, NOX2, NOX3, NOX4, NOX5, Duox1, and Duox2 have been identified. These NOX enzymes participate in a wide range of cellular processes including apoptosis, host defense, cellular signal transduction, oxygen sensing, and angiogenesis (1). While NOXs can be detected in many types of cells, the expression of different NOX isoforms is cell- or tissue-specific (2) allowing each NOX a distinct physiological and pathological function (3). NOX-produced superoxide is rapidly converted to hydrogen peroxide, which potentially regulates the target molecules through reversible or irreversible oxidation of redox-sensitive cysteine residues. For instance, NOX2-mediated ROS production plays an essential role in non-specific host defense against pathogens and NOX2 deficiency is responsible for the disorder, chronic granulomatous disease (CGD) in humans (4) and CGD-like immune defect in mice (5). It has been shown that NOX1-mediated ROS production regulates RANKL signaling and is required for BMDM differentiation into osteoclast (6). It has also been found that NOX4 overexpression-induced ROS upregulation promotes ovarian cancer growth and angiogenesis (7).

Macrophages play a role in the regulation of innate immune responses to acute and chronic
inflammation (8). Deregulation of macrophage differentiation may lead to defects in wound healing and diseases including autoimmune disorders and cancer (9). Circulating monocytes are versatile precursors with the ability to differentiate into a variety of macrophages (10). Stimulated monocytes activate pro-survival pathways, migrate to tissues and differentiate into macrophages (10). Granulocyte-macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF) induce monocyte-macrophage lineage differentiation in vivo and in vitro. Studies have shown that many signaling pathways are activated by GM-CSF or M-CSF. Specifically, the three mitogen-activated protein kinases (MAPKs), ERK, JNK and p38, are known to be activated by GM-CSF or M-CSF treatment and have been implicated in monocyte/macrophage differentiation (11,12). Our previous studies showed that JNK and ERK activation by GM-CSF or M-CSF are critical for monocyte survival and differentiation (13,14).

GM-CSF or M-CSF-differentiated macrophages can be further polarized to more specialized cells in response to additional stimuli (15). When M-CSF-differentiated macrophages are activated with bacterial products and Th1 cytokines such as lipopolysaccharide (LPS) and interferon-γ (INF-γ), they are polarized to classically activated macrophages, also known as M1 macrophages, which have immune-stimulatory properties and cytotoxic function against tumor cells (16). However, when M-CSF-differentiated macrophages are activated by Th2 cytokines such as IL-4 or immune-suppressors such as IL-10, they become alternatively activated or M2 macrophages, which have low cytotoxic function but high tissue-remodeling activity (16). Our previous work found that ROS are crucial for the differentiation of M2-type macrophages (13), but whether NOX enzymes are involved in monocytes to macrophage differentiation remains unclear. In addition, tumor associated macrophages (TAMs), abundantly found in the tumor microenvironment, play a critical role in promoting tumor progression and metastasis (17,18). However, the role of NOX enzymes in TAMs occurrence, tumor growth and metastasis had not been delineated. In the present study, we used the NOX1-KO, NOX2-KO and NOX1/2 double knockout mice (19) to study the role of NOXs in macrophage differentiation. We showed that ROS generation in M-CSF-treated monocytes was largely blocked by the deletion of both NOX1 and NOX2, but not by the single gene deletion of either NOX1 or NOX2. The deficiency of ROS production then led to the loss of ERK and JNK activation and resulted in the impaired differentiation of monocytes to macrophages and the polarization of M2 macrophages. The polarization of M1 macrophages is normal in NOX1/2-double knockout mice. In a wound healing model, NOX1/2-double knockout mice had less infiltration of M2-type macrophages to the wound edge and delayed wound healing compared to that in wild type mice. More importantly, in a LLC (Lewis lung carcinoma) tumor model, NOX1/2-DKO mice had significantly lower numbers of TAMs, reduced tumor growth and fewer metastatic lung foci as compared to wild type mice.

Results

Deletion of NOX1 and NOX2 resulted in the loss of ROS production during macrophage differentiation—To evaluate the expression levels of NOX isoforms in mouse bone marrow monocytes (BMMs) and bone marrow derived macrophages (BMDMs), we isolated BMMs from C57BL/6 wild type (WT), NOX1-knockout, NOX2-knockout and NOX1/2-double knockout mice (from here on refer to WT, NOX1-KO, NOX2-KO and NOX1/2-DKO). No difference was detected in the percentage, number, cell viability and phenotype of bone marrow monocytes (BMMs) from all four groups (data not shown and supplemental Fig. S1). BMMs were treated with M-CSF for 6 days to differentiate into BMDMs. Total RNA collected from BMMs and BMDMs was subjected to real-time PCR assay for measuring the expression levels of different NOX isoforms. We found that NOX1 and NOX2 are the main isotypes expressed in both BMMs and BMDMs (Fig. 1A). Interestingly, low levels of expression of NOX4 and Duox1 were also detectable in BMMs and BMDMs. The expression levels of the other NOX isoforms are not affected in NOX1-KO, NOX2-KO and NOX1/2-DKO mice (Fig. 1B). To address whether M-CSF treatment resulted in different ROS levels, we measured ROS levels in BMMs from WT, NOX1-KO, NOX2-KO and NOX1/2-DKO mice. BMMs were treated with M-CSF for varying times and the ROS positive BMMs populations of WT, NOX1-KO, NOX2-KO and NOX1/2-DKO cells were measured. NOX1/2-DKO cells had significantly lower ROS positive population as compared to wild type (Fig. 1C). Both NOX1-KO and NOX2-
KO cells had similar ROS positive populations as the wild type did. We then measured the NOX activity by measuring superoxide generation in M-CSF treated BMMs from WT, NOX1-KO, NOX2-KO and NOX1/2-DKO mice. The basal NOX activity in NOX1/2-DKO cells was significantly lower than that measured in WT cells and the M-CSF-induced NOX activity was almost completely lost in the NOX1/2-DKO cells (Fig. 1D). Meanwhile, loss of NOX1 or NOX2 had only partial effect on the basal and M-CSF-induced NOX activity (supplemental Fig. S2). These data suggest that NOX1 and NOX2 are predominately responsible for ROS production in monocytes treated with M-CSF.

**Loss of NOX1 and NOX2 affects the differentiation of monocytes to macrophages and the polarization of M2 macrophages**—To investigate the role of NOX1 and NOX2 on macrophage differentiation, BMMs from WT, NOX1-KO, NOX2-KO and NOX1/2-DKO mice were first differentiated into BMDMs and then further perturbed to generate M1 and M2 populations. All groups of BMMs differentiated normally when treated with M-CSF except the NOX1/2-DKO monocytes, which appear more rounded and less differentiated (Fig. 2A). There was no obvious difference in cell viability after 6 days of M-CSF-induced differentiation among all four groups of monocytes (supplemental Fig. S1). However, FACS analysis showed that the F4/80+ BMDM population is significantly lower from NOX1/2-DKO mice compared with that from WT, NOX1-KO and NOX2-KO mice, suggesting that NOX1/2 depletion affects the differentiation of monocytes to F4/80+ macrophages (supplemental Fig. S3). The BMDMs were further polarized to M1 macrophages by treatment with LPS and INF-γ or M2 macrophages when treated with IL-4. The M1 population was similar among WT, NOX1-KO and NOX2-KO BMDMs and the NOX1/2-DKO BMDMs were only slightly less differentiated. However, the M2 population from NOX1/2-DKO but not NOX1-KO or NOX2-KO mice were substantially reduced compared with that from WT (Fig. 2, A and B). No significant difference in the expression of the M1-specific marker iNOS and cytokines (TNF-α and IL-12p40) (18) were observed between the four groups of M1 polarized BMDMs (Fig. 2C and supplemental Fig. S4A). However, the expression levels of the M2-specific marker Arginase 1 and chemokines (mCCL17, mCCL24) (20) were significantly decreased in NOX1/2-DKO BMDMs, but not in NOX1-KO or NOX2-KO M2 polarized BMDMs when compared to WT BMDMs (Fig. 2C and supplemental Fig. S4A). Furthermore, we isolated peritoneal macrophage from untreated 8-week old WT and NOX1/2-DKO mice. Most of macrophages from WT and NOX1/2-DKO mice are viable although there is a slight increase of cell death in the macrophages of NOX1/2-DKO mice (supplemental Fig. S5A). However, the macrophages from NOX1/2-DKO mice were less differentiated compared to those from WT mice (supplemental Fig. S5B). Next, the peritoneal macrophages were polarized to M1 and M2 macrophages. The M2, but not M1, population polarized from NOX1/2-DKO macrophages was significantly smaller compared with that from WT macrophages (Fig. 2, D and E). Collectively, these results indicated that NOX1 and NOX2 double knockout, but not NOX1 or NOX2 single knockout, biased M-CSF dependent differentiation of monocytes to macrophages resulting in the diminished polarization to M2 macrophages, but not M1 macrophages.

To examine whether NOX1 and NOX2 affect the polarization of M2 macrophages in vivo, WT, NOX1-KO, NOX2-KO and NOX1/2-DKO mice were given intraperitoneal treatment of thioglycollate (TG) or combination of IL-4 and anti-IL-4 antibody (referred to as IL-4c) (21) to induce the accumulation of M1- or M2 macrophages to the peritoneal cavity, respectively. Peritoneal macrophages were collected 4 days after treatment and were analyzed by flow cytometry for M1 or M2 populations. Consistent with our in vitro observations, thioglycollate treatment resulted in no significant difference in M1 population when analyzed by flow cytometry and western blot for M1-specific marker, iNOS (Fig. 2F and supplemental Fig. S4B). The percentage of the RELMs which is a cell surface marker of M2 macrophages (16), positive population in IL-4c treated peritoneal macrophages was similar in WT, NOX1-KO, and NOX2-KO mice, but was dramatically decreased in NOX1/2-DKO mice (Fig. 2F). Expression of the M2 marker, Arginase 1, was similar in IL-4c treated peritoneal macrophages from WT, NOX1-KO, and NOX2-KO but was decreased in NOX1/2-DKO mice (Fig. 2F and supplemental Fig. S4B). While the expression levels of M1 cytokines in peritoneal macrophages of thioglycollate-treated WT, NOX1-KO, NOX2-KO and NOX1/2-DKO mice were similar, however, the expression amounts of M2-specific chemokines (mCCL17, mCCL24) in peritoneal
macrophages of IL-4c-treated NOX1/2-DKO mice were dramatically decreased compared to that in WT, NOX1-KO, and NOX2-KO mice (Fig. 2G). These results indicated that NOX1 and NOX2 are also required for the polarization of M2 macrophages in vivo.

Activation of MAPKs, ERK and JNK, is impaired in M-CSF-treated NOX1/2-DKO cells—Our previous studies showed that BHA (butylated hydroxyanisole), a ROS scavenger reagent, can significantly block M2, but not M1, macrophage polarization (13). ROS-mediated activation of MAP kinases, ERK and JNK, is involved in macrophage differentiation (22). Since we found that loss of NOX1 and NOX2 results in the decrease of ROS generation and affects macrophage differentiation and M2 macrophage polarization, we then investigated whether NOX1 and NOX2 are required for ERK and JNK activation during macrophage differentiation and polarization. Activation of ERK and JNK was measured with specific antibodies against phosphorylated ERK and JNK. As shown in Fig. 3A, the activation of ERK and JNK was significantly reduced in M-CSF-treated NOX1/2-DKO monocytes when compared to WT cells. Meanwhile, there is no detectable decrease of ERK and JNK activation in M-CSF treated NOX1-KO or NOX2-KO monocytes. Next, we found that when macrophages were polarized to M1 macrophages, LPS/INF-γ treatment induced ERK and JNK activation in all four types of cells including NOX1/2-DKO indicating that ERK and JNK were activated by LPS/INF-γ treatment during M1 polarization (Fig. 3B, left panel). However, ERK and JNK were not activated during M2 polarization with IL-4 treatment in all four types of cells (Fig. 3B, right panel). These data suggest that ERK and JNK activation in NOX1/2-DKO macrophage was restored during M1 polarization with LPS/INF-γ treatment. Previous reports indicated that Stat6 is activated by IL-4 treatment and is important for M2 polarization (23,24). We found that the Stat6 activation was similar in WT, NOX1-KO, and NOX2-KO, but was severely impaired in NOX1/2-DKO (Fig. 3B, right panel). Collectively, we found that ERK and JNK were activated during monocyte to macrophage differentiation and that NOX1/2 are required for the activation of these kinases. Importantly, the activation of these kinases by LPS/INF-γ treatment may overcome the defect of monocyte to macrophage differentiation to allow the cells to polarize to M1 macrophages.

In our earlier study, we found that while both ERK and JNK are involved in monocyte to macrophage differentiation, ERK activation is more critical for differentiation and JNK activation is essential for cell survival (13,14). The important role of ERK activation in monocyte to macrophage differentiation is supported by the experiments with ERK inhibitor, U0126. Pretreating WT BMM with U0126 on day 0 before differentiation with M-CSF significantly blocked monocyte to macrophage differentiation (Fig. 3C and supplemental Fig. S6), however, these cells were able to polarize to M1, but not M2 macrophages (Fig. 3D). LPS/INF-γ-induced ERK activation is also critical for M1 polarization since treating macrophages with U0126 on day 6 blocked M1 polarization (supplemental Fig. S7, A and B). Because the complete inhibition of JNK activation with JNK inhibitor leads to massive cell death during monocyte to macrophage differentiation, we did not evaluate the role of JNK in the process with JNK inhibitor. These experiments further support our conclusions that ERK is critical for monocyte to macrophage differentiation and that the loss of ERK activation in the differentiation process is only a burden for M2, but not M1 polarization.

Thus, our data indicated that NOX1/2 deletion attenuated ERK and JNK activation resulting in insufficient monocyte to macrophage differentiation. This ERK and JNK activation in NOX1/2-DKO macrophages can be rescued by LPS/INF-γ treatment. The defect of monocyte to macrophage differentiation only affects M2, but not M1, polarization.

Loss of NOX1 and NOX2 has no effect on macrophage inflammatory response—To compare the inflammatory response between WT and NOX1/2-DKO macrophages, lipopolysaccharide (LPS)-primed BMDMs were stimulated with nigericin, ATP, poly A:T or salmonella typhimurium. Both WT and NOX1/2-DKO had similar upregulated levels of IL-1β secretion and caspase-1 activation upon these treatments (Fig. 4, A and B). Similar to previous studies in NADPH oxidase-deficient mice, inflammasome activation is present despite lack of reactive oxygen species production (25). This data further indicated that the loss of both NOX1 and NOX2 did not affect M1-type macrophage differentiation and its pro-inflammatory function.
**Loss of NOX1 and NOX2 delays wound healing**—Since M2 macrophages are known to play a role in wound healing, we next evaluated the biological effect of NOX1/2 deletion on skin repair. We performed wound healing assay using WT and NOX1/2-DKO mice. No bacterial infection in the wounds was observed during the experiment. For WT mice, wound areas were reduced to approximately 21.8% and 15.9% by day 5 and 7 after injury respectively and wound closure was almost complete by day 10. On the contrary, wound area remained over 32.7% and 23.6% of the original wound area by day 5 and 7 after injury and wounds were not closed by day 10 in NOX1/2-DKO mice (Fig. 5, A and B). These results indicated that the wound healing process was delayed in NOX1/2-DKO mice. Two-color immunofluorescence analysis with anti-iNOS and anti-Arginase1 antibodies revealed that while the expression patterns of M1-specific marker iNOS were similar in the wound areas of WT and NOX1/2-DKO mice, the elevated level of M2-specific marker Arginase1 was dramatically lower in NOX1/2-DKO mice compared to WT mice (Fig. 5C). In addition, mRNA levels of M1 cytokines TNF-α and IL-1β in wounds tissues were similarly regulated in both types of mice, but the mRNA levels of M2 cytokines TGF-β1, IL-10, and VEGF and Arginase 1 in the wound tissues of NOX1/2-DKO mice was significantly lower than that in WT wound tissues (Fig. 5D). Therefore, these results indicated that fewer M2 macrophages in the wound areas of NOX1/2-DKO mice leads to the lower expression levels of IL-10, TGF-β1, and VEGF, which contribute to the delay in wound healing in NOX1/2-DKO mice.

**Tumor growth and metastasis are reduced in NOX1/2-DKO mice**—To explore the role of NOX enzymes in tumor growth and metastasis, we used a tumor model in which mouse Lewis lung carcinoma (LLC) cells were subcutaneously injected into WT and NOX1/2-DKO mice. The growth rate of LLC tumor measured by tumor volume was significantly reduced in NOX1/2-DKO mice compared with WT mice (Fig. 6C). Tumors were excised at Day 33 post-implantation and the average weight of tumors from NOX1/2-DKO mice was significantly lower compared to WT tumors (Fig. 6, A and B). Recruitment of M2 macrophages (RELMα positive) to the LLC tumors was significantly decreased in NOX1/2-DKO mice tumors as determined by flow cytometry analysis of single cell suspensions of the excised tumor, while the total percentage of F4/80+ macrophages infiltration was just slightly decreased in NOX1/2-DKO mouse tumors compared to WT mouse tumors (Fig. 6D). Similarly, two-color immunofluorescence analysis with anti-F4/80 and anti-RELMα antibodies confirmed the above observations: similar amounts of F4/80+ macrophages but fewer RELMα macrophages in NOX1/2-DKO as compared to WT mice (Fig. 6E). This result was further verified by real-time PCR analysis of total FACS sorted F4/80 positive cells from tumors. The marker profile revealed higher expression levels of M2-specific marker Arg1, CCL24, IL-10 and VEGF and lower expression levels of M1-specific marker (TNF-α and iNOS) in cells from WT tumors compared with that from NOX1/2-DKO tumors (Fig. 6F). These data demonstrated that NOX1/2 loss did not significantly affect the overall infiltration of macrophages but potently decreased the occurrence of M2-like macrophages.

Next, we examined the effect of NOX1/2 loss on lung metastasis. NOX1/2-DKO mice bearing LLC tumors displayed a marked decrease in the numbers of micro- and macroscopic lung metastases. Normalized counts of the metastatic nodules in the lungs of NOX1/2-DKO bearing mice compared to WT littermates revealed two-fold reduction in the overall numbers of detectable metastatic deposits (Fig. 6G). Taken together, these data demonstrated that NOX1/2 loss potently decreased the occurrence of M2-like TAMs but not overall infiltration of macrophage while inhibiting tumor growth as well as metastatic foci formation in the lung.

**Discussion**

In the present study, we demonstrate for the first time that NOX1 and NOX2 double knockout results in the impaired deficiency of macrophage differentiation and M2 polarization. Deletion of either NOX1 or NOX2 alone exerted only minor effects on macrophage differentiation and polarization. The reason behind this phenomenon is the reduction of ROS production, which is required during the monocyte to macrophage differentiation (13). The production of ROS during macrophage differentiation is mainly controlled by NOX isozymes. It was reported that NOX1 and NOX2 are the two NOX isozymes expressed in BMDM (6,26,27). Single depletion of either NOX1 or NOX2 was not
sufficient to significantly reduce ROS production and NOX activity as well as ERK and JNK signaling activation. NOX1 and NOX2 double knockout significantly, but not completely, decreases ROS production, suggesting that other enzymes may be involved in M-CSF-induced ROS production during monocyte to macrophage differentiation. Low expression levels of NOX4 and Duox1 were observed in BMDMs, and BMMs, implying NOX4 and/or Duox1 may be involved in ROS production in these cells after M-CSF treatment. As our data indicates that the loss of NOX1 and NOX2 does not affect the M1 population, we examined the pro-inflammatory response of the M1 macrophages derived from wild type or NOX1/2-DKO mice and found that M1 macrophages from NOX1/2-DKO mice have the normal inflammatory function.

The NOX family of homologs produces ROS in various cell types in response to stimuli including growth factors, cytokines, and calcium. Thus, NOX-generated ROS, at least in part, behave as second messenger-like molecules. Excess or less levels of NOX-generated ROS, can affect the balance of cellular homeostasis, leading to diseased states. In fact, the deregulation of NOX-dependent ROS generation is potentially associated with chronic diseases including atherosclerosis, hypertension, inflammation and cancer (28). Wound healing is a complex process involving the interactions between different kinds of resident and infiltrating cell types (1). Both the lack of and excess ROS in the wound may affect healing negatively. Skin wounding creates a local stress environment that favors NOX activation at the wound margin. NOX activation-induced ROS production has a positive role not only in regulation of resident cell proliferation, including fibroblasts, keratinocyte, and stellate cells, but also in platelet recruitment, and angiogenesis related growth factors secretion (29-32). The majority of ROS is released by neutrophils and macrophages during the inflammatory phase of healing (33). Pro-inflammatory M1-type macrophages infiltrate in the wound margin soon after injury, whereas M2 macrophages are the primary effectors of later stages of wound healing and tissue regeneration (34). Our data indicates that the loss of NOX1 and NOX2 effectively reduces the NADPH activity and ROS produced by macrophages infiltrating to the wound site and the simultaneous inhibition of M-CSF-stimulated M2 macrophage differentiation. Further, our results demonstrated that both NOX1 and NOX2 are necessary for efficient wound healing. The delay in wound healing maybe a direct result of decreased M2 macrophages present in the wound margin. Our data suggests that NOX1/2 depletion impaired production of ROS and the subsequent reduction of ERK and JNK activation, which are crucial for monocyte to macrophage differentiation, and the subsequent reduction of Stat6 activation, which is important for M2 macrophages polarization. In turn, there is lower expression of IL-10, TGF-1β, and VEGF in the wound margin compared to WT mice, which contributes to the delay in wound healing in NOX1/2-DKO mice.

The functional role of NOXs in cancer development is not clear, however, the over production of intracellular ROS is thought to be involved in the increased risk of cancer or tumor progression (35). Studies have indicated that the local tumor microenvironment appears to be dominated by cytokines and growth factors that cause M2-type immunosuppressive environment instead of M1-type inflammatory conditions (36). Eliminating TAMs can effectively block tumor growth, enhance response to chemotherapy, and inhibit metastatic seeding. In a previous study, we showed that the anti-oxidant, BHA, efficiently blocked the differentiation of M2 macrophages and the occurrence of TAMs. Interestingly, since the total macrophages present in the tumor are not significantly decreased in NOX1/2-DKO mice (Fig. 6D), the decrease of TAMs may lead to the increase of M1 polarization and the greater inhibition of tumor growth. The present study demonstrated that NOX1/2 depletion potently decreased the occurrence of M2-like TAMs and inhibited tumor growth and metastasis to the lung. Although we cannot exclude the possibility that NOX1/2-DKO may create an unfavorable condition for tumor growth and metastasis, we believe that the decreased M2-like TAMs occurrence is critical for mediating tumor cell survival and progression.

**EXPERIMENTAL PROCEDURES**

*Animals—*C57BL/6 wild type, NOX1-knockout, and NOX2-knockout mice (from here on refer to as WT, NOX1-KO, and NOX2-KO mice) with C57BL/6 background were purchased from Jackson Laboratory. NOX1/2-double knockout mice (from here on refer to as NOX1/2-DKO mice) mice with C57BL/6 background were a gift from the Engelhardt lab (19,37). All mice used were 8-14 weeks old. Mice were maintained under pathogen-free conditions, and experimental
protocols were approved by NCI, following NIH guidelines.

**Reagents and antibody**—LPS was obtained from Sigma; anti-phospho-ERK (9101) and anti-ERK (9102) from Cell signaling technology; Anti-phospho-JNK (44682G) and Lucigenin from Invitrogen; anti-Arginase 1 (SC-20150) from Santa Cruz; anti-iNOS (610431), anti-JNK (51-1570GR) from BD Biosciences; anti-F4/80 (14-4801-81), F4/80-APC (17-4801-82), anti-IL-4 (16-7041-85) from eBiosciences; anti-RELMα (500-P24) from Peprotech; recombinant mouse M-CSF (416-ML-050), IL-4 (404-ML-050), IFN-γ (485-MI-100) from R&D Systems.

**Mouse M1/M2 macrophage differentiation in vitro and in vivo**—Bone marrow derived monocytes were isolated from mouse femurs and tibias and cultured for 2 h. The attached monocyte enriched cells were cultured in RPMI-1640 medium supplemented with 10% (vol/vol) FBS and 2 mM glutamine, with penicillin (100 U/ml) and streptomycin (100 μg/ml). For differentiation, cells were cultured for 6 days in the presence of recombinant mouse M-CSF (20 ng/ml). Resident peritoneal macrophages were isolated by peritoneal lavage and cultured in DMEM overnight. For M1 polarization, bone marrow derived monocytes treated for 6 days with M-CSF were then treated with LPS (100 ng/ml) and INF-γ (20 ng/ml) for 24 hrs. For M2 polarization, bone marrow derived monocytes treated for 6 days with M-CSF were then treated with IL-4 (25 ng/ml) plus IL-10 (10 ng/ml) for 24 hrs. In some experiments, the macrophages were primed with 200 ng/ml lipopolysaccharide (LPS) from Escherichia coli 055:B5 (Invivogen) for 4 h before stimulation with 5 mM ATP for 30 min, 20 μM nigericin for 30 min. To generate M1 and M2 macrophages in vivo, mice were intraperitoneally injected with 1 ml 3% sterile thioglycolate or with 200 μl of a IL-4c (combination of a 2:1 ratio of recombinant mouse IL-4 (mIL-4; 5 μg) and anti-IL-4 Ab (clone 11B11; 25 μg in PBS), or PBS vehicle control on days 0 and 2. 4 days after injection, thioglycolate-activated M1 macrophages and IL-4c-induced M2 macrophages were isolated by peritoneal lavage with 10 ml of ice-cold PBS.

**O² generation assay**—The O² generation assay was measured by O²-dependent lucigenin chemiluminescence. 3 × 10⁵ viable cells in 1 ml HBSS were incubated 20 min with 400 mM NADPH. Lucigenin (200 mM) was added and chemiluminescence was measured before and after addition of M-CSF. Cells were maintained at 37°C during the experiment.

**ROS levels measurement assay**—Mouse BMMSs from WT, NOX1-KO, NOX2-KO and NOX1/2-DKO mice were treated with M-CSF (20 ng/ml) for different time points. Cells were stained with CM-H2DCFDA for 30 mins, and then washed and fixed. The percentage DCFDA positive population was quantified by using a flow cytometry.

**Flow Cytometry**—Cells were detached with 2mM EDTA in PBS and were re-suspended in ice-cold PBS. Mouse IgG was preincubated with mouse BMDMs to reduce nonspecific staining. Cells were incubated on ice with RELMα-FITC (Peprotech), iNOS-PE (eBioscience) or F4/80-APC (eBioscience) antibodies for 1 hour and cells were washed twice and analyzed in a flow cytometry. Samples were acquired on a BD Calibur1 (BD Biosciences) and analyzed with FlowJo software (Tree Star). FITC-, PE-, or APC-conjugated IgG stained cells were used to indicate background fluorescence and to set quadrants before calculating the percentage of positive cells. For propidium iodide (PI) staining, cells were washed and re-suspended in HEPES buffer containing PI. The stained cells were analyzed with flow cytometry.

**Mouse Xenograft Models**—Mice were housed and maintained in a pathogen-free environment. Lewis lung carcinoma (LLC) cells (5x10⁸) were mixed with Growth Factor Reduced Matrigel (BD Life sciences) and injected subcutaneously into the right flank of each animal. Tumor volumes were measured using electronic calipers. Tumor volume (TV) = (width)² × length/2. Primary tumors and lung were harvested from mice 33 days after injection. For determining tumor multiplicity and maximal sizes, whole lungs were inflated with and fixed in 4% paraformaldehyde for 24 hrs. Lungs were paraffin-embedded and serial sections at 400 microns were histologically examined with hematoxylin and eosin (H&E) stain. For quantitation of lung tumor, tumor numbers of 5 serial sections per lung were counted and totaled. The lung metastasis index for each mouse was calculated as the ratio of the number of foci colonies observed in the lungs divided by the mass of the primary tumor (in grams) and normalized to WT as fold changes. (Mean ± SEM; n = 8 per group).

**Wounding and macroscopic examination**—Mice were anesthetized with diethyl ether and their backs were shaved and cleaning with 70% ethanol. The dorsal skin was picked up
at the midline and punched through 2 layers of skin with a sterile disposable biopsy punch (4 mm). Four full-thickness excisional wounds per mouse were made. Each wound site was digitally photographed at days 3, 5, 7 and 10 after wounding, and wound areas were determined by Image J. Changes in wound area were expressed as the percentage of the initial wound area. In some experiments, wounds and their surrounding areas, including the scab and epithelial margins, were taken at the indicated time.

**TAM analysis in tumor tissues and Isolation of lymphocytes**—Tumor tissue was minced and digested with dissociation buffer (100 U/ml Collagenase type IV and 100 μg/ml DNase in RPMI + 10% FBS) in a shaking incubator at 37 °C for 30 min. Digested tissues were filtered through 70-μm cell strainers. Cells were incubated with Fc Block. To identify TAMs, cells were stained with RELMα at 37 °C for 30 min. Unstained control and single-stained cells were prepared in every experiment for gating. Dead cells were gated out by side-scatter and forward-scatter analysis.

To isolate lymphocytes from tumor tissues, single cell suspension was carefully layer on the top of Ficoll-Paque PLUS and centrifuged at 400 × g for 30 mins. Draw off the lymphocyte layer and washed twice with RPMI 1640 medium.

**RNA isolation and Real-time PCR**—Total RNA was extracted with Trizol according to the manufacturer’s guidelines (Invitrogen). Any remaining DNA was removed with the DNA-free kit (Ambion) and was re-purified with the RNeasy kit (Qiagen). Taqman real-time gene expression assays were run on an ABI StepOne Plus system according to manufacturer’s protocol (Applied Biosystems). Gene expression was normalized to that of GAPDH or β-actin. All Taqman real-time primers used for gene-expression analysis were pre-designed and confirmed from Integrated DNA Technologies: mouse NOX1 (29694286), NOX2 (13544805), NOX3 (8910254), NOX4 (8820983), Duox1 (9914770), and Duox2 (11283196), TNF-α (58740533), IL-10 (59929059), IL-12β (12409997), CCL-17 (9199490), CCL-24 (13396581), Arginase 1 (8651372), TGF-1β (11254750), VEGF (31754187), iNOS (79286383).

**Western blot analysis**—Cells were collected and lysed in M2 buffer (20 mM Tris at pH 7, 0.5% NP-40, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 2 mM DTT, 0.5 mM PMSF, 20 mM β-glycerol phosphate, 1 mM sodium vanadate, and 1 mg/ml leupeptin). Cell lysates were separated by SDS-PAGE and analyzed by immunoblot. The proteins were visualized by enhanced chemiluminescence (ECL, Peirce).

**Immunofluorescence Analysis**—Tissue samples were embedded in OCT (Tissue-Tek) and 10 μm thick sections were stained with primary antibodies after blocking with bovine serum albumin overnight. Samples were then incubated with PE-conjugated secondary antibody (red) or FITC-conjugated secondary antibody (green). Nuclear staining by DAPI (blue) Microscopic observation was performed by fluorescence microscope (Zeiss).

**Statistical analysis**—Results were analyzed using the version 13 SPSS statistical software (SPSS, Chicago, IL, USA). Quantitative variables were analyzed between two groups using Student’s t-test or among multiple groups using one-way analysis of variance (ANOVA). Differences were considered significant at p < 0.05.

**Authorship Contribution:** QX designed and performed the experiments. SC conducted mouse experiments and helped to supervise the project. QX, and SC wrote the manuscript. JHQ, MC, and JJ helped with experiments. BB and JFE provided NOX1/2 double knockout mice and JFE advised the project and helped writing the manuscript. ZGL conceived, supervised and directed the project and wrote the manuscript.

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On Day 6 the cells were analyzed by imaging and flow cytometry. Representative light microscopy treated with DMSO or ERK inhibitor (U0126, 5 \( \mu \)M) for 15 mins. Cell lysates were immunoblotted with the indicated antibodies.

**Figure legend:**

**FIGURE 1.** NOX1 and NOX2 depletion inhibited ROS production and O\(^2-\) generation in monocytes. (A) The expression levels of NOX family (NOX1, NOX2, NOX3, NOX4, Duox1, and Duox2) in mouse bone marrow monocytes (BMMs) and bone marrow derived macrophages (BMDMs) were detected by real-time PCR assay. (B) The expression levels of NOX1, NOX2, NOX4, and Duox1 in BMDMs from WT, NOX1-KO, NOX2-KO and NOX1/2-DKO mice were detected by real-time PCR assay. (C) Mouse BMMs from WT, NOX1-KO, NOX2-KO and NOX1/2-DKO mice were treated with M-CSF (20 ng/ml) for the indicated time points. Cells were stained with CM-H2DCFDA for 30 mins, and then washed and fixed. The percentage DCFRDA positive population was quantified by using a flow cytometry. (D) Mouse BMMs from WT and NOX1/2-DKO mice were collected. Lucigenin (200 mM) was added and chemiluminescence was measured after treatment with or without M-CSF (20 ng/ml) over the indicated time. Relative O\(^2-\) generation was determined relative to the RLUs at the 0 time point for each group. (* \( p < 0.05 \) at all time points when WT+MCSF compared to WT baseline; \# \( p < 0.05 \) at all time points when WT+MCSF compared to NOX1/2-DKO+MCSF; ** \( p < 0.05 \) at most time points when NOX1/2-DKO baseline compared to WT baseline)

**FIGURE 2.** Absence of both NOX1 and NOX2 affects monocyte to macrophage differentiation. (A) BMMs from C57BL/6 WT, NOX1-KO, NOX2-KO and NOX1/2-DKO mice were treated with M-CSF (20 ng/ml) for 6 days (M0). On day 6, the M0 cells were treated with LPS (100 ng/ml) and INF-\( \gamma \) (20 ng/ml) for M1 or IL-4 (20 ng/ml) for M2 for 24 h. Representative light microscopy image from 3 independent experiments is shown. (B) Cells from (A) were analyzed by flow cytometry with antibodies to iNOS and F4/80 (M1 population) and to RELM\( \alpha \) and F4/80 (M2 population). (C) Detection of M1 cytokines (TNF-\( \alpha \), IL-12\( \beta \)) and M2 chemokines (CCL17, CCL24) by real-time PCR of cells from A. (D, E) Mouse peritoneal macrophages from WT and NOX1/2-DKO were isolated and cultured overnight. Then macrophages were polarized M1 and M2. Cells were co-stained with the indicated antibodies and analyzed by flow cytometry. (D). The expression levels of M1 cytokines (TNF-\( \alpha \), IL-12\( \beta \)) or M2 chemokines (CCL17, CCL24) were detected by real-time PCR (E). (F, G) WT, NOX1-KO, NOX2-KO and NOX1/2-DKO mice (n=3 per group) were i.p. injected with either thioglycollate (TG) or IL-4c. Day 4 after injection, macrophages were isolated by peritoneal lavage and co-stained with the indicated antibodies and analyzed by flow cytometry (F). The expression levels of M1 cytokines (TNF-\( \alpha \), IL-12\( \beta \)) or M2 chemokines (CCL17, CCL24) were detected by real-time PCR (G). All results represent the mean \pm SD from three independent experiments. (* \( p < 0.05 \), versus WT).

**FIGURE 3.** NOX1 and NOX2 depletion inhibited ROS-mediated ERK and JNK activation. (A) Mouse BMMs from WT, NOX1-KO, NOX2-KO and NOX1/2-DKO mice were treated with M-CSF (20 ng/ml) for the indicated time points. The expression levels of p-ERK, ERK, p-JNK, and JNK were determined by western blotting. (B) Mouse monocytes from WT, NOX1-KO, NOX2-KO and NOX1/2-DKO mice were treated with M-CSF for 6 days. On day 6, cells were treated with LPS/INF-\( \gamma \) (left panel) or IL-4 (right panel) for 15 mins. Cell lysates were immunoblotted with the indicated antibodies. (C) On Day 0 Mouse monocytes from WT, NOX1-KO, NOX2-KO and NOX1/2-DKO mice were treated with DMSO or ERK inhibitor (U0126, 5 \( \mu \)M) for 1 h and then treated with M-SCF for 6 days. On Day 6 the cells were analyzed by imaging and flow cytometry. Representative light microscopy.
image are shown (upper panel). Macrophages were collected and stained with anti-F4/80 antibody and analyzed by flow cytometry (lower panel). (D,E) On Day 6 the macrophages from (C) were further treated for 24 hours with either LPS (100 ng/ml) and INF-γ (20 ng/ml) for M1 or IL-4 (20 ng/ml) for M2 polarization. M1 cell population (iNOS and F4/80) and M2 cell population (RELMα and F4/80) were detected by flow cytometry (D). Detection of M1 cytokines (TNF-α, IL-12β) or M2 chemokines (CCL17, CCL24) by real-time PCR (E). (* p < 0.05, or ** p < 0.001 versus WT).

**FIGURE 4. NOX1 and NOX2 depletion has no effect on macrophage pro-inflammatory function.** (A,B) Measuring production of IL-1β (A) or Western blot analysis of expression levels of Caspase1 and Caspase1p20 (B) of LPS-primed BMDMs treated with 5 mM ATP (30 mins), 20 μM nigericin (2 h), or transfected with poly A:T (6 h), or infected with *salmonella typhimurium* (10⁸ CFU for 6 h).

**FIGURE 5. Wounding healing is delayed in NOX1 and NOX2 double-knockout mice.** (A) Analysis of skin wound healing over time. Representative image from 2 independent experiments with 4 animals per time point for each group is shown. (B) Percentage of wound area at each time point relative to the original wound area for mice from (A). (C) Immunofluorescence staining of Arginase 1 (Red), iNOS (Green), nucleus (Blue) at wound sites from WT and NOX1/2-DKO mice at the indicated times. Representative images are shown. (D) The expression levels of mouse IL-1β, TNF-α, iNOS, Arginase 1, TGF-β, VEGF, and IL-10 at the wound sites were analyzed by real-time PCR for the indicated time points. (* p < 0.05, versus WT).

**FIGURE 6. NOX1 and NOX2 deficiency inhibits LLC tumor growth and metastasis.** (A-C) LLC cells were subcutaneously injected into WT and NOX1/2-DKO mice. Xenografts were removed 33 days after implantation and tumors were photographed. Representative images are shown (A). Tumor weights at 33 days (B) and tumor volume for the indicated times (C) were obtained and presented. (Mean ± SEM; n = 8 per group), p values are indicated. (D) The percentage of F4/80+ cells in the primary tumors on Day 33 was determined by flow cytometry. Quantification of the percentage of RELMα+ cells in the F4/80+ population (* p < 0.05). (E) Representative immunofluorescence images showing Arginase I (Red), F4/80 (Green), nucleus (Blue) staining of primary tumors from WT and NOX1/2-DKO mice on Day 33. (F) The expression levels of TNF-α, iNOS, Arginase 1, CCL24, VEGF, and IL-10 in lymphocytes isolated from Day 33 tumors from WT and NOX1/2-DKO mice were analyzed by real-time PCR (* p < 0.05). (G) Representative images of H&E staining of lung sections 33 days after LLC cell injection (upper panel). Whole lung sections were scanned and were scored for metastatic foci (lower panel). Metastasis index (= metastasis number divided by primary tumor weight) (Mean ± SEM; n = 8 per group).
Figure 2
Figure 3
Figure 4

A

WT □ NOX1/2-DKO

mL-1β (pg/ml)

Mock Nigercin ATP Poly A:T S. typhi

B

WT NOX1/2-DKO

Mock Nigercin ATP Poly A:T S. typhi Mock Nigercin ATP Poly A:T S. typhi

45kd 20kd

Caspase 1 Caspase 1 p20

Figure 4
Figure 5
Figure 6
NADPH oxidases are essential for macrophage differentiation
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