Role of NOX2-derived reactive oxygen species in NK cell-mediated control of murine melanoma metastasis

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Running title: NOX2 in regulation of melanoma metastasis

Key words: melanoma metastasis, NOX2, reactive oxygen species, NK cells, IFNγ, immunotherapy

List of abbreviations: HDC, histamine dihydrochloride; NOX2, NADPH oxidase isoform 2; ROS, reactive oxygen species

Financial support: This work was supported by the Swedish Research Council (2012-2047 to AM, 2012-3205 to KH), the Swedish Society for Medical Research (SSMF to AM), the Swedish Cancer Society (CAN 212/595 to AM, CAN 2015/411 to KH), the Swedish state via the ALF agreement (ALFGBG-436961 to AM), the Erna and Victor Hasselblad Foundation (to AM), the Torsten and Ragnar Söderberg Foundation (to KH), the Assar Gabrielsson Foundation (to EA), BioCARE - a National Strategic Research Program at University of Gothenburg and the Sahlgrenska Academy at University of Gothenburg (to AM).

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ABSTRACT

The NADPH oxidase of myeloid cells, NOX2, generates reactive oxygen species (ROS) to eliminate pathogens and malignant cells. NOX2-derived ROS also have been proposed to dampen functions of natural killer (NK) cells and other anti-neoplastic lymphocytes in the microenvironment of established tumors. The mechanisms by which NOX2 and ROS influence the process of distant metastasis have only been partially explored. Here we utilized genetically NOX2-deficient mice and pharmacological inhibition of NOX2 to elucidate the role of NOX2 for the hematogenous metastasis of melanoma cells. After intravenous inoculation of B16F1 or B16F10 cells, lung metastasis formation was reduced in B6.129S6-Cybb<sup>tm1DinK</sup> (Nox2-KO) vs. Nox2-sufficient wild-type (WT) mice. Systemic treatment with the NOX2-inhibitor histamine dihydrochloride (HDC) reduced melanoma metastasis and enhanced the infiltration of IFN<sub>γ</sub>-producing NK cells into lungs of WT but not of Nox2-KO mice. IFN<sub>γ</sub>-deficient B6.129S7-<i>Ifng</i><sup>tm1Ts</sup>/J mice were prone to develop melanoma metastases and did not respond to in vivo treatment with HDC. We propose that NOX2-derived ROS facilitate metastasis of melanoma cells by down-modulating NK cell function and that inhibition of NOX2 may restore IFN<sub>γ</sub>-dependent, NK cell-mediated clearance of melanoma cells.
INTRODUCTION

Reactive oxygen species (ROS) are short-lived compounds that arise from electron transfer across biological membranes where the electron acceptor is molecular oxygen and the initial product is superoxide anion (O$_2^-$). ROS refer to oxygen radicals such as O$_2^-$ and the hydroxyl radical (OH) along with non-radicals, including hydrogen peroxide, that share the oxidizing capacity of oxygen radicals and may be converted into radicals (1). ROS are generated as by-products of mitochondrial ATP generation in the electron transport chain but are also produced in a regulated fashion by the nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOX) and the dual oxidases (DUOX). This family of transmembrane proteins comprises NOX1-5 and DUOX1-2 whose only known function is to produce ROS (2).

The NOX proteins are structurally similar and utilize a similar principal mechanism of ROS generation but vary in cellular and subcellular distribution. NOX2 is expressed almost exclusively in cells of the myeloid lineage such as monocyte/macrophages and neutrophilic granulocytes (3, 4). These cells utilize NOX2-derived ROS to eliminate intra- and extracellular microorganisms (5). NOX2 also has been linked to immunosuppression in cancer: when released from myeloid cells into the extracellular space, ROS generated by NOX2 may trigger dysfunction and apoptosis of adjacent anti-neoplastic lymphocytes, including natural killer (NK) cells (6, 7, 8, 9). The strategy to target ROS formation by myeloid cells has been proposed to improve the efficiency of cancer immunotherapy (3, 10, 11, 12).
The role of ROS and NOX2 for the growth and metastatic spread of cancer cells is, however, complex and controversial. Thus, although the genetic disruption of Nox2 reduces the subcutaneous growth of murine melanoma and lung carcinoma, it does not affect sarcoma growth or prostate cancer growth in mice (13, 14). Also, the in vivo administration of scavengers of ROS such as N-acetyl-cysteine reduces the tumorigenicity of murine melanoma cells (15) but enhances lymph node metastasis in other melanoma models, accelerates tumor progression in mouse models of B-RAF- and K-RAS-induced lung cancer and accelerates the metastasis of xenografted human melanoma cells in immunodeficient mice (16, 17, 18).

The detailed mechanisms of relevance to the discrepant impact of ROS for the growth and spread of cancer cells remain to be elucidated. Further understanding of the role of ROS for cancer progression requires experimental models that address a distinct phase of tumor progression, define the source of ROS and take mechanisms of immunosurveillance into account. For the present study, we aimed at determining the impact of genetic and pharmacological inhibition of NOX2 in a murine NK cell-dependent model of melanoma metastasis.
MATERIALS AND METHODS

Culture of cell lines. B16F1 and B16F10 murine melanoma cells were obtained in 2013 from the Cell Culture Laboratory at the Department of Virology, University of Gothenburg where cells were authenticated by melanotic morphology and checked for absence of mycoplasma using PCR before freezing aliquots. Each aliquot was thawed and cultured for no more than one week for each experiment. Cells were cultured in Iscoves’ medium containing 10% FCS (Sigma-Aldrich), 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 mg/ml streptomycin at 37°C, 5% CO₂ for 1 week before inoculation into mice.

Induction of lung metastasis in Nox2-KO and Ifng-KO mice. All animal experiments were approved by the Research Animal Ethics Committee in Gothenburg. Mice were maintained under pathogen-free conditions according to guidelines issued by the University of Gothenburg. C57BL/6 mice were obtained from Charles River Laboratories (Sulzfeld, Germany). B6.129S6-Cybb<sup>imDin</sup> (Nox2<sup>−/−</sup> or Nox2-KO) mice that lack the myeloid gp91<sup>phox</sup> subunit NOX2 and, thus, a functional ROS-forming NOX2 were obtained from Jackson laboratories (Maine, USA). B6.129S7-Ifng<sup>imTs/J</sup> (Ifng<sup>−/−</sup> or Ifng-KO) mice that do not produce IFNγ (19) were kindly provided by Prof. Nils Lycke, MIVAC at University of Gothenburg. Naïve C57BL/6, Nox2-KO and Ifng-KO mice (6-12 weeks of age) were treated intraperitoneally (i.p.) with PBS (control), HDC (Sigma, 1,500 μg/mouse), IL15 (0.04 μg/mouse), alone or combined, on the day before, the day after and 3 days after intravenous (i.v.) inoculation of B16F10 cells (5-15x10⁴ cells/mouse) or B16F1 cells.
(20 x10^4–30 x10^4 cells/mouse). Three weeks after tumor inoculation, mice were euthanized by cervical dislocation followed by harvesting of lungs and spleens. Lung metastasis was determined by counting visible pulmonary metastatic foci under a light microscope. The experimental design is outlined in Fig. 1A.

For assessment of the impact of NOX2 inhibition on immune parameters during the early phase of tumor progression, mice received HDC at 1,500 μg/mouse or PBS (control) one day before the inoculation of B16F10 cells followed by dissection of lungs at 30 min or 24 hours after tumor cell inoculation as shown in Fig. 2A. In the latter experiments, naïve mice and HDC-treated mice that did not receive melanoma cells were used as additional controls.

**Preparation of single cell suspensions from lungs and spleens.** Lung tissues were dissociated into single cells by combining enzymatic degradation of extracellular matrix with mechanical dissociation using gentle MACS® Technology (Miltenyi Biotech, Auburn) based on instructions provided by the manufacturer. Single-cell suspensions of splenocytes were prepared by mashing the spleens through a 70μm cell strainer followed by depletion of erythrocytes using RBC Lysing buffer (Sigma-Aldrich, Steinheim).

**Flow cytometry.** The following fluorochrome-labeled antimouse mAbs were purchased from BD Biosciences: anti-CD45 (30-F11), anti-CD11c (HL3), anti-IaIe (2G9), anti-CD3 (145-2311), anti-CD4 (RM4-5), anti-CD8 (53-6.7), anti-NK1.1 (PK136), anti-CD19 (1D3), anti-CD11b (M1/70), anti-Gr1 (RB6-8C5), anti-CD40 (3/23) and anti-Ly6C (AL-21). Anti-CD33 (29A1.4) was from Ebiosciences
(Stockholm); anti-F4/80 (BM8) and anti-CD69 (H1.2F3) were from BioLegend (Stockholm). LIVE/DEAD Fixable Yellow Dead Cell Stain Kit or DAPI (both from Invitrogen, Oregon) were used as cell viability markers in flow cytometry analyses. A minimum of 100,000 gated live cells were collected on a four-laser BD LSRFortessa (405, 488, 532, and 640 nm). Data were analyzed using FACSDiva Version 8.0.1 software (BD Biosciences).

Detection of ROS. Superoxide anion production was determined by use of the isoluminol-electrogenerated chemiluminescence technique as described elsewhere (20). Briefly, single cell suspensions of lungs were diluted to $10^7$ cells/ml in Krebs-Ringer glucose buffer supplemented with isoluminol (10 mg/ml; Sigma-Aldrich) and horseradish peroxidase (HRP, 4 U/ml, Boehringer Mannheim, Germany) and added to 96-well plates that were incubated at 37°C. PMA myristate acetate (PMA, $5 \times 10^{-8}$ M, Sigma-Aldrich, Missouri, USA) or the formyl peptide receptor agonist WKYMVm ($10^{-5}$ M, Tocris Bioscience, Bristol, UK) were added for induction of ROS production. Light emission was recorded continuously using a FLUOstar Omega plate reader (BMG, Ortenberg, Germany). In some experiments HDC (10-1,000 $\mu$M, final concentrations) was added 5 min prior to the addition of WKYMVm.

Depletion of Gr1+ and NK cells in vivo. Gr1+ cells were depleted by i.p injections of 400 $\mu$g anti-Gr1 antibody (BioXCell, West Lebanon, USA, Clone RB6-8C5) 2 days before B16 cell inoculation. This procedure depletes >95% of Gr1+ cells in blood and other tissues (21). NK cells were depleted by i.p injections of 250 $\mu$g anti-NK1.1 antibody (BioXCell, West Lebanon, USA, Clone PK136) 4 days and 2 days before B16F10 cell inoculation. NK cell depletion was confirmed by flow cytometry on
lungs and spleen tissue harvested on day 1, day 3 and day 6 after antibody injection.

**NK cell isolation and adoptive transfer.** Spleens were harvested from WT C57BL/6 mice and single cell suspensions were prepared. Splenocytes were enriched for NK cells by passage through nylon wool columns (Polysciences, Warrington, USA). NK cells were then negatively selected using an NK cell isolation kit II (Miltenyi Biotech) according to the manufacturer’s instructions to a purity of > 70%. Five million enriched NK cells were injected i.v. 12 hours before inoculation of B16F10 cells. WT NK cells in *Ifng*−/− mice were detected 2 days after adoptive transfer by collecting peripheral blood followed by DNA extraction and PCR. The primer pair used for detection of WT *Ifng* was 5’AGAAGTAAGTGGAAAGGGCCAGAAG 3’ and 5’AGGGAAACTGGGAGAGGAAATAT 3’. For detection of the disrupted IFNγ gene (*Ifng*−/−) the primer pair 5’TCAGCGCAGGGCCCGGTTCTTT 3’ and 5’ATCGACAAGACCGGCTTCCATCCGA 3’ was used (19).

**Detection of IFNγ.** Mice were pre-treated with HDC (1,500 μg) or PBS on the day before i.v. inoculation of B16F10 cells. Thirty minutes after tumor cell inoculation mice were sacrificed and single cell lung cell suspensions were prepared. Lung cells were co-cultured overnight with B16 cells (500,000 cells/mL) in flat bottom 96-well plates at effector: target cell ratios of 1:1 to 50:1. Supernatants were collected after 24h and the IFNγ content was determined by ELISA (Mouse IFNγ DuoSet ELISA, R&D Systems).
Statistics. Two-tailed paired or unpaired *t*-tests were used for statistical calculations. For multiple comparisons, one-way ANOVA followed by the Holm-Sidak multiple-comparison test was used.

RESULTS

Inhibition of NOX2 reduces hematogenous melanoma metastasis

To elucidate the role of NOX2-derived ROS in murine melanoma metastasis, we utilized genetically modified mice that lack the myeloid gp91phox subunit NOX2 and thus a functional ROS-producing NOX2 in myeloid cells (Nox2-KO mice). Over a range of amounts of i.v. inoculated B16F10 cells, it was observed that the establishment of melanoma metastases was less pronounced in lungs of Nox2-KO mice compared with WT B6 mice (Fig. 1B). We next evaluated effects of HDC, a NOX2-inhibitor (22), on melanoma metastasis in WT and Nox2-KO mice. These experiments were performed using loads of injected B16F10 cells that produced comparable numbers of metastases in WT and Nox2-KO animals, *i.e.* 100,000 B16F10 cells for WT mice and 150,000 cells for Nox2-KO mice. In agreement with a previous report (23) systemic treatment of mice with HDC (1,500 µg/mouse i.p.) during the initial phase of melanoma engraftment (days -1, 1 and 3 after tumor cell inoculation) decreased the number of lung metastases in WT mice. These effects were not observed in Nox2-KO mice (Fig. 1C). The NK cell-activating cytokine IL15 (24) (0.04 µg/mouse on days -1, 1 and 3) exerted antimetastatic activity *in vivo* in WT and Nox2-KO mice (Fig. 1C). Combined treatment with HDC and IL15 additively reduced B16F10 metastasis in WT mice but not in Nox2-KO mice (Fig. 1C). Experiments using the B16F1 strain of melanoma cells (25) confirmed the reduced level of
metastasis in Nox2-KO mice and the NOX2-dependent, antimetastatic effect of HDC

\textit{in vivo} (Fig. 1D).

**HDC targets ROS formation \textit{in vitro} and \textit{in vivo}**

CD11b^{+}Gr1^{+} myeloid cells express NOX2 and constitute the principal source of extracellular ROS in blood and tissue (26, 27). Accordingly, CD11b^{+}Gr1^{+} cells isolated from the lungs of naive WT mice, but not from Nox2-KO mice, produced extracellular ROS upon stimulation, whereas the Gr1^{-} fraction of lung cells produced minute extracellular ROS (Fig. 2A). ROS formation from WT lung cells was dose-dependently suppressed by HDC \textit{in vitro} (Fig. 2B).

In experiments designed to assess the dynamics of ROS-producing myeloid cells in lungs after B16F10 cell inoculation (Fig. 2C), we observed a pronounced and transient influx of CD11b^{+}Gr1^{+} myeloid cells into lungs at 30 minutes after i.v. inoculation of tumor cells (Fig. 2D-E). Systemic treatment with HDC prior to melanoma cell inoculation did not alter the degree of influx of myeloid cells into lungs (Fig. 2E) but reduced the ROS formed \textit{ex vivo} in lung cell suspensions (Fig. 2F). To further clarify the impact of CD11b^{+}Gr1^{+} cells on melanoma metastasis, Gr1^{+} cells were depleted from WT mice before treatment of mice with HDC and i.v. infection of B16F10 cells. The extent of lung metastasis was reduced in the absence of Gr1^{+} cells. Systemic treatment with HDC did not affect metastasis in Gr1^{+}-depleted mice (Fig. 2G).

**Role of NK cells for melanoma metastasis in WT and Nox2-KO mice**
NK cell function is reportedly critical to limit lung metastasis in experimental models of murine melanoma (23, 28). To define the role of NK cells in the context of NOX2 inhibition, WT and Nox2-KO mice were depleted of NK cells by anti-NK1.1 antibody treatment prior to melanoma cell inoculation. NK cell depletion more than doubled metastasis formation in WT and Nox2-KO mice. HDC did not inhibit melanoma metastasis in animals depleted of NK cells (Fig. 3A). In experiments designed to clarify whether the reduced ROS levels in lungs following administration of HDC translated into altered NK cell function at the site of tumor expansion, it was observed that treatment of mice with HDC entailed increased NK cell counts in lungs, but not in spleen, at 3 weeks after tumor cell inoculation (Fig. 3B). Unexpectedly, we detected fewer NK cells in lungs and spleens of Nox2-KO mice than in WT animals (Fig. 3B). Also, as shown in Fig. 3A, the degree of metastasis was strikingly enhanced in NK cell-depleted Nox2-KO mice, which may point towards the possibility of increased functionality of NK cells in the absence of NOX2.

**NOX2 inhibition enhances the capacity of lung NK cells to produce IFNγ**

As the antimetastatic functions of NK cells in the B16 model reportedly rely on the formation of IFNγ (29, 30), we assessed the IFNγ production of pulmonary NK cells from Nox2-KO and WT mice. Lung cells were isolated 30 min after B16F10 cell inoculation and IFNγ production was then assessed upon co-culture of lung cells with B16F10 cells *in vitro*. Only minor amounts of IFNγ (<25 pg/ml) were detected when lung cells or B16 cells were cultured alone. Also, minute levels (<10 pg/ml) of IFNγ were produced in co-cultures of lung cells and B16 cells after the depletion of NK cells *in vivo* using anti-NK1.1, thus supporting that the IFNγ produced in these cell cultures was contributed by NK cells (Fig. 3C). It was further observed that lung NK
cells from Nox2-KO mice produced significantly higher amount of IFNγ \textit{ex vivo} at a lung cell to melanoma cell ratio of 50:1 compared with lung NK cells from WT mice (WT vs. Nox2-KO lungs; 297±81 vs 749±27 pg/ml, respectively; \( P = 0.004 \), \( t \)-test). A similar experimental design was adopted to assess the impact of pharmacological NOX2 inhibition by HDC on the formation of IFNγ in lungs. Lung cells were isolated from HDC-treated or control WT mice at 30 min after B16F10 cell inoculation. When lung cells were co-cultured with the B16 cells, higher concentrations of IFNγ were produced \textit{ex vivo} by lung NK cells isolated from mice treated with HDC \textit{in vivo} (Fig. 3C).

**Role of IFNγ in NOX2-mediated control of melanoma metastasis**

We next assessed the capacity of B16F10 cells to form metastases in Ifng-KO vs. WT mice. In accordance with earlier studies (29, 30) melanoma metastasis was enhanced in IFNγ-deficient mice (Fig. 4A). Systemic treatment with HDC did not reduce melanoma metastasis in Ifng-KO mice (Fig. 4B). The adoptive transfer of IFNγ-producing WT NK cells, but not the transfer of Ifng-KO NK cells, to Ifng-KO mice significantly restored the antimitastatic efficacy of HDC (Fig. 4B). Presence of cells with Ifng\textsuperscript{+/-} genotype in blood of Ifng-KO mice was confirmed by PCR at 2 days after the adoptive transfer of WT NK cells (Fig. 4C).
DISCUSSION

We report that genetic inhibition of NOX2, which mediates oxidative stress by generating ROS from myeloid cells, reduced the capacity of two strains of murine melanoma cells (B16F1 and B16F10) to form lung metastases after i.v. inoculation, apparently by facilitating NK cell-mediated clearance of malignant cells. Also, treatment of mice with the NOX2 inhibitor HDC reduced melanoma metastasis in WT but not in Nox2-KO mice. Our results concur with a study showing that HDC reduces the subcutaneous growth of EL-4 thymoma tumors in WT but not in Nox2-KO mice (31), thus underscoring that the anti-neoplastic efficacy of HDC depends on the availability of NOX2.

Our results also show that the establishment of melanoma metastases was associated with a rapid and transient accumulation of ROS-forming CD11b^+Gr1^+ myeloid cells in the lung parenchyma and that the ROS-forming capacity of infiltrating myeloid cells *ex vivo* was suppressed by the *in vivo* administration of HDC. Pharmacological inhibition of NOX2 also entailed increased numbers of lung NK cells in tumor-bearing mice. In agreement with earlier studies (29, 30), the availability of IFNγ was critical for NK cell-mediated clearance of B16 melanoma cells from lungs. Furthermore, the antimetastatic effect of HDC was absent in Ifng-KO mice but could be reconstituted by the adoptive transfer of Ifng^+/+ NK cells. Collectively, these results imply that the antimetastatic properties of HDC rely on the availability of NK cell-derived IFNγ. We observed that despite the more efficient NK cell-mediated clearance of melanoma cells in Nox2-KO, rather than in WT mice, higher counts
of NK cells were detected in the lung parenchyma of WT mice. This finding implies that NK cells were more efficient effector cells on a per cell basis in Nox2-KO mice. In agreement with this hypothesis, we observed that pulmonary NK cells from Nox2-KO mice showed enhanced formation of IFNγ ex vivo.

From these results, we hypothesize that NOX2-derived ROS produced by myeloid cells may exert oxidative stress with ensuing reduction of NK cell–mediated clearance of melanoma cells and aggravation of metastasis. In agreement with our findings, the subcutaneous growth of murine melanoma and lung carcinoma was reduced in Nox2-KO mice (13). However, several studies report contradictory effects of ROS on the course of experimental melanoma and other forms of cancer, in particular regarding direct effects of ROS on tumor cells. Cancer cells thus display elevated ROS concentrations due to enhanced metabolism and mutations that trigger oxidative processes (32, 33, 34). The increased ROS may promote mutagenesis and may also render tumor cells more prone to expand and produce distant metastases (34, 35, 36, 37). In agreement, over-expression of the antioxidant SOD3 inhibits murine breast cancer cell metastasis (38), and the ROS scavenger N-acetyl-cysteine reduces the tumorigenicity of murine melanoma cells (15).

High endogenous ROS concentrations in malignant cells may also render these cells more vulnerable to further stresses (34, 39, 40). Hence, anticancer therapies that trigger a further increase in ROS formation may induce cell death in cancer cells compared with their non-malignant counterparts (34, 41, 42). In addition, ROS can limit malignant growth by triggering activation of p53, whereas antioxidants enhanced tumor progression in a p53-dependent manner (17). Antioxidants can
enhance lymph node metastasis in a model of genetically related melanoma (16). In immunodeficient NOD-SCID-Il2rg<sup>−/−</sup> mice, oxidative stress reduces the ability of primary melanoma cells to metastasize, whereas treatment with antioxidants enhanced metastasis (18).

We hypothesize that at least two mechanisms of relevance to melanoma metastasis and ROS-mediated oxidant stress are operable in immunocompetent mice, i.e. direct effects of ROS on tumor cells that may either inhibit or enhance melanoma cell expansion, and oxidant-induced immunosuppression that may promote tumor growth and metastasis. The relative significance of these partly opposing mechanisms may relate to the sensitivity of melanoma cells to the growth-promoting or toxic effects of ROS as well as to the sensitivity of melanoma cells to immune-mediated clearance. This view may, at least in part, explain the contradiction that the in vivo administration of ROS-scavenging antioxidants such as N-acetylcysteine promotes as well as prevents murine melanoma metastasis (15, 16, 17).

The source of ROS may be critical for its capacity to promote or inhibit tumor progression. In our model, only ROS derived from NOX2-sufficient myeloid cells were targeted. In contrast, ROS scavengers such as N-acetyl-cysteine may also neutralize ROS generated from other sources, including those formed in mitochondria during cell respiration (16). The notion that NOX2<sup>+</sup> myeloid cells may facilitate melanoma metastasis is supported by the findings that neutrophil infiltration of human primary melanomas heralds early metastatic spread (43) and that the exposure of murine cutaneous melanomas to UV light or chemical carcinogens triggers neutrophil-dependent inflammation that promotes metastasis (44, 45). Indeed, the
adoptive transfer of CD11b^Ly6G^ neutrophilic granulocytes enhances the formation of lung metastases after i.v. inoculation of murine carcinoma cells (46). The effect was secondary to granulocyte-induced inhibition of NK cell function. Neutrophil secretion of IL1β and matrix metalloproteinases contributed to tumor cell extravasation but the detailed mechanism of NK cell inhibition was not defined (46).

Although further studies are required to clarify the detailed impact of granulocytes and other NOX^+ myeloid cells for the course of cancer, our results imply that the release of NOX2-derived ROS from these cells may constitute a mechanism of NK-cell inhibition during metastasis of relevance to these previous reports, and that strategies to target NOX2-derived ROS may facilitate NK cell-mediated clearance of metastatic cells. This assumption gains further support by the results of the present study showing that the depletion of Gr1^+ cells reduced melanoma metastasis, and that NOX2 inhibition using HDC did not affect metastasis in Gr1^+ -depleted animals. Additionally, the finding that IL15, an NK cell-activating cytokine, improved the antimetastatic efficacy of pharmacological NOX2 inhibition in WT animals suggests a combinatorial immunotherapy to reduce metastasis formation.

In summary, our results suggest that NOX2 function impacts NK cell-mediated control of murine melanoma metastasis. We propose that pharmacological inhibition of NOX2, alone or combined with immunostimulatory strategies, should be further evaluated in preventing melanoma metastasis.

**Author contribution**
Authors E.A., K.H. and A.M. designed the study. Authors E.A., J.J. and F.H.N. performed experiments. Authors E.A. and A.M. analyzed experiments. Authors E.A., K.H. and A.M. wrote the manuscript. All authors read and approved the manuscript prior of submission.

Acknowledgements

We are grateful to Prof. Nils Lycke, MIVAC, University of Gothenburg, for providing the B6.129S7-Ifng<sup>tm1Ts</sup>/J mice.

Footnotes

Abbreviations used:

- histamine dihydrochloride (HDC)
- nicotinamide adenine dinucleotide phosphate (NADPH)
- myeloid cell NADPH oxidase (NOX2)
- reactive oxygen species (ROS)
References


Figures and legends

**Fig. 1. Impact of genetic and pharmacological inhibition of NOX2 on B16 melanoma metastasis.** Panel (A) shows the experimental design. Panel (B) shows the number of metastatic foci formed in lungs of WT and Nox2-KO (Nox2^{+/−}) mice at 3 weeks after i.v. inoculation of 50,000, 100,000 or 150,000 B16F10 cells. Medians and quartiles are indicated by boxes. Error bars show the min to max values (n = 6 for each group; t-test; two independent experiments). Panel (C) shows the number of metastatic foci in lungs of WT or Nox2-KO mice after systemic treatment with HDC and/or IL15. In these experiments, 100,000 B16F10 cells were injected into WT mice and 150,000 cells into Nox2^{+/−} mice to achieve comparable levels of baseline metastasis (n = 15 for all groups of WT mice; n = 8 for control, HDC and IL15 groups of Nox2^{+/−} mice and n = 3 for HDC + IL15). Combined treatment with IL15 and HDC was significantly more effective than IL15 alone to reduce metastasis formation in WT mice, when analyzed by t-test, P = 0.01 (up to five independent experiments). Panel (D) shows results from lung metastasis formation by the B16F1 melanoma cell line. This cell line is less metastatic compared to B16F10 and therefore 200,000 B16F1 cells were injected into WT mice and 300,000 cells into Nox2^{+/−} mice. The number of metastatic foci in lungs of WT and Nox2-KO mice after systemic treatment with HDC or IL15 was determined after 3 weeks. (n = 4 for all groups except n = 3 for control group of WT mice). The results shown in Fig 1C-D were evaluated by Repeated Measures ANOVA. Non-significant values where P > 0.05 represented with ‘n.s’ whereas ‘*’ represents P ≤ 0.05; ‘**’ represents P ≤ 0.01 and ‘***’ represents P ≤ 0.001.
Fig. 2. Effect of HDC on the ROS production in mouse lungs after melanoma cell inoculation. Panel (A) shows the extracellular ROS production of PMA-stimulated WT Gr1$^+$ (black solid line), WT Gr1$^-$ (black dashed line) or Nox2$^{-/-}$ Gr1$^+$ mouse lung cells (blue line) in a representative experiment out of three performed. Panel (B) shows the ROS production of WT lung cells triggered by $10^{-7}$ M WKYMVm in response to HDC at indicated final concentrations. The mean ROS production +/- SEM is displayed ($n = 3$, $t$-test). Panel (C) represents the experimental design designed to assess the dynamics of ROS-producing myeloid cells in lungs after B16 cell inoculation. Panel (D) shows a representative dot plot of CD11b$^+Gr1^+$ cells out of live CD45$^+$ lung cells before (0 h) or 0.5 h after tumor cell inoculation. Panel (E) shows the fraction of CD11b$^+Gr1^+$ cells out of live CD45$^+$ cells in lungs at indicated time points after tumor cell injection, with or without pre-treatment of mice with PBS (control) or HDC 24 h before analysis, as determined in single cell lung suspensions ($n = 18$ in each group; four independent experiments). Panel (F) shows the ROS formation (area under curve, AUC) ex vivo in response to PMA stimulation of single lung cell suspensions from mice pretreated with HDC or PBS on the day before tumor cell inoculation. ROS production was determined at 30 min and 24 h after inoculation of 100,000 B16 cells ($n = 5-10$, $t$-test; three independent experiments). Panel (G) shows a reduced B16F10 metastasis formation and lack of effects of systemic treatment with HDC on metastasis formation in animals depleted of Gr1$^+$ cells prior to melanoma cell inoculation ($n = 4-5$ for each group, one way ANOVA). Non-significant values where $P > 0.05$ represented with ‘n.s’ whereas ‘*’ represents $P \leq 0.05$; ‘**’ represents $P \leq 0.01$ and ‘***’ represents $P \leq 0.001$. 

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Fig. 3. Antimetastatic effects of HDC rely on NK cells and NK cell-derived IFNγ.

Panel (A) shows the effects of systemic treatment with HDC on B16F10 metastasis formation in WT and Nox2−/− animals depleted of NK cells (n = 7 for untreated WT mice with and without NK cells (two independent experiments); n = 3 for HDC-treated WT mice with and without NK cells; n = 4 for each group of Nox2−/− mice, one way ANOVA). Panel (B) shows effects of systemic treatment with HDC on NK cell numbers in lungs and spleens of WT and Nox-KO (Nox2−/−) mice at 3 weeks after tumor cell inoculation. The percentage of NK cells out of live CD45+ cells was determined by flow cytometry (WT mice n = 9-11; Nox2−/− mice n = 9-13, t-test; three independent experiments). Panel (C) shows IFNγ levels produced in lung cells from HDC-treated and control WT mice that were incubated with B16 melanoma cells at indicated effector to target cell ratios. Mice received HDC or PBS (control) 24 hours before i.v. inoculation of B16 cells. Lungs were recovered 30 min after tumor cell inoculation (n = 11 for the control group, n = 6 for the other groups, two-way ANOVA; two independent experiments). Non-significant values where P > 0.05 represented with ‘n.s’ whereas ‘*’ represents P ≤ 0.05; ‘**’ represents P ≤ 0.01 and ‘***’ represents P ≤ 0.001.

Fig. 4. Impact of IFNγ in B16 melanoma metastasis. Panel (A) shows box plots of B16F10 metastasis at 3 weeks after i.v. inoculation of 50,000, 100,000 or 150,000 B16 melanoma cells into WT and Ifng−/− mice (n = 6 for each group, t-test; two
independent experiments). The left part of panel (B) shows the lack of efficacy of systemic treatment with HDC on metastasis formation at 3 weeks after inoculation of B16 melanoma cells into Ifng−/− mice (n = 19-21, t-test; four independent experiments). The right part of panel (B) shows effects of systemic treatment with HDC on metastasis formation (% of control) in Ifng−/− mice that received the adoptive transfer of purified NK cells from WT mice (n = 9 for each group, t-test) or purified NK cells from Ifng−/− mice (n = 6, t-test; two independent experiments). Panel (C) shows the presence of WT Ifng in peripheral blood collected from six representative Ifng−/− mice who had received adoptive transfer of WT NK cells (lanes 1-3) or Ifng−/− NK cells (lanes 4-6) 2 days earlier. PCR was performed for WT Ifng and the disrupted IFNγ gene of Ifng−/− mice. Non-significant values where P > 0.05 represented with ‘n.s’ whereas ‘*’ represents P ≤ 0.05; ‘**’ represents P ≤ 0.01 and ‘***’ represents P ≤ 0.001.
Figure 1

A

6-10 week old WT, Nox2⁻/⁻ or Ifng⁻/⁻ mice

Day (-1) Day (0) Day (+1) Day (+3) Day (+20)

HDC (1500 µg) (i.p.) B16 cells (i.v.) HDC (1500 µg) (i.p.) HDC (1500 µg) (i.p.) Analysis

B

B16F10

C

Metastatic foci

WT Nox2⁻/⁻

5x10⁴ 10x10⁴ 15x10⁴

D

B16F1

Metastatic foci

HDC IL-15

WT Nox2⁻/⁻
Figure 2

A

ROS production (MCPx10^2)

WT Gr1^+
WT Gr1^-
Nox2^/- Gr1^+

Time (min)

B

ROS production (AUCx10^4)

HDC (μM)

***

C

6-10 week old WT mice

-24 h 0 h 0.5 h 24 h

HDC (1500 μg) (i.p)
B16F10 cells (i.v)
Analysis
Analysis

D

0 h

5.2%

0.5 h

23.1%

Gr1

E

%CD11b^+Gr1^+

0 0.5 24

Time after injection (h)

Control HDC

F

ROS production (AUCx10^4)

0 0.5 24

Time after injection (h)

Control HDC

G

Metastatic foci

300

Gr1^+ cells

HDC

n.s
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Updated version
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doi:10.1158/2326-6066.CIR-16-0382

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