Additional file 1

Dual oxidase 1 limits the IFNγ-associated antitumor effect of macrophages

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Running title: DUOX1 is involved in the antitumor effect of macrophages

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Author contributions: L.M. designed the study, performed the experiments, analyzed the results and wrote the manuscript. M. G-d-T. performed experiments; P.H. performed experiments and reviewed the manuscript, and S.B. performed experiments. R.A.L., C.C., R.C. and W.L. provided technical support. C.D. provided the *Duox1*$^{-/-}$ mice and reviewed the manuscript. M.M. supervised the study and wrote the manuscript. E.D. supervised the study and reviewed the manuscript.

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Supplemental methods

Human tissue samples

Sample collection was performed from the local tumor bank approved by the Ministry of Research (AC-2016-2844). The study was approved by the local scientific and ethical board and waived the need for specific consent. Samples have been already described (Meziani et al., 2018).

Histopathological analyses and immunohistochemistry

For detection of human macrophages and DUOX1, paraffin human lung sections (irradiated (n=4) and nonirradiated (n=4)), were processed for heat-induced antigen retrieval, incubated with mouse monoclonal anti-human CD163 antibody (*Diagnostic BioSystems, USA,* 1:100) and anti-DUOX1 (home-made previously described (Ameziane-ElHassani *et al*., 2015). Staining was visualized using the peroxidase/diaminobenzidine Mouse PowerVision kit (*ImmunoVision Technologies*) and examined using microscopy virtual slide system (Olympus VS120).

Cytokine profiling

For the simultaneous determination of the relative levels of secreted cytokines and chemokines, the Mouse Cytokine Array Panel A kit (R&D Systems) was used to analyse the culture supernatant from M-CSF-treated BMDMs. The assay was performed according to the manufacturer's instructions. The reactive proteins were visualized using chemoluminescence detection. The images were acquired and quantified using GeneSys imagers coupled to Synoptic 1.4MP cameras (Ozyme).

Phagocytosis assay

Intranasal administration of FluoSpheres: The mice were anesthetized (2% isoflurane) and 20µL of diluted FluoSpheres (1:2, in Physiological serum) were administered intra-nasally for WT and *Duox1*$^{-/-}$ mice. Four hours later, lungs were harvested and digested. FluoSpheres uptake by both CD45^{+} CD11b^{-} Ly6G^{-} CD11c^{+} SiglecF^{+} CD64^{+} alveolar macrophages (AMs) and CD45^{+} CD11b^{+} Ly6G^{-} CD11c$^{-/+}$ SiglecF^{-} CD64^{+} interstitial macrophages (IMs) was assessed via flow cytometry.

H_2O_2 production

H_2O_2 generation was measured in GM-CSF-induced proinflammatory BMDMs by the Amplex Red/HRP assay (Molecular Probes, Invitrogen), which detects the accumulation of a fluorescent oxidized product.

For extracellular H_2O_2 generation, 10^5 cells were suspended in Dulbecco's phosphate-buffered saline (D-PBS) with $CaCl_2$ and $MgCl_2$ and incubated with D-glucose (1mg/ ml), horseradish peroxidase (0.5U/ml), and Amplex red (50µM), and the fluorescence was immediately measured in a microplate reader (Victor3; PerkinElmer) at 30°C for 40min using excitation at 530nm and emission at 595nm. H_2O_2 production was quantified using standard calibration curves. The specific enzymatic activity was expressed as nanomol of H_2O_2 per hour.

Lung cell dissociation

Lungs from WT and *Duox1*$^{-/-}$ mice were digested using Lung Dissociation Kit (Miltenyi Biotec) during 30 minutes at 37°C and 1500rpm. Cells from lungs were filtered through a cell strainer (70µm, Miltenyi biotec) and cells were used for flow cytometry experiments.

Supplemental Figure legends

Figure S1. DUOX1 is expressed in macrophages infiltrating Human lung tissue. Immunohistological assessment of DUOX1 expression (anti-DUOX1, red staining) in human lung tissue-infiltrating macrophages (anti-CD163, brown staining), by DAB (3',3-Diaminobenzidine) in paraffin-imbibed sections of non-irradiated (**A**) and irradiated (**B**) human lung parenchyma between 25Gy and 60Gy. Arrows indicate stained macrophages. (i) Scale bars=1mm. (ii) Scale bars=100um. Data information: human biopsies were obtained from patients receiving thoracic radiotherapy between 1981 and 2012.

Figure S2. DUOX1 deficiency alters H_2O_2 production in GM-CSF-treated macrophages *in vitro*. Extracellular H_2O_2 production activity was measured in GM-CSF treated BMDMs. Data were from one experiment and represented as mean±SEM. n=4-5, **: $P<0.01$ (Student's *t*-test). Associated with Figure 1.

Figure S3. DUOX1 deficiency had no effect on M-CSF-treated macrophages *in vitro*. (**A**) Supernatant from cultured bone marrow derived-macrophages (BMDMs) with M-CSF were analyzed for cytokine secretion. (**B**). Supernatant from M-CSF treated BMDMs was analysed using cytokine arrays and densitometric analysis of signal presented as pixel density and representative blot. Data were obtained from one experiment and represented as mean±SEM. n=3. (**C**) Expression of cultured BMDM proteins were analyzed by flow cytometry. Data were obtained from two independent experiments and represented as mean±SEM. n=4. Associated with Figure 1.

Figure S4. DUOX1 controls macrophage phagocytotic function in the lung. (**A**) FluoSpheres were injected intranasally in WT and *Duox1*$^{-/-}$ mice. (**B**) Four hours later, alveolar macrophages (AMs) and interstitial macrophages (IMs), which have captured the FluoSpheres have been analyzed by flow cytometry. (**C**) The uptake capacity of FluoSpheres by AMs and IMs was represented as mean fluorescence intensity (ΔMFI= MFI of FluoSpheres^{+} AMs/IMs -MFI of control AMs/IMs). Data were obtained from two independent experiments and represented as mean±SEM. n=2-3. Associated with Figure 2.

Figure S5. Tumor growth assessment in WT and *Duox1*$^{-/-}$ mice. (A) MC38 cells were injected subcutaneously in WT and *Duox1*$^{-/-}$ mice and tumor growth was monitored. (**B**) MC38 cells were injected subcutaneously in WT and *Duox1*$^{-/-}$ mice. At day 8, tumors were irradiated (RT) at 8Gy and tumor growth was monitored. Associated with Figure 3 and 4, respectively.

Figure S6. Expression of the inhibitory costimulatory recpetors CTLA4 and PD-1 on lymphoid cells. (**A**) Mean fluorescence intensity (ΔMFI= mean fluorescence intensity of antibody-mean fluorescence intensity of isotype control) of CTLA4 expression on tumor Tregs, $CD4^+$ T cells, $CD8^+$ T cells and NKs and representative histograms of $CTLA4^+$ tumor-infiltrating Tregs, $CD4^+$ T cells, $CD8^+$ T cells and NK cells are shown. (**B**) Mean fluorescence intensity (ΔMFI= mean fluorescence intensity of antibody-mean fluorescence intensity of isotype control) of PD-1 expression on tumor Tregs, $CD4^+$ T cells, $CD8^+$ T cells and NKs and representative histograms of $PD\text{-}1^+$ tumor-infiltrating Tregs, $CD4^+$ T cells, $CD8^+$ T cells and NKs are shown. Data were obtained from two independent experiments and are represented as the mean±SEM. n=7-8, p=0.059, p=0.08 (two-way ANOVA). Associated with Figure 5.

Figure S7. Cytokine profile analysis in the tumor tissue. Tumor tissue from mice treated with BMDMs and radiotherapy were analyzed for cytokine secretion. Data were obtained from two independent experiments and represented as mean±SEM. n=7-8.