Polymorphonuclear myeloid-derived suppressor cells limit antigen cross-presentation by dendritic cells in cancer

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Abstract

Dendritic cells (DCs) are critical component of immune responses in cancer primarily due to their ability to cross-present tumor associated antigens. Cross-presentation by DCs in cancer is impaired, which may represent one of the obstacles for the success of cancer immunotherapies. Here, we report that polymorphonuclear myeloid derived suppressor cells (PMN-MDSC) blocked cross-presentation by DCs without affecting direct presentation of antigens by these cells. This effect did not require direct cell-cell contact and was associated with transfer of lipids. Neutrophils (PMN) and PMN-MDSC transferred lipid to DCs equally well, however PMN did not affect DC cross-presentation. PMN-MDSC generate oxidatively truncated lipids previously shown to be involved in impaired cross-presentation by DCs. Accumulation of oxidized lipids in PMN-MDSC was dependent on myeloperoxidase (MPO). MPO deficient PMN-MDSC did not affect cross-presentation by DCs. Cross-presentation of tumor associated antigens in vivo by DCs was improved in MDSC depleted or tumor-bearing MPO KO mice. Pharmacological inhibition of MPO in combination with checkpoint blockade reduced tumor progression in different tumor models. These data suggest MPO-driven lipid peroxidation in PMN-MDSC as a possible non-cell autonomous mechanism of inhibition of antigen cross-presentation by DCs and propose MPO as potential therapeutic target to enhance the efficacy of current immunotherapies for cancer patients.
**Introduction**

Dendritic cells (DCs) are highly specialized antigen presenting cells (APC) (1, 2). They can be broadly divided into three subsets: classical DCs (cDC), plasmacytoid DCs (pDCs), and inflammatory DCs. Classical DCs consist of two large groups: cDC type 1 (cDC1) and type 2 (cDC2). cDC1 are key players in the regulation of cancer immune responses due to their ability to cross present antigens to CD8⁺ T cells and to generate cytotoxic effector T cell responses (3-6). Their presence is critically important for the success of immunotherapies, such as adoptive T cell transfer and checkpoint blockade (7-9). Defective function of cDC1 was described in several types of cancer, which contributed to ineffective immune responses (3, 5). We and others have found that accumulation of lipids dramatically affected the antigen cross-presentation by cDC1 resulting in an impaired induction of specific anti-tumor CD8⁺ T cell responses (10-13). Recent studies demonstrated specific role of lipid bodies (LB) containing oxidatively truncated species of lipids in negative regulation of cross-presentation by cDC1 (10). However, the source of oxidized lipids in DCs remained unclear since the machinery for lipid oxidation is largely missing in cDC1.

Myeloid derived suppressor cells (MDSC) are pathologically activated neutrophils (PMN) and monocytes (Mon) with potent immune suppressive activity. These cells are vital component of the immunosuppressive network that supports tumor progression and limits the efficacy of cancer therapies (14, 15). MDSC consist of two main subsets of cells: monocytic (M-MDSC) and polymorphonuclear (PMN-MDSC) cells. PMN-MDSC are most abundant population of MDSC. We and others have shown that accumulation of lipids in PMN-MDSC is a critical driver of their suppressive functions (16-19). PMN-MDSC are characterized by high production of reactive oxygen species, as well as elevated level of myeloperoxidase (MPO), which may contribute to
formation of various species of oxidized lipids. In this study we tested the hypothesis that PMN-MDSC could regulate cross-presentation by cDC1.
Results

We generated CD172a^+CD103^+ cDC1 \textit{in vitro} from bone marrow (BM) hematopoietic progenitor cells (HPC) of naïve mice with GM-CSF and FLT3L (Fig. 1A). cDC1 were cultured with PMN from control mice, PMN-MDSC, or M-MDSC from spleen of Lewis Lung Carcinoma (LLC) tumor bearing (TB) mice. Immune suppressive activity of PMN-MDSC was confirmed in antigen-specific suppression assay (Fig. S1). After 24h, cDC1 were isolated from the co-culture with MDSC and loaded with OVA-derived long peptide (Pam)2-KMFVESIINFEKL, which requires processing and cross-presentation or OVA-derived short peptide (SIINFEKL) that directly binds to MHC class I H2Kb (pMHC). Co-culture of cDC1 with PMN did not affect cross-presentation or direct presentation of antigens by DC as compared to DC cultured alone (Fig. S2A). In contrast, PMN-MDSC dramatically reduced the ability of cDC1 to present OVA-derived antigens to peptide-specific OT-1 CD8^+ T cells (Fig. 2B) without impairing the direct presentation of short peptide (Fig. 2C). We asked whether co-culture with DCs affected the viability of PMN and PMN-MDSC differently. We assessed the number of granulocytes at start of the culture and after 24 hr culture with DCs. No differences in the total number of granulocytes or fold changes from baseline were found (Fig. S2B). Similarly, no differences from background (DCs cultured alone) were found in the number of DCs or cDC1 after co-culture with PMN-MDSC or PMN (Fig. S2C).

cDC1 after co-culture with PMN or PMN-MDSC stimulated allogeneic CD8^+ T cells equally well (Fig. 1D). No differences were seen in the expression of molecules involved in T-cell stimulation: MHC I, CD40, CD80, CD86 and PD-L1 on the surface of cDC1 cultured with PMN and PMN-MDSC (Fig. 1E). M-MDSC did not affect cross-presentation of DCs as compared to control monocytes (Fig 1F). Thus, only PMN-MDSC, but not PMN or M-MDSC selectively inhibited DC cross-presentation. We asked, whether direct cell-cell contact was required for PMN-MDSC
mediated inhibition of DC cross-presentation. Separation of PMN-MDSC and CD103⁺ cDC1 using semi-permeable Transwell (0.4 µm) membrane did not abrogate the negative effect of PMN-MDSC on cross-presentation by cDC1. Direct presentation of peptide was not changed (Fig. 1G). The data with Transwells suggested that PMN-MDSC affected DC cross-presentation via soluble factors. PMN-MDSC release different cytokines and factors (arginase, S100A8/A9, IL-6, PGE2, etc) that can inhibit DC function. However, they are known to affect broad functional activity of cells (20-23), rather than selectively cross-presentation as observed in our experiments. We did not find changes in the ability of DCs to activate T-cell proliferation after direct presentation of short peptide or stimulation of allogeneic T cell proliferation (a hallmark of DC activity), as well as expression of MHC class I and major costimulatory molecules or PD-L1 (Fig. 1C-E).

Since, the accumulation of lipids was previously shown to be responsible for the defective antigen cross-presentation by DC (10) and for the regulation of PMN-MDSC suppressive functions (18), we hypothesized that PMN-MDSC might inhibit antigen cross-presenting ability of DC by acting as a source of transferable lipids. To test this hypothesis, we assessed the lipids in cDC1 after co-culture with PMN or PMN-MDSC and BODIPY 493/503 staining. By using confocal microscopy (Fig. 2A) and flow cytometry (Fig. 2B) we found that co-culture with PMN or PMN-MDSC caused very substantial, but similar accumulation of lipids in cDC1. To confirm these data, we stained PMN or PMN-MDSC with BODIPY 493/503 prior to the co-culture with DC, washed the excess of BODIPY and cultured for 24h with DCs. Both PMN and PMN-MDSC were able to transfer lipids to cDC1 (Fig. 2C). Transfer of lipids did not require direct cell-cell contact. PMN-MDSC were stained with BODIPY 493/503. Excess of BODIPY was washed away and cells then were co-cultured with cDC1 separated by Transwells (0.4 µm). Transwells did not prevent the transfer of lipids from PMN-MDSC to DC (Fig. 2D).
To quantitatively characterize the transfer of lipids from PMN-MDSC to DCs, we loaded PMN-MDSC with deuterium labeled linoleic acid (LA-d4) and assessed the incorporation of LA-d4 into different types of lipids in DCs. PMN-MDSC derived free LA-d4 and its elongation product, free arachidonic acid (AA-d4), were found in DCs as non-oxidized fatty acids as well as their mono-oxygenated species (Fig. 2E, Fig. S3). Moreover, LA-d4 and AA-d4 esterified into non-oxidized TAGs and oxidized TAGs were detected in both PMN-MDSC and, importantly, in DCs (Fig. 2F, Fig. S3). Thus, taken together, these data indicate that although both PMN and PMN-MDSC can transfer lipids to CD103⁺ cDC1, only PMN-MDSC affected cross-presentation by DCs. This suggested that the nature of lipids released from PMN-MDSC could be a factor that affects cross-presentation.

Previous studies implicated accumulation of lipid bodies (LB) in defective DC cross-presentation (10, 11). We evaluated whether PMN and PMN-MDSC were able to facilitate accumulation of LB in DCs. Co-culture of cDC1 with either PMN or PMN-MDSC resulted in accumulation of LB in cDC1 (Fig. 2A) indicating that appearance of large LB was not enough for defective cross-presentation. Previous studies demonstrated that only LB containing oxidatively truncated lipids, particularly TAGs, impaired DC cross-presentation(10). Thus, it appears that the chemical nature rather than the amounts of accumulated lipids may have an impact on DC cross-presentation. Therefore, we employed quantitative LC-MS analysis to assess the amounts of oxidatively-truncated TAGs in PMN and PMN-MDSC. We found that PMN-MDSC from TB mice had markedly higher amounts of oxidatively truncated lipids than PMN from tumor free mice (Fig. 3A). Similar species of lipids accumulated in DCs in the presence of tumor explant supernatants (TES) (Fig. S4) consistent with the previous observation (10).
PMN-MDSC have efficient oxidative machinery with MPO and NADPH oxidase as its key components. To determine whether MPO or NADPH oxidase are responsible for the generation of oxidized lipids in PMN-MDSC, we analyzed lipid profile in PMN-MDSC isolated from spleen of EL4 TB WT, MPO, or GP91 (component of NADPH oxidase complex) KO mice. We found that the amount of oxidatively truncated TAG was dramatically reduced to essentially the same low levels in both types of KO mice. In contrast only modest decrease in the amount of total TAG was detected (Fig. 3B).

Next we sought to determine the impact of MPO and GP91 on the ability of PMN-MDSC to block cross-presentation by DCs. We co-cultured cDC1 with PMN-MDSC obtained from spleen of LLC and EL4 TB MPO KO or GP91 KO mice. cDC1 were then loaded with OVA long or short peptides and used to stimulate OT-1 CD8+ T cells. The amount of lipids transferred to cDC1 by WT and KO PMN-MDSC was the same (Fig. S5A). Co-culture with WT PMN-MDSC altered cross-presenting ability of cDC1. However, co-culture with PMN-MDSC from MPO KO or GP91 KO mice did not have an effect cross-presentation of DCs (Fig 3C,D). The direct presentation of short peptide by cDC1 (Fig. 3C,D) and the expression of MHC I, CD40, CD80, CD86 and PDL-1 molecules on DC surface were not affected (Fig. S5B).

Next, we sought to assess the impact of MDSC on DC cross-presentation in vivo by depleting MDSC using anti-DR5 antibody described previously (24, 25). Lung cancer cells with expression of OVA (LLC-OVA) were injected s.c. Two weeks later when tumors reached 0.5 cm, mice were treated with control immunoglobulin or DR5 antibody twice with 3 days interval. Reduction of PMN-MDSC and M-MDSC in spleens was verified by flow cytometry (Fig. S6A). Short treatment with DR5 antibody did not significantly affect tumor growth (Fig. S6B). cDC1 (CD11c+MHCI+CD103+CD11b-CD172a+) and cDC2 (CD11c+MHCI+CD103-
CD11b+CD172a+) were isolated from draining lymph nodes (dLNs) and used to stimulate OT-1 CD8+ T cells. cDC1 isolated from mice with MDSC depletion showed a higher ability to stimulate the proliferation of specific CD8+ T cells, compared to cDC1 isolated from untreated mice (Fig. S6C).

DR5 antibody reduced both populations of MDSC. Although those results were suggestive of potential role of MDSC in DC cross-presentation, we tested more specific mechanism associated with PMN-MDSC function and formation of oxidized lipids by using MPO KO mice. We did not test this hypothesis in GP91 KO mice because superoxide is important for the DC cross-presentation and GP91 KO DC showed defective cross-presentation (26). Thus, it would have made difficult the interpretation of data.

LLC-OVA tumors were established s.c. Two weeks later when tumors reached 1 cm in diameter (tumor sizes in WT and MPO KO mice were similar) (Fig. 4A), cDC1 and cDC2 were isolated from dLNs and their ability to stimulate OVA-specific OT-1+ T cells was assessed. cDC1 isolated from MPO KO LLC-OVA TB mice had markedly higher ability to cross-present OVA-derived peptide than cDC1 from wild-type mice (Fig. 4B). As expected, cDC2 had low cross-presenting activity and it was similar in WT and MPO KO mice (Fig. 4B). Both DC populations from WT and KO mice showed similar stimulation of allogeneic T cells (Fig. 4C). PMN-MDSC from KO mice were not able to suppress antigen specific CD8+ T cell responses in vitro compared to WT PMN-MDSC (Fig. 4D).

These results suggested that deletion of MPO in PMN-MDSC may have strong effect on cross-presentation by DCs. Therefore, we investigated whether the pharmacological inhibition of MPO affected the efficacy of immunotherapy in TB mice. Mice were injected s.c with LLC (Fig. 5A) or B16F10 (Fig. 5B) tumor cells and treated with MPO inhibitor 4-ABAH (4-Aminobenzoic
hydrazide 95%) twice per day starting from day 5 and PD1 antibody twice per week, starting from
day 10. Neither PD1 antibody nor MPO inhibition had anti-tumor effects in these models. However
the combination, markedly decreased tumor growth in both models (Fig. 5 A,B). The antitumor
effect of combination of MPO inhibitor and PD1 antibody was abrogated in mice depleted of CD8+ 
T cells (Fig. 5C) indicating that observed antitumor effect was dependent on generation of
antitumor CD8+ T cells. To ascertain that observed effect was dependent on cross-presentation,
we used BATF3 KO mice, which are depleted of cDC1(27). In the absence of cDC1 in BATF3 
KO mice the effect of combined treatment was abrogated (Fig. 5D).
Discussion

Our study suggested novel mechanism of negative regulation of cross-presentation by DCs in cancer, involving possible transfer of oxidized lipids from PMN-MDSC to cDC1. PMN-MDSC are dramatically expanded in many tumor models in mice and different types of human cancers and infiltrate tumors and lymphoid organs (15, 28). These cells are characterized by immune suppressive activity, distinct biochemical and transcriptomics profile compared to normal neutrophils (15). Classical PMN respond to various pathogens and trauma by respiratory burst and degranulation with the release of anti-bacterial and antiviral enzymes. They rapidly die in the process. As a result, even activated classical PMN usually lack immune suppressive activity. However, their antitumor activity is also difficult to detect. In contrast, PMN-MDSC, produce number of factors (arginase 1, prostaglandin E2, sustained level of superoxide and peroxynitrite, etc.) that inhibit function of T cells and other cells of immune system (15). As a result, these cells may blunt antitumor response and promote tumor progression and limit the effect of immune therapy of cancer. In mice, tumor PMN-MDSC are more suppressive than in spleens due to up-regulation of Nos2 expression and several other suppressive mechanisms regulated by hypoxia as well as other factors present in tumors (28). It is known that spleen PMN-MDSC have relatively weak suppression of T cells function as compared to M-MDSC and tumor associated macrophages (14, 28). Therefore, well established association between increased presence of PMN-MDSC in blood and negative outcomes in cancer patients was rather puzzling. Our study showed that PMN-MDSC can blunt the ability of DCs to cross-present antigens and thus demonstrated potential role of PMN-MDSC in blocking the priming phase of immune responses in cancer by affecting cross-presenting cDC1 and thus contributing to the resistance to checkpoint blockade therapy.
The conclusion that lipid transfer from PMN-MDSC to DCs can inhibit cross-presentation is based on several previous and current findings.

1). LB containing oxidatively truncated lipids blocked cross-presentation of DCs (10, 11). LB containing electrophilic oxidatively truncated lipids covalently bound to chaperone heat shock protein 70. This interaction prevented the translocation of peptide-MHC class I complexes to cell surface by causing their accumulation inside late endosomes/lysosomes. As a result, DCs were no longer able to stimulate adequate CD8$^+$ T cells responses (10). Loading of DCs with oxidized but not with non-oxidized fatty acids inhibited cross-presentation by DCs (10).

2). PMN-MDSC and PMN transfer lipids to DCs, but only PMN-MDSC blocked cross-presentation.

3). PMN-MDSC in contrast to PMN are enriched for oxidized lipids. This effect depends on MPO and NADPH oxidase activity also known to be substantially increased in PMN-MDSC as compared to PMN (14, 29, 30).

4). In the absence of MPO or NADPH oxidase, PMN-MDSC lose the ability to block cross-presentation and inhibition of MPO in PMN-MDSC in TB mice dramatically improved cross-presentation by DCs and antitumor effect of immunotherapy.

Although MPO is detected in monocytes, its amount and activity are much higher in neutrophils, which may explain why M-MDSC lack the ability to block cross-presentation by DCs. MPO expression is generally lost during differentiation of monocytes to macrophages (31, 32). MPO is not expressed in DCs, which further supports the role of PMN-MDSC in blocking DC cross-presentation. It is important to point out that MPO deficiency also affect PMN-MDSC suppressive activity. However, at the time of evaluation, MPO KO and WT mice had similar tumor size, which excludes the effect of differences in tumor burden on DC cross-presentation. Moreover, previous
study demonstrated that MPO can directly inhibit expression of activation markers in DC and the ability of DC to stimulate allogeneic responses (33). The fact that PMN-MDSC had selective effect only on cross-presenting ability of DCs strongly suggested that this effect was not mediated via release of MPO. MPO and NADPH oxidase may affect not only lipids, but also various proteins, so it is possible that regulation of cross-presentation by PMN-MDSC is more complex process than lipid transfer. However, unlike oxidized lipids, oxidized proteins are not released from live PMN-MDSC. Our data indicate that most of PMN-MDSC remained alive during co-culture with DCs, which make this mechanism less likely. More studies will be needed to clarify this mechanism. However, regardless of the specific mechanism, our findings suggest that targeting of MPO enhanced the effect of check-point inhibitor, which may suggest novel therapeutic opportunity.
Methods

Mouse models. Animal experiments were approved by The Wistar Institute Animal Care and Use Committee. Balb/c or C57BL/6 mice (female, 4–6 week old) were obtained from Charles River, OT-I TCR-transgenic mice (C57Bl/6-Tg(TCRαTCRβ)1100mjb) (female, 4–6 week old), PMEL TCR-transgenic mice (B6.Cg-Thy1a/Cy Tg(TcraTcrob)8Rest/J) (female, 4–6 week old), MPO KO (B6.129X1-Mpotm1Lus/J) (female, 4–6 week old), GP91 KO (B6.129S-Cybbtm1Din/J) (female, 4–6 week old), CD204 KO (B6.Cg-Msr1tm1Csk/J) (female, 4–6 week old), Batf3 KO (129S-Batf3tm1Kmm/J) (female, 4–6 week old) were purchased from Jackson Laboratory.

Reagents and cell lines. Tumor cell lines including EL4 lymphoma, CT26 (colon carcinoma) and LLC (Lewis Lung Carcinoma) were maintained in DMEM medium supplemented with 10% fetal bovine serum (FBS, Atlanta) at 37 °C, 5% CO2. LLC-OVA were maintained in complete DMEM supplemented with 0.5 mg/ml of G418 (Gibco, USA). Tumors were injected subcutaneously (s.c.) on the flank of the animal at $5 \times 10^5$ cells per mouse. Tumor cell lines were tested for mycoplasma contamination by using Universal Mycoplasma detection kit (ATCC). SIINFEKL peptide and control peptide RAHYNIVTF were obtained from American Peptide Company (Vista, CA), (Pam)2–KMFVESINFEKL peptide (derived from OVA) and (Pam)2-KMFVKVPRNQDWL (derived from gp100) were obtained from DBA Synthetic biomolecules (San Diego). Anti-mouse CD103 biotin conjugated beads were purchased from BioLegend, and used for CD103+DC purification. APC conjugated anti mouse CD11c, Percp 5.5 conjugated anti-mouse MHC I, APC-cy7 conjugated anti-mouse CD11b, Pe conjugated anti-mouse Ly6G, Apc conjugated anti-mouse Ly6C, Pe-cy7 conjugated anti-mouse CD3 and AF700 and FITC anti-mouse CD45 antibodies were purchased from BD Bioscience. FITC conjugated anti-mouse F4/80 and Percp 5.5 conjugated anti-mouse CD11c were purchased from Invitrogen. Pe-cy7 anti-mouse MHC II, CD172 and CD40,
AF700 conjugated anti-mouse CD86, BV421 conjugated anti-mouse CD103 or XCR and APC conjugated anti-mouse PDL-1 were purchased from BioLegend. Aqua live was obtained from Invitrogen. BODIPY lipid dye 493/503 was obtained from Invitrogen. List of antibodies is provided in Table S1.

**Cell phenotype and lipid contents by flow cytometry.** DC were incubated with FC-block (BD Biosciences) for 5 min and surface staining was performed at 4 °C for 15 min. Cells were run on Celesta flow cytometer (BD Biosciences) and data were analyzed by FlowJo (Tristar). For lipid staining, cells were re-suspended in 500 μl of Bodipy 493/503 at 0.25 μg/ml in PBS. Cells were stained for 15 min at room temperature in the dark, then washed twice, re-suspended in PBS and run immediately on Celesta. For lipid transfer staining, PMN MDSC (or other cells) were re-suspended in 500 μl of Bodipy 493/503 at 0.25 μg/ml in PBS, then stained for 15 min at room temperature in the dark, washed twice, re-suspended in complete media and co-cultured with unstained DC. After 24h cells were stained for surface staining. At least 10,000 cells were collected for subsequent analysis.

**Generation of DCs.** Mouse DCs were generated from enriched BM hematopoietic progenitor cells (HPCs) with 10 ng/ml of GM-CSF (Peprotech) and 100 ng/ml of FLT3-L (Peprotech). Briefly, HPCs were isolated from mouse BM by using Lineage depletion kit (Miltenyi), according to manufacturer’s instructions. Cells were seeded at 3x10⁴ cell/ml in 24 well plates; GM-CSF (10 ng/ml) and FLT3-L (100 ng/ml) were added to the culture at day 0 and day 3. At day 5, cells were split and cultured with fresh media with cytokines at 7.5x10⁵ cell/ml in 6 well plates. Cytokines were then added every 3 days. Cells were finally harvested and used between day 11 and day 14. DC phenotype was analyzed before performing each experiment to check the percentage of CD103⁺ DC (80-90%).
Co-cultures. Harvested DCs were co-cultured with FACS sorted (BD FACS Aria II) PMN-MDSC (CD45+CD11b+Ly6G+) from spleen or tumor of TB mice, PMN naïve (CD45+CD11b+Ly6G+) from BM or spleen of naïve mice, mMDSC (CD45+CD11b+Ly6C+) from spleen of TB mice, naïve Monocytes (CD45+CD11b+Ly6C+) from BM or spleen of naïve mice, in the ratio 1:4 (DC : other cells). Sorted cells were obtained from both LLC and EL-4 TB mice, sacrificed between day 21 and day 28 after tumor challenge. When comparing WT TB mice with KO TB mice, animals with similar sized tumors were used. Spleens were mechanically dissociated. BM was flushed with precision needle and 1ml syringes. Cells were plated at the density of 1x10⁶ cells/ml of complete media containing GM-CSF (10 ng/ml) and FLT3-L (100 ng/ml). For co-cultures with Transwell system, 0.4 μm pore size Transwell inserts for 6 well plate (Corning) were used. DC were plated in the lower compartment, while other cells were plated in the upper compartment. Cells were plated at the density of 1x10⁶ cells/ml of complete media containing GM-CSF (10 ng/ml) and FLT3-L (100 ng/ml). After 24h of co-culture, cells were harvested and CD103⁺ DC were isolated with anti-mouse CD103 biotin conjugated beads (BioLegend) and streptavidin Microbeads (Milteny). The purity has been checked for each cell type co-culture (≥95%).

Cross-presentation of OVA derived long peptides. Isolated CD103⁺ DCs were loaded for 16–18 h with 5 μg/ml of long peptides. Splenocytes were isolated from spleens of responder mice by using mechanical digestion and then plated at 2.5-10 x 10⁴ splenocytes per well (depending on the experiment). DC and splenocytes were mixed at different ratios. DC were loaded with 0.5 μg/ml of SIINFEKL or Gp100 control peptide for 1 h at 37 °C. After antigen loading, DCs have been washed twice in complete media to remove the excess of peptide. In some experiments, DC were used to stimulate allogeneic splenocytes isolated from spleen of Balb/e mice. Before mixing DCs with splenocytes, some DCs for each condition has been analyzed for surface activation markers.
After 30h (for direct presentation) or 48h (for cross-presentation) or 96h (for allogenic MLR), 
$[^{3}\text{H}]$-thymidine was added at 1 μCi per well for an additional 18 h followed by cell harvesting and a radioactivity count on liquid scintillation counter.

**Cross-presentation in vivo.** LLC OVA was injected subcutaneously (s.c.) on both the flank of the mice at $5 \times 10^5$ cells per mouse. After 20 days, mice were euthanized and draining lymph nodes (for each flank: 2 axillar, 1 inguinal) were collected. LN were then digested for 30 min at 37 °C with collagenase A (0.5 mg/ml; Sigma Aldrich), Dnase I (0.2 mg/ml, Roche), diluted in HBSS with Ca$^{2+}$/Mg$^{2+}$ and 20 mM EDTA (Invitrogen) was added 5 min at room temperature to stop the reaction. Single suspensions were prepared and then DC were stained and sorted on BD FACS AriaII (BD Biosciences). CFSE labeled OT1 CD8+ T cells, isolated by using EasySep Mouse CD8+ T Cell Enrichment Kit (STEMCELL), were used as responders at 1:5 ratio (5,000 DC : 25,000 T cells). Proliferation was checked 3 days after by FACS Celesta. Allogenic stimulation of CFSE labeled CD8+ T cells isolated from spleen of Balb/c mice was used as control.

**Confocal microscopy.** Dendritic cells were washed twice with PBS, resuspended in complete RPMI and 50,000 cells were seeded on poly-L-lysine cellware 12 MM round coverslips (Corning) for 45 min at 37 °C. After that time, cells were washed with PBS and were stained for surface markers. Briefly, cells were incubated with Fc-block (BD Biosciences) for 10 min, stained with unconjugated antibodies for 15 min at 4 °C, washed twice with PBS before incubation with fluorochrome associated secondary antibodies. Afterwards, cells were fixed and permeabilized with Fixation & Permeabilization Buffers (BD Biosciences) for 15 min at RT, washed twice with wash buffer (BD Biosciences), and then blocked with PBS containing 5% FBS for 45 min. Cells were incubated with Fc-block for 5 min at RT and stained with different primary antibodies, at 4 °C for 16–18 h. Cells were washed three times and incubated with fluorochrome associated
antibodies for 45 min at RT. After that time cells were washed three times and then stained with BODIPY, to detect lipid bodies for 15 min at RT. Cells were washed and incubated with DAPI and mounted on slides using Prolong Gold antifade reagent (Life Technology). The cells were imaged with a Leica TCS SP5 laser scanning confocal microscope (Leica Microsystems).

**Suppression assay.** Single cells suspensions from spleen and tumors were prepared as described above. Then cells were stained and sorted on BD FACS Aria BD (Biosciences). PMN-MDSC (CD45⁺CD11b⁺Ly6G⁺Ly6Clo) and M-MDSC (CD45⁺CD11b⁺Ly6G⁻Ly6Chi) were plated in U-bottom 96-well plates (3 replicates) in RPMI with 10% FBS and co-cultured at different ratios with splenocytes from Pmel or OT-1 transgenic mice in the presence of cognate peptides: OT-1 (SIINFEKL; 0.1 ng/ml), Pmel (EGSRNQDWL; 0.1 μg/ml). After 48 h, cells were incubated with [3H]-thymidine (PerkinElmer).

**α-DR5 treatment, MPO inhibitors and checkpoint blockade.** Mice were challenged (day 0) with LLC OVA on both the flanks at 5 x 10⁵ cells per mouse. 14 days after the challenge (when the tumor reached 0.5 cm of diameter), the animals were randomly allocated in 2 different groups. One of those received α-DR5 (anti-mouse DR5 (CD262), MD5-1, Bio Cell) intraperitoneally (i.p.), every 3 days starting from day 5 after the challenge, at a concentration of 100 μg/mouse in 100 μl of PBS (Corning). The other group was treated with the control immunoglobulin (in 100 μl of PBS). Percentage of Mon and PMN out of live cells was assessed in blood.

Mice were challenged (day 0) with LLC on the flanks at 5 x 10⁵ cells per mouse. 4 days after the challenge, mice were randomly allocated in 4 different groups. MPO inhibitor 4-ABAH (4-Aminobenzoic hydrazide 95%, Sigma Aldrich) was given i.p. at a dose of 40 μg/g in 400 μl of HBSS 1X (Hank’s Balanced Salt Solution, Gibco), twice a day starting from day 4 after tumor challenge. α-PD1 (anti-mouse PD1 (CD279), RMP1-14, Bio Cell) was given i.p. at a dose of 200
μg per mouse in 100 μl of PBS, every 3 days starting from day 8 after tumor challenge. One group received the vehicle, one group received α-PD1 + vehicle, one group received 4-ABAH and the last group received the combination of 4-ABAH and α-PD1. The same experiment was repeated with the addition of α-CD8 treatment. α-CD8 (anti-mouse CD8a, 53-6.7, Bio Cell) was given i.p. at a dose of 100 μg per mouse, twice a week starting from day 1 after tumor challenge.

**Liquid chromatography and mass-spectral analysis of deuterated and non-deuterated free fatty acids and neutral lipids.** Lipids were extracted by Folch procedure with slight modifications, under nitrogen atmosphere, at all steps. Free fatty acids were analyzed using a Dionex Ultimate™ 3000 HPLC system coupled on-line to Q-Exactive hybrid quadrupole-orbitrap mass spectrometer (ThermoFisher) using a C18 column (Acclaim PepMap RSLC, 300 μm 15 cm, ThermoFisher). Gradient solvents (A: Methanol (20%)/Water (80%) (v/v) and B: Methanol (90%)/Water (10%) (v/v) both containing 5 mM ammonium acetate was used. The column was eluted at a flow rate of 12 μL/min using a linear gradient from 30% solvent B to 95% solvent B over 70 min, held at 95%B from 70 to 80 min followed by a return to initial conditions by 83 min and re-equilibration for an additional 7 min. Spectra were acquired in negative ion mode. The scan range for MS analysis was 150-600 m/z with a maximum injection time of 100 ms using 1 microscan and a resolution of 140,000. An isolation window of 1.0 Da was set for the MS and MS² scans. Capillary spray voltage was set at 2.6 kV, and capillary temperature was 250 °C. The S-lens Rf level was set to 60. Analytical data were acquired and analyzed using Xcalibur software.

TAGs were analyzed as molecular ammonium adducts (TAG+NH₄⁺) using a Dionex Ultimate™ 3000 HPLC system coupled on-line to Q-Exactive hybrid quadrupole-orbitrap mass spectrometer (ThermoFisher) using a Luna 3 μm C18 (2) 100A, 150 × 1.0 mm column (Phenomenex) at a flow rate of 0.065 ml/min. The column was maintained at 35 °C. The analysis was performed using
gradient solvents (A and B) containing 0.1% NH₄OH. Solvent A was methanol and solvent B was propanol. The column was eluted for 2 min from 0% B to 2% B (linear), from 3 to 6 min with a linear gradient from 2% solvent B to 3% solvent B, then isocratically from 3 to 18 min using 3% solvent B, 18 to 35 min with a linear gradient from 3% solvent B to 40% solvent B, 35 to 60 min using a linear gradient from 40 to 55% solvent B, then isocratically from 60 to 65 min at 55% solvent B then from 65 to 80 min from 55 to 0% B (linear) followed by equilibration from 80 to 90 min at 0% B. MS analysis was performed in positive ion mode at a resolution of 140,000 for the full MS scan and 17,500 for the MS² scan in a data-dependent mode with an inclusion list for TAG. The scan range for MS analysis was 300–1200 m/z with a maximum injection time of 128 ms using one micro scan. A maximum injection time of 500 ms was used for MS² (high-energy collisional dissociation (HCD)) analysis with collision energy set to 24. An isolation window of 1.0 Da was set for the MS and MS² scans. Capillary spray voltage was set at 4.5 kV, and capillary temperature was 320 °C. Sheath gas was set to eight arbitrary units and the S-lens Rf level was set to 60.

**Statistical analysis.** Statistical analysis was performed using unpaired two-tailed Student's t test and ANOVA with corrections for multiple comparison when more than 2 groups were compared, with significance determined at p < 0.05. Estimation of variation within each group of data was performed and variance was similar between groups that were compares. Animal experiments were not blinded.

**Data availability.** All relevant data are available within the articles and its supplementary information and from the authors upon reasonable request.
**Authors contributions**

AU, VT, ET, LD, – performed experiments,

Y.T. – performed data analysis,

VEK – designed experiments, analyzed data, wrote manuscript,

DIG – designed overall concept and experiments, analyzed data, wrote manuscript

FV – performed and designed experiments, analyzed data, wrote manuscript

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**Figure legends**

**Figure 1. PMN-MDSC inhibit antigen cross-presentation by CD103⁺DC.** A. Example of gating strategy for the identification of cDC1 (CD172⁻CD103⁺DC) and cDC2 (CD172a⁻CD103⁺DC) in culture of DC differentiated in presence of GM-CSF and FLT3L. B. Proliferation of OT1 CD8⁺ T cells in the presence of CD103⁺DC loaded with OVA-derived long peptides after co-culture with PMN or PMN-MDSC (n=5, each experiment has been performed in triplicate). C. Proliferation of OT1 CD8⁺ T cells after stimulation with CD103⁺DC loaded with OVA-derived short peptide after co-culture with PMN or PMN-MDSC (n=5, each experiment has been performed in triplicate). D. Proliferation of allogenic CD8⁺ T cells after stimulation with CD103⁺DC after co-culture with PMN or PMN-MDSC (n=3, each experiment has been performed in triplicate). E. Analysis of surface markers by flow cytometry in CD103⁺DC after co-culture with PMN (n=6) or PMN-MDSC (n=6). F. Proliferation of OT1 CD8⁺ T cells after stimulation with CD103⁺DC loaded with OVA-derived long peptides after co-culture with monocytes or M-MDSC (n=3, each experiment has been performed in triplicate). G. Proliferation of OT1 CD8⁺ T cells after stimulation with CD103⁺ DC loaded with OVA-derived long peptides (left Panel) or short derived peptides (right panel) after co-culture with PMN-MDSC separated in Transwell system. (n=3). B-D and F-G: representative experiments are shown. Proliferation was measured by ³[H]-thymidine uptake in triplicates. In all experiments mean and SEM are shown *p < 0.05, **p < 0.01 ***p<0.001 in unpaired two-tailed Student's t test or ANOVA with corrections for multiple comparisons when more than two groups were compared.

**Figure 2. Lipid transfer from PMN-MDSC to DCs.** A. Confocal image of lipid bodies (LB) using BODIPY in cDC1 after co-culture with PMN or PMN-MDSC. Scale bar = 50 µm. B. Flow cytometric analysis of lipid content in cDC1 stained with BODIPY after co-culture with PMN and
PMN-MDSC and expressed as fold change to the geometric mean of Bodipy expression in DCs alone (n=3). **C.** Flow cytometric analysis of lipid transfer from PMN-MDSC to cDC1 after coculture with BODIPY labeled PMN and PMN-MDSC. Left panel – representative example of staining; Right panel – cumulative results of the experiments (n=3). **D.** Flow cytometric analysis of lipid content in cDC1 stained with BODIPY after co-culture with PMN-MDSC with or without Transwell system (n=3). **B-D.** In all experiments mean and SD are shown *p < 0.05, **p < 0.01, ***p<0.001 in ANOVA test with corrections for multiple comparisons. **E.** Heat map showing the content of non-oxidized fatty acids LA, LA-d4, AA and AA-d4 elongated from LA-d4 (upper panel) and their mono-oxygenated species (lower panel) in PMN-MDSC and DC after co-culture DC with PMN-MDSC LA-d4. **F.** Heat map showing the content of non-oxidized TAGs molecular species containing LA-d4 and elongated AAd4 (upper panel) and their oxygenated species (lower panel) in PMN-MDSC and DC after co-culture DC with PMN-MDSC LA-d4.

**Figure 3. MPO and NADPH oxidase in PMN-MDSC affect cross-presentation by CD103⁺DC.**
**A.** Content of oxidatively truncated TAGs molecular species in PMN-MDSC from indicated tumor-bearing and PMN from tumor free mice (n=3). ONA - 9-oxononanoic acid. **B.** Content of oxidatively truncated TAGs molecular species (left panel) and total TAGs (right panel) in PMN-MDSC isolated from spleen of EL4 tumor-bearing WT, GP91 KO, MPO KO and PMN from tumor free mice. Please note 1000-fold difference in the scales between panels. (n=3). ONA - 9-oxononanoic acid. **C.** Proliferation of OT1 CD8⁺ T cells after stimulation with CD103⁺DC after co-culture with WT PMN MDSC or MPO KO PMN-MDSC and loading with OVA-derived long peptides (left panel) or OVA-derived short peptide (right panel). (n=3, each experiment has been performed in triplicate). **D.** Proliferation of OT1 CD8⁺ T cells after stimulation with CD103⁺DC after co-culture with WT PMN MDSC or GP91 KO PMN-MDSC and loading with OVA-derived...
long peptides (left panel) or OVA-derived short peptide (right panel). (n=3, each experiment has been performed in triplicate). **C, D.** Representative experiments are shown. Proliferation was measured by $^3$H-thymidine uptake in triplicates. In all experiments mean and SD are shown *p < 0.05, **p < 0.01, ***p<0.001 in two-sided Student’s t-test. In Fig. 3A and 3B ANOVA test with correction for multiple comparisons was performed.

**Figure 4. Effect of MPO deletion on cross-presentation by DCs. pharmacological inhibition of MPO on tumor growth.** **A.** LLC-OVA tumor growth in MPO KO mice (n=3). **B.** Proliferation of OT1 CD8$^+$ T cells stimulated with DC1 (CD11c$^+$MHCII$^+$CD103$^+$CD11b$^+$CD172a$^+$) and DC2 (CD11c$^+$MHCII$^+$CD103$^+$CD11b$^+$CD172a$^+$) isolated from draining lymph nodes of WT and MPO KO mice bearing LLC-OVA tumor (n=4). **C.** Proliferation of allogeneic CD8$^+$ T cells stimulated with CD103$^+$DC (cDC1) and CD172a$^+$DC (cDC2) isolated from draining lymph nodes of WT and MPO KO mice bearing LLC-OVA tumors. Proliferation of CD8$^+$ T cells measured at 72 h by CFSE dilution in triplicates (n=4). **D.** Suppressive activity of PMN-MDSC isolated from spleen of WT or MPO KO tumor bearing mice. Dashed line represented the proliferation of CD8$^+$ T cells in absence of MDSC. Proliferation was measured by $^3$H-thymidine uptake in triplicates. Typical example of three performed experiments is shown. In all experiments mean and SD are shown *p < 0.05, **p < 0.01 in unpaired two-tailed Student's t test between compared groups.

**Figure 5. Effect of pharmacological inhibition of MPO on tumor growth.** **A.** Tumor growth in LLC TB mice, treated with anti-PD1 and MPO inhibitor (each group n=5). **B.** Tumor growth in B16F10 TB mice, treated with anti-PD1 and MPO inhibitor (each group n=8). **C.** Tumor growth in LLC bearing mice depleted of CD8$^+$ T cells and treated with anti-PD1 and MPO inhibitor (each group n=4). **D.** Tumor growth in LLC bearing BATF3 KO mice, treated with anti-PD1 and MPO inhibitor (each group n=5).
inhibitor (each group n=4). Mean with SEM are shown. ***p<0.001, ****p<0.0001. Two-way ANOVA with corrections for multiple comparison were used.
Figure 1
Figure 2
Figure 3: Bar charts and line graphs showing the effect of different cell ratios on protein expression and cell proliferation. The graphs compare the expression levels under various conditions, with labels and statistical significance markers indicating differences between experimental groups.
Figure 4
Figure 5

(A) Tumor Area (mm²) over Days after tumor injection for different treatments: Vehicle, MPOi, Vehicle + αPD1, MPOi + αPD1.

(B) Tumor Growth (mm²) over Days after tumor injection for different treatments: Vehicle, MPOi, Vehicle + αPD1, MPOi + αPD1.

(C) Tumor Area (mm²) over Days after tumor injection for different treatments: Vehicle + αPD1, MPOi + αPD1 + αCD8.

(D) Tumor Growth (mm²) over Days after tumor injection for different treatments: Control, MPOi + αPD1.