Loss of phagocytic and antigen cross-presenting capacity in aging DCs is associated with mitochondrial dysfunction

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Abstract

Impaired functionality of dendritic cells (DCs) significantly contributes to decreased adaptive immune responses in aged hosts. The expression of MHC-peptide on the DC surface is the critical first step in T cell priming, but few studies have addressed the effect of aging on antigen (Ag) acquisition, processing, and presentation by DCs. Here we show that aged murine DCs were less efficient in the cross-presentation of cell-associated Ag and subsequently in the cross-priming of CD8^{+} T cells than their young counterparts.

The decreased cross-presentation was associated with a reduction in the frequency of CD8αDCs and merocytic (CD8α−CD11b−)DCs that could endocytose cell-associated Ag, as well as the number and the size of the endocytosed particles in the DC that did internalize cell-associated materials.

Mechanistically, phagocytic capacity has been associated with mitochondrial activity and membrane potential (ψ_m). Aged DCs exhibited profound signs of mitochondrial dysfunction, illustrated by lower ψ_m, reduced ATP turnover and coupling efficiency, decreased baseline oxidative phosphorylation (OXPHOS), and greater proton leak and ROS production. Mimicking the aged metabolic phenotype in young DCs by pharmacologic manipulation indicated that the reductions in ψ_m and ATP impeded the phagocytic capacity while ROS interfered with a later step in the cross-presentation process. Conversely, in vitro scavenging of ROS partially restored cross-presentation by aged DCs.

Together, these data suggest that improvement of aged DC functionality might be feasible in the elderly, by targeting metabolic dysfunction, or its downstream sequelae, thereby opening new avenues for enhancing vaccine efficiency in this population.

Keywords

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Introduction

Aging has a profound negative impact on the capacity of the immune system to develop efficient effector responses against a vast array of antigens (Ag). It is well established that vaccines are poorly immunogenic in older individuals compared to younger individuals (reviewed in (1)). Similarly, in pre-clinical models, cancer vaccination was less effective in old mice than in young mice, with short lasting and weak T cell responses to tumor-associated Ags (rev. in (2)).

Although age-related intrinsic defects in T cells and B cells, and notably the paucity of naïve T cells in old mice, are critical, it has become clear that alterations within the innate immune system significantly impact the development of adaptive immunity in the aging host. Recently impaired functionality of dendritic cells (DCs) has been identified as a significant contributor in this decreased response (3). Notably, young TCR-transgenic CD4+ and CD8+ T cells exhibited poor expansion in an aged environment in response to their cognate Ags (4, 5). Similarly, the transfer of Ag-pulsed aged DCs into young recipients resulted in the defective induction of Ag-specific endogenous CD8+ T cell responses compared to the transfer of young DCs (6–8).

DCs are a phenotypically and functionally heterogeneous population of leukocytes with distinct functions. Their capacity to activate T cells critically depends on the level of MHC-peptide complexes they display on their surface. High levels of MHC-peptide/TCR interaction have been proposed to lower the T cell's need for additional costimulation to reach full activation. Several groups have studied the effect of aging on the expression of membrane-associated and soluble costimulatory molecules (5, 6, 9–18), but very few studies have addressed the effect of aging on Ag acquisition, processing, and presentation by DCs. Decreases in phagocytic capacity have been reported in aging murine macrophages and CD11c+ cells as well as DCs generated from mouse bone marrow or human monocytes (4, 9, 19–23). However, the molecular mechanisms that confer this defect in phagocytosis remain completely unknown.

Mechanistically, phagocytic capacity and endosomal trafficking have recently been associated with mitochondrial activity and mitochondrial membrane potential (ψ_m) in fibroblasts and bone-marrow derived murine macrophages (24). Park et al showed that uptake of apoptotic cells increased ψ_m and ATP, and that genetic or chemical reduction of ψ_m and/or ATP levels resulted in accelerated trafficking of phagocytosed materials to low pH organelles (24). Importantly, aging is associated with a decline in mitochondrial function, including decreases in mitochondrial number, mitochondrial DNA, protein levels and protein activity (25, 26), as well as a reduced oxidative phosphorylation capacity (OXPHOS) resulting in decreased ATP levels and increased Reactive Oxygen Species (ROS) production (27, 28). Exploring how the mitochondrial function is affected, and its relationship with decreased DC functionality in aging, is thus of particular interest.

To address these important and unresolved questions, we used a well-established model of cross-presentation (the presentation of exogenous Ag on MHC class I) to determine the capacity of the aged DCs to cross-present cell-associated Ags in vitro and *in vivo*. We also

studied whether age-related changes in mitochondrial functions are linked with DC impaired functionality.

Materials and methods

Mice, cells, and peptides

Young (2–3 months) and aged (16–20 months) C57BL/6J mice were obtained from the NIA aging colony. Mice expressing OVA under the actin promoter (actmOVA) were crossed to the $K^{b-/-}$ background in our facility. Mice were maintained under specific pathogen-free conditions in accordance with guidelines by the Association for Assessment and Accreditation of Laboratory Animal Care International.

DCs were isolated from spleens of naive mice as described before (29). Briefly, DCs were first enriched by negative selection using anti-biotin-beads and biotinylated Ab to TCR, CD19, IgM, IgD and NKp46, followed by positive selection with CD11c-beads (Miltenyi). Enriched DCs were either used directly or further (sub)sorted by flow cytometry based on their expression of CD11c, CD11b, and CD8α, using CD3, CD19, IgM, IgD, and NKp46 as lineage/dump markers (MoFlow, Coulter, X). Overall DC purity after flow cytometric sorting was 98% and after bead isolation 80%. In all studies viability was >95% (7-aminoactinomycin D [7-AAD] staining).

$OVA_{257-264}$-specific B3Z hybridoma cells were cultured in IMDM supplemented with 10% FCS, 50 μM 2-ME, 2 mM L-glutamine, 20 U/ml penicillin, and 20μg/ml streptomycin (30). $OVA_{257-264}$ (SIINFEKL) and control peptide LCMV gp_{33-41} (KAVYNFATC) were obtained from A&A Laboratories (San Diego, CA).

DC characterization, endocytosis, and pH studies

Enriched DCs were analyzed for the expression of CD4, CD8α, CD11b, CD11c, XCR1, CD40, CD80, CD86, CD273, CD274, CD275, K^b, D^b, and I-A/E by flow cytometric analysis (Abs/isotype controls: eBioscience/BioLegend, San Diego, CA; using a LSRII, Becton-Dickinson, CA).

For endocytosis studies, enriched DCs were incubated with Cell-Trace Violet-labeled irradiated splenocytes (1:3 ratio) at 4°C and 37°C in the absence or presence of N-acetylL-cysteine (NAC, 0.5mM), Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP, 1 nM), and Oligomycin (50nM). After 4–6 hrs DCs were stained with Abs to CD11c, MHCII, CD11b, CD8α, to identify DC populations together with a fixable live/dead staining and analyzed by flow cytometry or ImageStream (Amnis, Seattle, WA) (29, 31, 32). At least 5,000 events were acquired for each condition. Images of fixed cells were analyzed using IDEAS 6.0. The number and size of phagocytosed particles were determined using the spot counting and spot size features after tight masking on the brightfield image to exclude membrane-associated extracellular particles (29, 31, 32). The measure of phagosomal pH over time in enriched DCs was performed as described in Savina et al (33). Briefly, DCs were cultured with dually-labeled beads (pH sensitive FITC and pH insensitive FluoProbes 647) and analyzed at different time points by flow cytometry, using a gating FCS/SSC selective for cells containing one bead. The ratio of the mean fluorescence intensity (MFI)

emission between the two dyes was determined and compared with a standard curve (ranging from pH 5.5 to 8).

Metabolism parameters

To determine mitochondrial mass, mitochondrial membrane potential (Ψ m), and ROS production, enriched and subsorted DCs were stained with CD11c, CD11b, CD8α and combinations of Mitrotracker-Green, Mitotracker-Deep Red, JC1, and H2D-CFDA (Invitrogen). Cellular ATP was determined using an ATP Bioluminescent Assay Kit (Sigma).

For real-time analysis of oxidative phosphorylation, DCs were analyzed using an XF-24 Extracellular Flux Analyzer (Seahorse Bioscience) (34). 5×10^5 purified DCs were plated in XF Running Buffer (FX media, 10mM Dextrose, 2mM L-glutamax, 1mM sodium pyruvate) per the manufacturer's instructions to obtain real-time measurements of the oxygen consumption rate (OCR) and the extracellular acidification rate (ECAR). Where indicated, ECAR and/or OCR were analyzed in response to 10μM oligomycin, 4μM FCCP and 1μM rotenone plus 10μM antimycin A (all Sigma-Aldrich).

In vitro cross-presentation by DCs

Flow cytometry sorted DC subtypes or total DCs were incubated with irradiated actmOVA-$K^{b-/-}$ cells (1500 rad/3×10^5 cells/well) together with 1×10^5 OVA$_{257-264}$-specific B3Z hybridoma cells in 96-well U-bottom plates in the presence or absence of NAC (0.5 mM). In parallel experiments DCs were incubated with irradiated actmOVA-$K^{b-/-}$ in the absence or presence of FCCP (1 nM) or oligomycin (50 nM) for 4 hrs after which the DCs were repurified and cultured with B3Z cells in the absence or presence of NAC (0.5 mM). B3Z activation was determined 24 h later by chlorophenol red-β-D-galactopyrannoside conversion assay (30). OVA$_{257-264}$-pulsed DCs were used as positive controls.

DC transfer studies

Total DCs from young and aged mice were incubated with irradiated actmOVA-$K^{b-/-}$ cells as described above followed by sorting of CD8α^-CD11b$^-$DCs. 5×10^5 purified young or aged DCs were (i.v.) transferred into young and aged wt Bl/6 recipients. Seven days after the DC transfer, endogenous Ag-specific CD8$^+$ T cell responses were assessed by intracellular cytokine production after a 5-hr stimulation with OVA$_{257-264}$ peptide (cognate) or GP$_{33-41}$ (control) in the presence of brefeldin A. Surface staining and intracellular cytokine staining for IFN-γ, IL-2, and TNF-α were performed using a Cytofix/Cytoperm kit (BD Pharmingen, San Diego, CA) according to the manufacturer's directions. Capacity for secondary expansion in vitro was determined by stimulating the splenocytes on irradiated MEC.B7.SigOVA cells for 6 days and dividing the absolute number of endogenous Ag-specific CD8$^+$ T cells at the beginning of the culture by the absolute number of endogenous Ag-specific CD8$^+$ T cells at the end of the culture as described before (35, 36).

Statistical analysis

Unless stated otherwise, the data are expressed as means ± SEM. and evaluated using an ANOVA followed by a Dunnett test. A *p* value of <0.05 was considered statistically significant.

Results

Increased DC frequency but poorer T cell priming capacity in aged mice

To assess the effect of aging on the DCs' capacity to cross-prime cell-associated Ags, we first analyzed the DC composition in young and aged C57Bl/6 mice. Aged mice contained higher frequencies as well as absolute numbers of total DCs (CD11c+ MHCII+ CD3−CD19−NKp46−) in the spleen, LN, and bone marrow. No differences were observed in the frequencies of splenic pDCs, CD11bDCs, and CD8αDCs. However, DCs with the CD8α−CD11b− (mcDCs) surface phenotype were significantly expanded (fig. 1a–c, and not shown).

As these CD8α−CD11b− mcDCs, along with CD8αDCs, have recently been reported to induce strong CD8$^+$ T cell responses to cell-associated Ags in both tumor and autoimmunity settings (29, 32), we assessed the effect of aging on their functionality. We first tested their capacity to cross-present cell-associated OVA in MHC class I, H-2K^b. Although we did not observe significant differences in costimulatory molecule expression between young and aged DCs (fig. S1a) as previously reported (18), we used the OVA$_{257-264}$-specific B3Z hybridoma that does not require additional costimulation as reporter system for MHCI-peptide density on the DC surface. Young and aged CD8αDCs, mcDCs, and CD11bDCs were first incubated with irradiated actmOVA-K$^{b-/-}$ cells, purified again and cultured with the OVA$_{257-264}$-specific B3Z hybridoma. As shown before, CD8αDCs and mcDCs, but not CD11bDCs showed capacity for cross-presentation of cell-associated Ags. Aged CD8αDCs and mcDCs were both less efficient at B3Z activation than their young counterparts (fig. 1d). This was not a result of decreased MHCI expression or viability as both young and aged DC subsets showed comparable MHCI expression (fig. 1e, **and S.1b**) and *in vitro* survival for the duration of the experiment (>95%, data not shown).

We next tested whether aging affected the DCs potential for cross-priming in vivo. As mcDC were the most dysregulated in number in old animals, we focused on this subset. McDCs from young and aged mice were purified and cultured with irradiated actmOVA-K$^{b-/-}$ cells, repurified and transferred into young and aged wt Bl/6 recipients. Transfer of young mcDCs induced a ~4 fold higher frequency and absolute number of endogenous OVA$_{257-264}$-specific CD8$^+$ T cells than transfer of aged mcDCs into young hosts (fig. 2.a–c). Moreover, young endogenous OVA$_{257-264}$-specific CD8$^+$ T cells induced by young mcDCs underwent significant expansion upon secondary encounter with Ag *in vitro* (fig. 2.d). In contrast, young endogenous OVA-specific CD8$^+$ T cells induced by aged mcDCs failed to undergo secondary expansion (fig. 2.d). Similar results were obtained when aged mice received young or aged mcDCs. While overall priming in aged mice was significantly lower than in young mice, transfer of young mcDCs into aged mice induced significantly more endogenous OVA$_{257-264}$-specific CD8$^+$ T cells than transfer of aged mcDCs (fig. 2.a–

c). Importantly, aged endogenous CD8^{+} T cells primed by young mcDCs retained some of their potential for secondary expansion, while aged endogenous OVA$_{257-264}$-specific CD8^{+} T cells primed by aged mcDCs contracted upon secondary encounter with Ag (fig. 2.d). Together these data reveal an intrinsic defect in the aged DCs' capacity to prime CD8^{+} T cells to cell-associated Ags.

Altered endocytosis of cell-associated Ags in aged DCs

To identify the underlying mechanisms for this decreased cross-priming, we first determined whether aging affected uptake of cell-associated Ags. Young and aged DCs were cultured with Cell-Trace Violet labeled irradiated splenocytes and the endocytic process for each DC population was analyzed using classic and imaging flow cytometry (29).

Aged CD8αDCs and mcDCs were less efficient at endocytosing irradiated cells than their young counterparts, as determined by flow cytometry (fig. 3a). Detailed analysis by imaging flow cytometry showed that aging also reduced the number and the size of the endocytosed particles in the DC (fig. 3b–d). Indeed, the majority (>75%) of endocytosing young CD8αDCs and mcDCs contained more than 2 particles, with approximately 45% of the DCs containing more than 4 particles (fig. 3c). In contrast, less than half of the aged CD8αDCs and mcDCs contained more than 2 particles, and less than 15% contained more than 4 particles. In addition, the size of endocytosed particles in the young DCs was approximately double of those in the aging DCs (fig. 3d). As we had shown before, CD11bDC were poorly endocytic (fig. 3.a–c *item (iii)*) (37), but again, the size of the endocytosed particles by these aged CD11bDC was significantly smaller than those in young CD11bDC (fig. 3d).

Aging alters DC metabolism

Endocytic capacity has recently been associated with mitochondrial activity and mitochondrial membrane potential (ψ_m) in bone-marrow-derived murine macrophages and fibroblasts (24). Since aging has been associated with impaired mitochondrial function and decreased ψ_m (25, 27, 38–41), we explored whether decreased mitochondrial function could be the mechanism underlying the decreased endocytic capacity in aged DCs.

We first used Seahorse FX24 analyses to assess mitochondrial function and cellular respiration in purified young and aged DCs. Young DCs showed higher baseline oxygen consumption rates (OCR) than aged DCs (fig. 4.a, c). Basal respiration is strongly controlled by ATP turnover and partly by substrate oxidation and proton leak. To dissect which of these 3 processes was altered in aged DCs, we sequentially added the ATP synthase inhibitor oligomycin to assess ATP turnover and proton leak ("b" and "c", respectively in fig. 4b), then the uncoupler FCCP to assess maximal respiration ("d" in fig. 4b), and finally antimycin/rotenone to assess the non-mitochondrial OCR rate ("e" in fig. 4b). The non-mitochondrial OCR rate was comparable between young and aged DCs (fig. 4a, young, 134.8 ± 17.2: aged, 124.1 ± 14.7) and these values were subtracted from all other values to strictly assess mitochondrial functions.

Addition of oligomycin resulted in a significantly greater reduction of OCR in young DCs compared to aged DCs ("b" values in fig. 4c), indicating decreased ATP turnover in aged

DCs. Moreover, the coupling efficiency, i.e. the fraction of basal mitochondrial oxygen consumption used for ATP synthesis ("b/a" values in fig. 4c), was significantly higher in young DCs. In addition, aged DCs showed a greater oligomycin-insensitive respiration (proton leak, "c" in fig. 4c) than young DCs. The decreased ATP turnover and lower coupling efficiency suggested a deficiency in the ATP synthesis machinery of the aged DCs.

Using flow cytometry, we next assessed the mitochondrial mass and ψ_m in the different subsets of young and aged DCs. Mitotracker Green staining intensity was similar between young and aged DCs, for all subsets, suggesting comparable total mitochondrial mass in the young and aged DCs (fig. 4d). We next stained DCs with JC-1, a lipophilic cationic dye that can selectively enter into mitochondria and reversibly change color as the mitochondrial membrane potential (ψ_m) increases. As shown in fig. 4d, all subsets of aged DCs showed significantly decreased ψ_m. In addition, all subsets of aged DCs had increased ROS levels (fig. 4d), further suggesting mitochondrial dysfunction.

Genetic or chemical reduction of ψ_m and/or ATP levels was recently shown to accelerate trafficking of phagocytosed materials to low pH organelles in the LR73 cell line (24). Accelerated acidification in DCs would significantly reduce the amount of Ag available for MHCI loading and could therefore reduce cross-presentation. To determine whether the decrease in endocytosed material in aged DCs resulted from accelerated degradation of the fluorescently-labeled cellular material, we assessed the endosomal acidification rate in young and aged DC subsets using beads that were dual-labeled with a pH sensitive and pH resistant dye (fig. S2) (33). While there were large differences in endosomal acidification rates between DC subsets, no differences between young and aged DCs were found, suggesting that increased degradation was not the dominant process reducing the Ag availability in aging DCs.

Decreases in mitochondrial functionality reduce cross-presentation by young DCs

We next tested whether creating an "aged" phenotype in young DCs by reducing ATP, ψ_m or increasing ROS were sufficient to affect their phagocytic and T cell priming capacity. Young DCs were incubated with Cell-Trace Violet labeled irradiated actmOVA-K$^{b-/-}$ cells in the absence or presence of combinations of chemicals that affect distinct aspects of mitochondrial function. We used low concentrations of FCCP (reduces ψ_m, increases ROS, little effect on ATP), or oligomycin (little effect on ψ_m, increases ROS, inhibition of ATP). As shown in fig. S2, these drugs had the expected effect in the young DCs. They were used alone or in combination with N-acetyl-L-cysteine (NAC, scavenges ROS, no effect on ψ_m or ATP, fig. S3). Endocytosis (fig. 5a) and cell viability (fig. S3) was assessed 4 hrs later. Both FCCP and oligomycin used alone significantly reduced the phagocytic capacity of CD8αDCs and mcDCs (black bars). NAC alone during the endocytic period did not alter the DC phagocytic activity nor did it alter the inhibitory effects of FCCP and oligomycin on this phagocytic capacity (white bars *vs* black bars). Together these data indicate that ψ_m and ATP, but not ROS, are important for the endocytic process (fig. 5a).

To determine the effect of the same drugs on the young DCs cross-presentation, young DCs were incubated with irradiated actmOVA-K$^{b-/-}$ cells in the presence of FCCP or oligomycin, followed by sorting and coculture with the B3Z cells in the absence or presence

of NAC. As expected due to their inhibitory effects on endocytosis, FCCP and oligomycin-treated young DCs showed poorer capacity to activate B3Z cells than untreated DCs. Interestingly, addition of NAC to the FCCP and oligomycin-treated DCs partly restored their ability to activate B3Z cells (fig. 5b). These data suggest that in conditions where ROS generation is augmented, such as in aged DCs, DC cross-presentation is impaired, although not through impaired endocytosis.

Scavenging of ROS partially restores cross-presentation by aged DCs

We next tested whether ROS scavenging could improve the poor cross-presenting capacity of aged DCs. Addition of NAC to cultures of young DCs did not significantly improve endocytosis, DC survival, or the B3Z response to irradiated actmOVA-K$^{b-/-}$ cells (not shown). Similarly, NAC did not enhance endocytosis or survival in aged DCs (fig. 6a). However, addition of NAC to aged DCs significantly improved the B3Z response (fig. 6b). As B3Z cells are relatively resistant to oxidative stress (not shown), these data imply that the inhibitory effect of ROS on cross-presentation resulted from a direct effect on the DCs.

Discussion

Aging has been shown to reduce DC functionality but the underlying mechanisms are poorly understood. Here we show that aging affected DC numbers as well as DC subpopulation composition. Moreover, aging significantly reduced the DCs' capacity to phagocytose and cross-present cell-associated Ags and this impairment was associated with decreased ATP production, decreased ψ_m, and increased ROS production. Our data further indicate that decreased ATP and ψ_m conferred the defect in phagocytic capacity while ROS impaired DCs at a later phase of the cross-presentation process.

Although various groups have studied DCs in aging, there is little consensus about the impact of aging on DCs numbers, composition, and function. Moreover, it is currently unclear whether the observed changes result from intrinsic defects in the DCs or its precursors, extrinsic factors associated with the aged environment, or a combination of both. Several studies reported normal DC numbers and subset composition in lymphoid tissues from young and aged mice, while others suggest that aging decreases CD8α$^+$DCs and increases CD8α$^-$ DC(4, 14, 15). Our data are in partial agreement with these latter studies, as we found the total number and frequency of splenic DCs to be increased, predominantly due to increased CD8α$^-$CD11b$^-$ DCs. These discrepancies likely arise from the study of different background strains, as well as differences in staining and analysis strategies (3). The increase of the CD8α$^-$CD11b$^-$ DCs is of high interest as this population has significant phenotypic and functional overlap with mouse CD8αDCs in young animals, as well as having a human equivalent (BDCA3+ DCs) that is the most potent at priming T cells to cell-associated Ags (42–46). Consequently, CD8αDCs and mcDCs are very potent inducers of anti-tumor responses, but can also play a role in autoimmunity, as both processes are driven by cross-presentation of cell-associated self-Ags (29, 32, 47, 48). However, despite their functional importance, nothing is currently known how aging affects their functionality, and particularly how they cross-present cell-associated Ags.

Cross-presentation of cell-associated Ags is a coordinated process, starting with the recognition of cell-associated materials and their subsequent internalization into endosomes that eventually fuse with acidic lysosomes where the materials are degraded. To facilitate cross-presentation, DCs need to actively delay endosomal acidification in order to allow the transport of the endocytosed Ags from endosomal vesicles into the cytosol, where they are processed by the proteasome and loaded on MHC class I molecules in the endoplasmic reticulum (49).

Our data indicate that aging affects multiple aspects of the cross-presentation process. Indeed, we found that aging significantly reduced the endocytic capacity of CD8DCs and mcDCs, resulting in a reduced frequency of phagocytosing DCs as well as a reduction in the number and size of the endocytosed particles. Importantly, our data suggest that reduced endocytosis resulted from the disruption of the active internalization process and not from defective binding/recognition, as tethering of cellular materials was similar in aged and young DCs. Age-associated attenuation of phagocytosis has been reported for a variety of cell types, including primary and in vitro generated macrophages and DCs. Importantly, our data strongly suggest that the age-associated endocytic defect is related to decreased ATP production and a lower ψ_m in aged DCs, which, to our knowledge, has never been reported.

Phagocytosis requires ATP for actin polymerization and activation of myosin motor proteins that drive all stages of endocytosis, including ruffle formation, membrane delivery, closure of the phagocytic cup, as well as short range movement of newly formed vesicles (50). Active phagocytosis is therefore often considered to be an ATP drain that significantly taxes cellular energy (51). In agreement with these previous data, our studies show that young DCs with chemically reduced ATP levels (similar to levels found in aged DCs) showed significantly reduced phagocytic capacity, thus suggesting a similar mechanism underlying deficient phagocytic capacity by aged DCs.

Besides a role for ATP, we also found a relation between lower ψ_m and reductions in phagocytic capacity. Park et al (24) showed that ψ_m increased after uptake of apoptotic cells in BM-derived macrophages without detectable effects on total cellular ATP production. How changes in ψ_m affect phagocytic capacity remains unclear, but some studies suggest that changes in ψ_m may alter mitochondrial Ca^{2+} accumulation and mobilization, thereby affecting phagocytosis, which is a Ca^{2+}-sensitive process (52–54). While this is an intriguing hypothesis, especially in light of the dysregulated Ca^{2+} homeostasis in aged cells (55) and the possible suppression of ψ_m by high levels of Ca^{2+}, more research is needed to causally link these different phenomena.

Another important finding of our studies is that aged DCs expressed significantly higher levels of ROS than young DCs, and that in vitro ROS scavenging significantly improved cross-presentation by aged DCs. ROS has an important role as a secondary messenger in several signaling pathways, but if the production exceeds its deactivation it leads to oxidative damage to proteins, lipids, and nucleic acids (28, 33, 56, 57). How ROS affect cross-presentation in aged DCs is not completely clear. Our data show that ROS scavenging did not improve phagocytic capacity in aged DCs or young DCs. Moreover, increased ROS

levels in aged DCs did not affect early endosome trafficking and lysosomal fusion, as there were no differences in endosomal acidification rate between young and aged DCs. These findings thus suggest that the ROS affects later steps in the Ag processing/presentation pathway in aged DCs. A possible step that ROS could affect is endosomal acidification, as suggested by the fact that sustained low production of ROS at the endosomal lumen has been shown to prevent endosomal acidification and promote cross-presentation (33). However, in our experiments, scavenging of ROS by NAC did not alter the acidification rate or cross-presentation by young DCs, which is in agreement with our previous findings (29). It is therefore likely that overproduction of ROS in aged DCs directly damages molecules involved in the cross-presentation pathway. Further studies aimed at the dissection of the molecular processes that drive mitochondrial dysfunction, and their differential impact on the process of cross-presentation, are thus warranted. Furthermore, the ability of ROS scavengers such as NAC to restore the cross presentation ability in aged mice will need to be rigorously tested in an in vivo setting of cross priming, to test whether this avenue could be targeted to increase immune responses in aged individuals.

Together, our study shows that different components associated with age-related mitochondrial dysfunction – reduced ATP synthesis, reduced ψ_m and increased ROS production – have specific deleterious effects on the cross-presenting capacity of aging DCs. Cross-presentation is only the first step in the cross-priming of cells to cell-associated Ags. Importantly, high levels of MHC-peptide can lower the requirement of costimulation by lowering the threshold for T cell activation (58). Given that aging has been reported to negatively affect expression of costimulatory molecules and pro-inflammatory cytokines (3, 4, 7, 14, 59), lowering the threshold for T cell activation by increasing MHCI-peptide levels is of clear interest. While more research is needed to dissect how aging affect DC-T cell communication, our data thus suggest that improvement of DC functionality might be feasible in the elderly, by targeting metabolic dysfunction, or its downstream sequelae, thereby opening new avenues for enhancing vaccine efficiency in this fragile population.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations

Ag   antigen

ATP	adenosine triphosphate
DC	dendritic cell
ψ_m	mitochondrial membrane potential
ECAR	extracellular acidification rate
FCCP	carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone
mcDC	merocytic DC
MFI	mean fluorescent intensity
NAC	N-acetyl-L-cysteine
OCR	oxygen consumption rate
OXPHOS	oxidative phosphorylation
ROS	reactive oxygen species
TCR	T cell receptor

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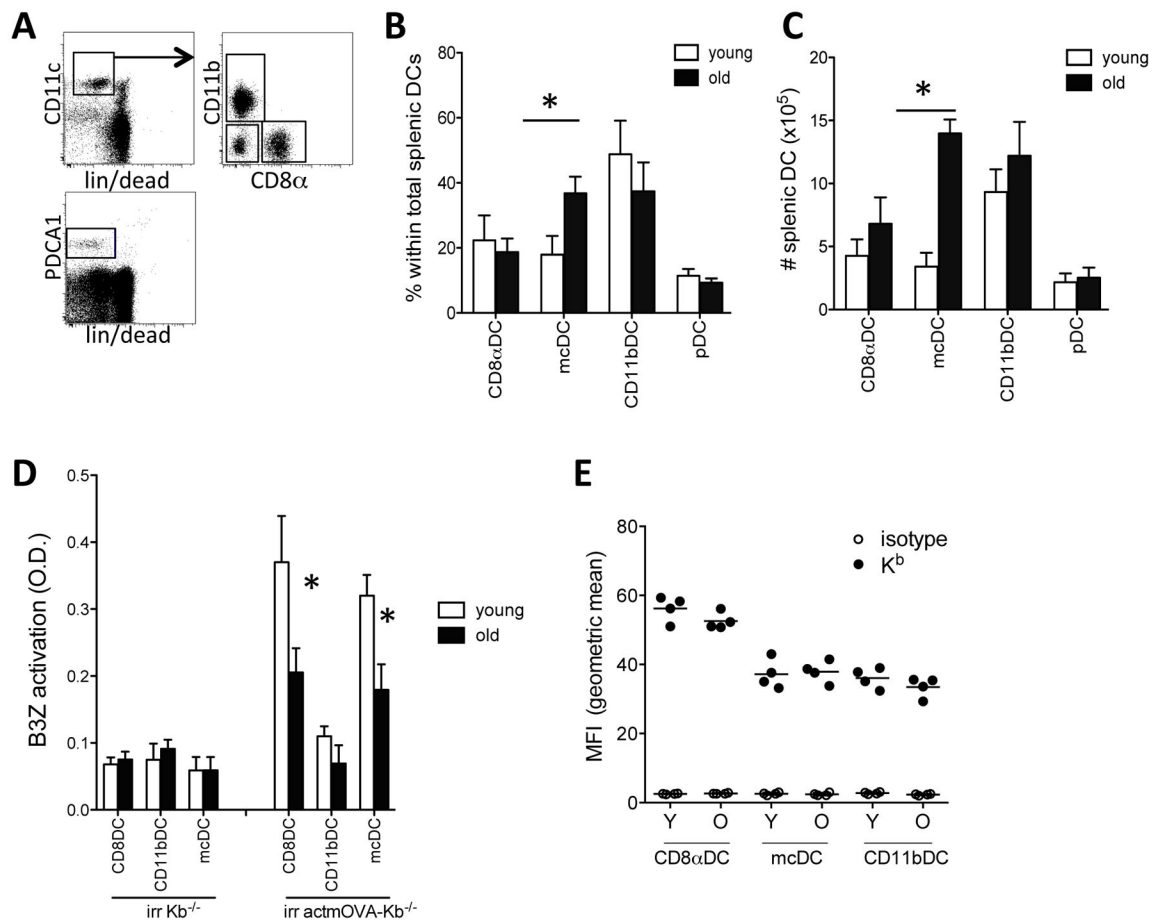
Figure 1. Altered DC composition and cross-presenting capacity in aged mice

Spleens were harvested from young (white bars) and aged (black bars) mice and stained for CD11c, MHCII, CD11b, CD8α, in combination with a lineage stain (CD3, CD19, IgM, IgD, and NKp46) and fixable live/dead stain. **A.** Representative image of the gating strategy to assess DC numbers and composition. **B.** Frequency of DC subpopulations within the total splenic DC population. **C.** Absolute number of DC subpopulations per spleen. Data expressed as mean ± s.e.m. with n=9–11/group.*, p<0.05.

D. Purified young (white bars) and aged (black bars) CD8αDCs, mcDCs, and CD11bDCs were incubated with irradiated actmOVA-K$^{b-/-}$ cells, resorted and cultured with the OVA$_{257-264}$-specific B3Z hybridoma. B3Z activation was determined 24 hrs later by CPRG conversion assay. Data from one experiment (of 3) are expressed as mean ± s.e.m. with n=3/group.*, p<0.05. **E.** Fluorescent intensity if K^b on young and aged DCs. Black circles, K^b; open circles, isotype control. N=4/group.
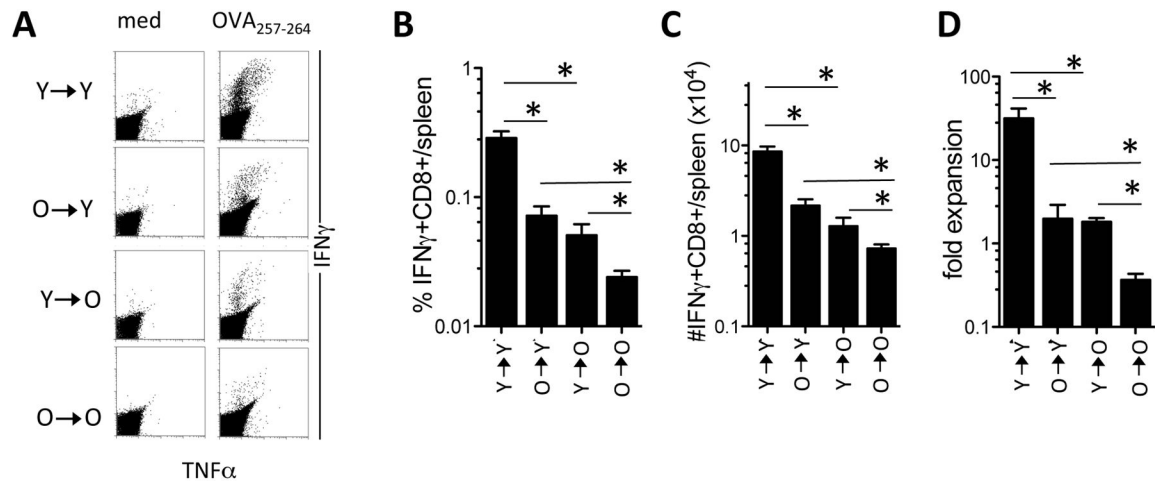
Figure 2. Decreased cross-priming to cell-associated Ags by aged mcDCs in vivo
CD11c+CD11b–CD8α– mcDCs from young (Y) and aged (O) mice were isolated and cultured with irradiated actmOVA-K$^{b–/–}$ cells, repurified and transferred into young and aged recipients. Seven days later the spleens were harvested and CD8$^+$ T cell responses to OVA were examined. **A.** Representative flow cytometric dot-plot of CD8$^+$ T cells producing IFNγ and TNFα upon stimulation with medium or OVA$_{257–264}$ peptide. **B.** The frequency of OVA$_{257–264}$ specific CD8$^+$ T cells per spleen. **C.** The absolute number of OVA$_{257–264}$ specific CD8$^+$ T cells per spleen. **D.** Capacity for secondary expansion in vitro of CD8$^+$ T cells primed by young or aged mcDC in young and aged recipients. CD8$^+$ T cells were restimulated on irradiated MEC.B7.SigOVA cells and the fold expansion was determined by dividing the absolute number of Ag-specific CD8$^+$ T cells at the beginning of the culture by the absolute number of Ag-specific CD8$^+$ T cells at the end of the culture. Data from one experiment (of 2) are expressed as mean ± s.e.m. with n=4–5/group.*, p<0.05.
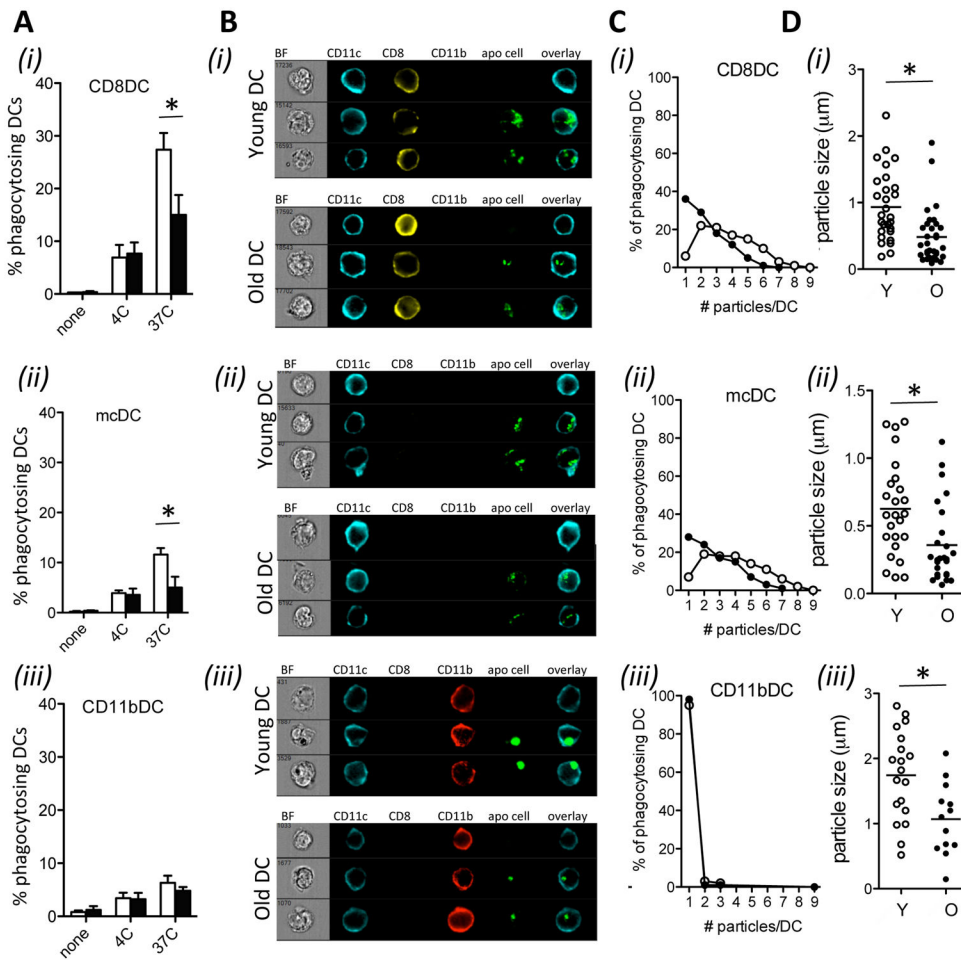
Figure 3. Aging reduces phagocytic capacity in DCs
Purified DCs from young (white bars/circles) and aged mice (black bars/circles) were cultured with Cell-Trace Violet-labeled irradiated splenocytes. After 4 hrs DCs were stained with Abs to CD11c, MHCII, CD11b, CD8α, and a fixable live/dead dye. CD8αDC (*i*), mcDC (*ii*), and CD11bDC (*iii*) were analyzed by flow cytometry (**A**) or imaging flow cytometry (**B–D**). **A.** Flow cytometric analysis of the frequency of phagocytic cells in each DC population. Data are expressed as mean ± s.e.m. with n=3/group. **B.** Representative imaging flow cytometry images of different DC populations upon co-culture with irradiated cells. BF = Bright field, Blue = CD11c, yellow = CD8, red = CD11b, green = irradiated cell material. **C.** Number of particles per DC within the phagocytosing DC population. The number of phagocytosed particles was determined using the spot counting features after tight masking on the brightfield image to exclude membrane-associated extracellular particles. **D.** Size distribution of phagocytosed particles with the Cell-Trace Violet+ DC populations. Particle size was determined using a combination of the spot mask, combined with spot size feature and area feature. Representative data of 3 independent experiments are shown. Each condition contained 500–1000 viable, focused DCs.
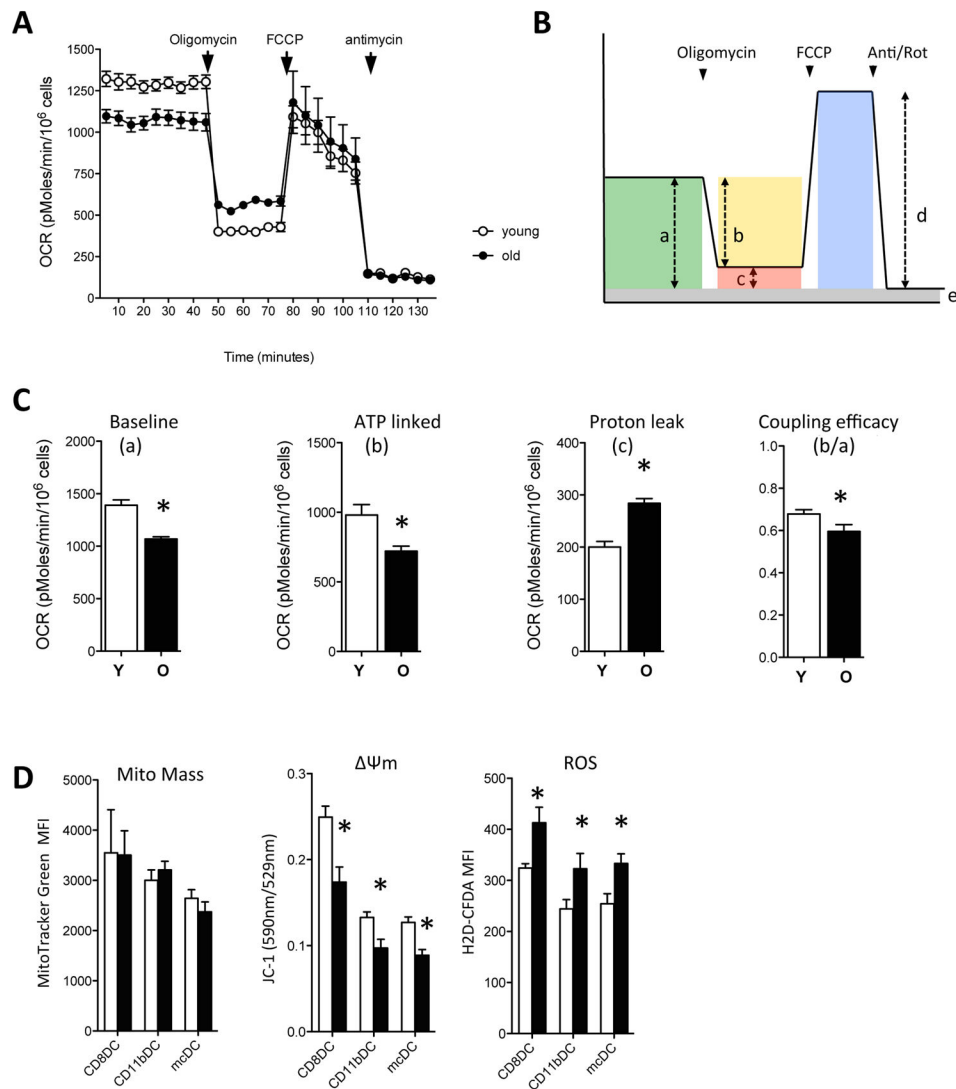
Figure 4. Reduced mitochondrial functionality in aged DCs

Young (white circles/bars) and aged (black circles/bars) DCs were probed for their mitochondrial function. **A.** DCs were seeded into Seahorse Bioscience plates and oxygen consumption rates (OCR) were determined under basal conditions followed by the sequential addition of oligomycin, FCCP, and antimycin/rotenone. A representative experiment (of 3) is shown. Data are expressed as mean ± s.e.m. with n=2 (Y) or 3 (O).*, p<0.05. **B.** Scheme outlining the approach to identify parameters for the calculation of the relative contribution of non-respiratory chain oxygen consumption, ATP-linked oxygen consumption, proton-leak, and coupling efficiency. **C.** Decreased baseline OCR, ATP-linked OCR and coupling efficiency and increased proton leak in added DCs. **D.** Flow cytometric analysis of mitochondrial mass, mitochondrial membrane potential (ψ_m) and ROS production at steady state in young and aged DCs. A representative experiment (of 3) is shown. Data is expressed as mean ± s.e.m. with n=3.*, p<0.05.
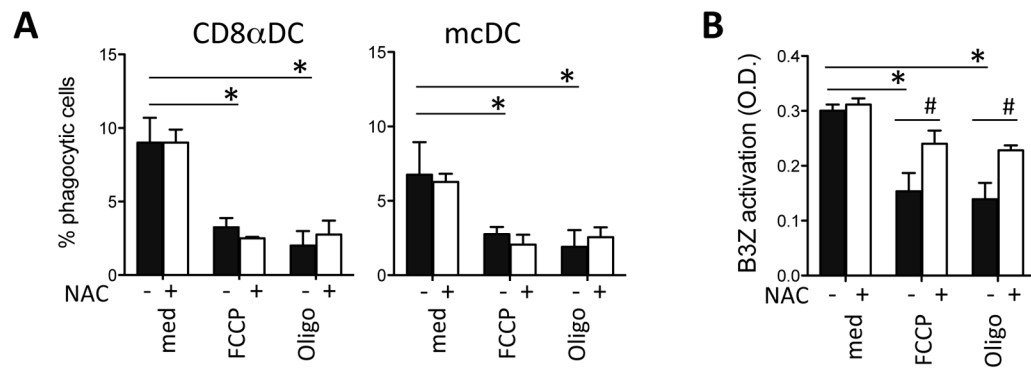
Figure 5. Distinct roles for ATP, ψ_m and ROS in endocytosis and cross-presentation
A. Young DCs were cultured with Cell-Trace Violet-labeled irradiated actmOVA-K$^{b-/-}$ cells in the presence of vehicle, FCCP, or oligomycin in the absence (black bars) or presence of ROS scavenger NAC (white bars). After 4 hr DCs were stained with Abs to CD11c, MHCII, CD11b, CD8α, and live/dead staining and the frequency of Cell-Trace Violet+ DCs was determined by flow cytometry. **B.** Young DCs were cultured with irradiated actmOVA-K$^{b-/-}$ cells in the presence of vehicle, FCCP, or oligomycin and repurified before being cultured with the OVA$_{257-264}$-specific B3Z hybridoma in the absence (black bars) or presence (white bars) of NAC. B3Z activation was determined 24 h later by CPRG conversion assay. Data are expressed as mean ± s.e.m. with n=3/group.*, p<0.05.

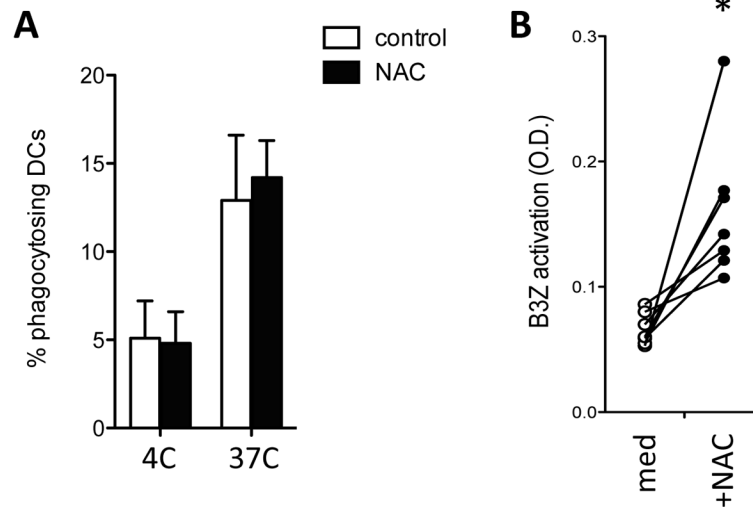**Figure 6. ROS scavenging partially restores cross-presentation in aged DCs**

Purified DCs from and aged mice were cultured with Cell-Trace Violet-labeled irradiated actmOVA-$K^{b-/-}$ cells. **A.** Flow cytometric analysis of the frequency of phagocytic cells in each DC population after 4h of co-culture. Data are expressed as mean ± s.e.m. with n=3/group. **B.** $OVA_{257-264}$-specific B3Z hybridoma response to aged DCs in the absence (white circles) or presence (black circles) of ROS scavenger NAC. B3Z activation was determined after 24 h by CPRG conversion assay. Data of 7 individual DC sorts are shown. *, p<0.05.