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Loss of Phagocytic and Antigen Cross-Presenting Capacity in Aging Dendritic Cells Is Associated with Mitochondrial Dysfunction

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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 Ag acquisition, processing, and presentation by DCs. In this study, 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 were their young counterparts. The decreased cross-presentation was associated with a reduction in the frequency of CD8α DCs 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 (ΔΨm). Aged DCs exhibited profound signs of mitochondrial dysfunction, illustrated by lower ΔΨm, reduced ATP turnover and coupling efficiency, decreased baseline oxidative phosphorylation, and greater proton leak and reactive oxygen species (ROS) production. Mimicking the aged metabolic phenotype in young DCs by pharmacologic manipulation indicated that the reductions in ΔΨm and ATP impeded the phagocytic capacity whereas ROS interfered with a later step in the cross-presentation process. Conversely, in vitro scavenging of ROS partially restored cross-presentation by aged DCs. Taken 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. The Journal of Immunology, 2015, 195: 2624–2632.
production (27, 28). Exploring how the mitochondrial function is affected, as well as 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 [MHC 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 mo old) and aged (16–20 mo old) C57BL/6 mice were obtained from the National Institute on Aging aging colony. Mice expressing OVA under the actin promoter (actmOVA) were crossed to the K<sup>b</sup>-<sup>+/−</sup> 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 Biotec). Enriched DCs were either used directly or further (sub)sorted by flow cytometry based on their expression of CD11c, CD11b, and CD8<sup>a</sup> by using CD3, CD19, IgM, IgD, and NKp46 as lineage/dump markers (MoFlo, Beckman Coulter). Overall DC purity after flow cytometric sorting was ≥98% and after bead isolation was ≥95% (anti-NKp46 D staining).

**OVA<sub>257–264</sub>-specific B23 hybridomas were cultured in IMDM supplemented with 10% FCS, 50 μM 2-ME, 2 mM l-glutamine, 20 U/ml penicillin, and 20 μg/ml streptomycin (30). OVA<sub>257–264</sub> (SIINFEKL) and control peptide LCMV gp33–41 (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, CD8<sup>a</sup>, CD11b, CD11c, XCR1, CD40, CD80, CD86, CD273, CD275, K<sup>d</sup>, K<sup>d</sup>, and I-A<sup>e</sup> by flow cytometric analysis (Abs/isotype controls were from eBioscience/BioLegend [San Diego, CA] using an LSR II from BD Biosciences [San Jose, CA]).

For endocytosis studies, enriched DCs were incubated with CellTrace Violet–labeled irradiated splenocytes (1:3 ratio) at 4°C and 37°C in the absence or presence of N-acetyl-l-cysteine (NAC, 0.5 mM), carbonyl cyanide 3-(trifluoromethoxy)phenylhydrazone (FCCP, 1 nM), and oligomycin (50 mM) for 4 h, after which the DCs were repurified and cultured with B23 cells in the absence or presence of NAC (0.5 mM). B23 activation was determined 24 h later by a chlorophenol red-β-galactosidase conversion assay (30). OVA<sub>257–264</sub>-pulsed DCs were used as positive controls.

**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 <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 capacity of DCs to cross-prime cell-associated Ags, we first analyzed the DC composition in young and aged C57BL/6 mice. Aged mice contained higher frequencies of both CD8<sup>a</sup> DCs and mcDCs, but not CD11b DCs, compared with young mice. Aged mice showed capacity for cross-presentation of cell-associated Ags. Aged DCs, mcDCs, and CD11b DCs were first incubated with irradiated actmOVA-K<sup>b</sup>-<sup>+/−</sup> cells, purified again, and cultured with the OVA<sub>257–264</sub>-specific B3Z hybridoma that does not require additional costimulation as a reporter system for MHC I–peptide density on the DC surface. Young and aged CD8<sup>a</sup> DCs, mcDCs, and CD11b DCs were then incubated with irradiated actmOVA-K<sup>b</sup>-<sup>+/−</sup> cells, purified again, and cultured with the OVA<sub>257–264</sub>-specific B3Z hybridoma. As shown before, CD8<sup>a</sup> DCs and mcDCs, but not CD11b DCs, showed capacity for cross-presentation of cell-associated Ags. Aged CD8<sup>a</sup> DCs and mcDCs were both less efficient at B3Z activation than were their young counterparts (Fig. 1D). This was not a result of decreased MHC I expression or viability, as both young and aged DC subsets showed comparable MHC I expression (Fig. 1E, Supplemental Fig. 1B) and in vitro survival for the duration of the experiment (>95%, data not shown).

In vitro cross-presentation by DCs

Flow cytometry–sorted DC subtypes or total DCs were incubated with irradiated actmOVA-K<sup>b</sup>-<sup>+/−</sup> cells (1500 rad/3 × 10<sup>5</sup> cells/well) together with 1 × 10<sup>5</sup> OVA<sub>257–264</sub>-specific B23 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<sup>b</sup>-<sup>+/−</sup> in the absence or presence of FCCP (1 μM) or oligomycin (50 μM) for 4 h, after which the DCs were repurified and cultured with B23 cells in the absence or presence of NAC (0.5 mM). B23 activation was determined 24 h later by a chlorophenol red-β-galactosidase conversion assay (30). OVA<sub>257–264</sub>-pulsed DCs were used as positive controls.
We next tested whether aging affected the DC potential for cross-priming in vivo. As mcDCs 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 actmOV A-K\textsubscript{b}/2 cells, repurified, and transferred into young and aged wild-type BL/6 recipients. Transfer of young mcDCs induced a ∼4-fold higher frequency and absolute number of endogenous OVA\textsubscript{257–264} -specific CD8\textsuperscript{+} T cells than did transfer of aged mcDCs into young hosts (Fig. 2A–C). Moreover, young endogenous OVA\textsubscript{257–264} -specific CD8\textsuperscript{+} T cells induced by young mcDCs underwent significant expansion upon secondary encounter with Ag in vitro (Fig. 2D). In contrast, young endogenous OVA\textsubscript{257–264} -specific CD8\textsuperscript{+} T cells primed by aged mcDCs failed to undergo secondary expansion (Fig. 2D). Similar results were obtained when aged mice received young or aged mcDCs. Whereas overall priming in aged mice was significantly lower than in young mice, transfer of young mcDCs into aged mice induced significantly more endogenous OVA\textsubscript{257–264} -specific CD8\textsuperscript{+} T cells than did transfer of aged mcDCs (Fig. 2A–C). Importantly, aged endogenous CD8\textsuperscript{+} T cells primed by young mcDCs retained some of their potential for secondary expansion, whereas aged endogenous OVA\textsubscript{257–264} -specific CD8\textsuperscript{+} T cells primed by aged mcDCs contracted upon secondary encounter with Ag (Fig. 2D). Taken together, these data reveal an intrinsic defect in the capacity of aged DCs to prime CD8\textsuperscript{+} 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 CellTrace Violet–labeled irradiated splenocytes, and the endocytic process for each DC population was analyzed using classic and imaging flow cytometry (29).

Aged CD8\textsuperscript{a} DCs and mcDCs were less efficient at endocytosing irradiated cells than were 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, most (≥75%) endocytosing young CD8\textsuperscript{a} DCs and mcDCs contained more than two particles, with ∼45% of the DCs containing more than four particles (Fig. 3C). In contrast, less than half of the aged CD8\textsuperscript{a} DCs and mcDCs contained more than two particles, and <15% contained more than four particles. Additionally, the size of endocytosed particles by these aged CD11b DCs was significantly smaller than those in young CD11b DCs (Fig. 3D).

**FIGURE 1.** Altered DC composition and cross-presenting capacity in aged mice. Spleens were harvested from young (open bars) and aged (filled bars) mice and stained for CD11c, MHC II, CD11b, and CD8\textsuperscript{a} 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 ± SEM with n = 9–11/group. *p < 0.05. (D) Purified young (open bars) and aged (filled bars) CD8\textsuperscript{a} DCs, mcDCs, and CD11b DCs were incubated with irradiated actmOV A-K\textsubscript{b}/2 cells, resorted, and cultured with the OVA\textsubscript{257–264}–specific B3Z hybridoma. B3Z activation was determined 24 h later by CPRG conversion assay. Data from one experiment (of three) are expressed as mean ± SEM with n = 3/group. *p < 0.05. (E) Fluorescence intensity of K\textsuperscript{b} on young and aged DCs (\bullet, K\textsuperscript{b}; ○, isotype control; n = 4/group). MFI, mean fluorescence intensity.
Aging alters DC metabolism

Endocytic capacity has recently been associated with mitochondrial activity and $\Delta \psi_{\text{mit}}$ in bone marrow–derived murine macrophages and fibroblasts (24). Because aging has been associated with impaired mitochondrial function and decreased $\Delta \psi_{\text{mit}}$ (25, 27, 28, 38–40), 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 OCRs than did aged DCs (Fig. 4A, 4C). Basal respiration is strongly controlled by ATP turnover and partly by substrate oxidation and proton leak. To dissect which of these three 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 nonmitochondrial 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 with aged DCs (“b” values in Fig. 4C), indicating decreased ATP turnover in aged DCs. Moreover, the coupling efficiency, that is, the fraction of basal mitochondrial oxygen consumption used for ATP synthesis (“b/a” values in Fig. 4C), was significantly higher in young DCs. Additionally, aged DCs showed a greater oligomycin-insensitive respiration (proton leak, “c” in Fig. 4C) than did 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 $\Delta \psi_{\text{mit}}$ 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 $\Delta \psi_{\text{mit}}$ increases. As shown in Fig. 4D, all subsets of aged DCs showed significantly decreased $\Delta \psi_{\text{mit}}$. Additionally, all subsets of aged DCs had increased ROS levels (Fig. 4D), further suggesting mitochondrial dysfunction. Genetic or chemical reduction of $\Delta \psi_{\text{mit}}$ 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 MHC I 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 (Supplemental Fig. 2) (33). Although 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, $\Delta \psi_{\text{mit}}$, or increasing ROS were sufficient to affect their phagocytic and T cell priming capacity. Young DCs were incubated with CellTrace 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 $\Delta \psi_{\text{mit}}$, increases ROS, little effect on ATP) or oligomycin (little effect on $\Delta \psi_{\text{mit}}$, increases ROS, inhibition of ATP). As shown in Supplemental Fig. 2, these drugs had the expected effect in the young DCs. They were used alone or in combination with NAC (scavenges ROS, no effect on $\Delta \psi_{\text{mit}}$ or ATP; Supplemental Fig. 3). Endocytosis (Fig. 5A) and cell viability (Supplemental Fig. 3) was assessed 4 h later. Both FCCP and oligomycin used alone significantly reduced the phagocytic capacity of CD8α DCs and mDCs (filled 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 (open bars versus filled bars). Taken together, these data indicate that $\Delta \psi_{\text{mit}}$ and ATP, but not ROS, are important for the endocytic process (Fig. 5A).
To determine the effect of the same drugs on the cross-presentation of young DCs, young DCs were incubated with irradiated actmOVA-K<sup>b</sup>-/- 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 owing to their inhibitory effects on endocytosis, FCCP- and oligomycin-treated young DCs showed poorer capacity to activate B3Z cells than did 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<sup>b</sup>-/- 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. In this study, we show that aging-affected DC numbers as well as DC subpopulation composition. Moreover, aging significantly reduced the capacity of DCs to phagocytose and cross-present cell-associated Ags, and this impairment was associated with decreased ATP production, decreased Δψ<sub>m</sub>, and increased ROS production. Our data further indicate that decreased
ATP and \( \Delta \psi_m \) conferred the defect in phagocytic capacity whereas 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 DC numbers, composition, and function. Moreover, it is currently unclear whether the observed changes result from intrinsic defects in the DCs or their 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, whereas others suggested that aging decreases CD8\( ^{+} \) DCs and increases CD8\( ^{+} \) 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 CD8\( ^{+} \)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 CD8\( ^{+} \)CD11b\(^{-} \) DCs is of high interest, as this population has significant phenotypic and functional overlap with mouse CD8\( ^{+} \) DCs 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 (41–45). Consequently, CD8\( ^{+} \) DCs and mcDCs are very potent inducers of anti-tumor responses, but they can also play a role in autoimmunity, as both processes are driven by cross-presentation of cell-associated self-Ags (29, 32, 46, 47). However, despite their functional importance, nothing is currently known how aging affects their functionality, and particularly how they cross-present cell-associated Ags.

**FIGURE 4.** Reduced mitochondrial functionality in aged DCs. Young (open circles/bars) and aged (filled circles/bars) DCs were probed for their mitochondrial function. (A) DCs were seeded into Seahorse Bioscience plates and OCRs were determined under basal conditions followed by the sequential addition of oligomycin, FCCP, and antimycin/rottenile. A representative experiment (of three) is shown. Data are expressed as mean \( \pm \) SEM 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 nonrespiratory 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, \( \Delta \psi_m \), and ROS production at steady-state in young and aged DCs. A representative experiment (of three) is shown. Data are expressed as mean \( \pm \) SEM with \( n=3 \). *\( p < 0.05 \). MFI, mean fluorescence intensity; O, aged; Y, young.
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 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 I molecules in the endoplasmic reticulum (48).

Our data indicate that aging affects multiple aspects of the cross-presentation process. Indeed, we found that aging significantly reduced the endocytic capacity of CD8 DCs 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 $\Delta \psi_{\text{m}}$ 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 (49). Active phagocytosis is therefore often considered to be an ATP drain that significantly taxes cellular energy (50). 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 $\Delta \psi_{\text{m}}$ and reductions in phagocytic capacity. Park et al. (24) showed that $\Delta \psi_{\text{m}}$ increased after uptake of apoptotic cells in bone marrow–derived macrophages without detectable effects on total cellular ATP production. How changes in $\Delta \psi_{\text{m}}$ affect phagocytic capacity remains unclear, but some studies suggest that changes in $\Delta \psi_{\text{m}}$ may alter mitochondrial Ca$^2+$ accumulation and mobilization, thereby affecting phagocytosis, which is a Ca$^2+$-sensitive process (51–53). Whereas this is an intriguing hypothesis, especially in light of the dysregulated Ca$^2+$ homeostasis in aged cells (54) and the possible suppression of $\Delta \psi_{\text{m}}$ 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 did 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 when the production exceeds its deactivation it leads to oxidative damage to proteins, lipids, and nucleic acids (28, 33, 55, 56). How ROS affects 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 affect 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

![FIGURE 5.](image1.png)

Distinct roles for ATP, $\Delta \psi_{\text{m}}$, and ROS in endocytosis and cross-presentation. (A) Young DCs were cultured with CellTrace Violet–labeled irradiated actmOVA-Kb$^+/-$ cells in the presence of vehicle, FCCP, or oligomycin and repurified after 4 h by CPRG conversion assay. Data are expressed as mean ± SEM with $n = 3$. (B) OVA$^{257-264}$–specific B3Z hybridoma response to aged DCs in the absence (open bars) or presence (filled bars) of ROS scavenger NAC. B3Z activation was determined after 24 h by CPRG conversion assay. Data are expressed as mean ± SEM with $n = 7$. $^p < 0.05$.

![FIGURE 6.](image2.png)

ROS scavenging partially restores cross-presentation in aged DCs. Purified DCs from 4C and 37C mice were cultured with CellTrace Violet–labeled irradiated actmOVA-Kb$^+/-$ cells. (A) Flow cytometric analysis of the frequency of phagocytic cells in each DC population after 4 h of coculture. Data are expressed as mean ± SEM with $n = 3$. (B) OVA$^{257-264}$–specific B3Z hybridoma response to aged DCs in the absence (open bars) or presence (filled bars) of ROS scavenger NAC. B3Z activation was determined after 24 h by CPRG conversion assay. Data are expressed as mean ± SEM with $n = 7$. $^p < 0.05$. 

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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, as well as 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.

Taken together, the present study shows that different components associated with age-related mitochondrial dysfunction, that is, reduced ATP synthesis, reduced ∆Ψmt, 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 (57). Given that aging has been reported to negatively affect expression of costimulatory molecules and proinflammatory cytokines (3, 4, 7, 14, 58), lowering the threshold for T cell activation by increasing MHC I–peptide levels is of clear interest. Although more research is needed to dissect how aging affects 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 efficacy in this fragile population.

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Disclosures

The authors have no financial conflicts of interest.

References

Supplemental Figure 1.

**Surface marker expression on young and aged DCs.** Spleens for young and aged mice were stained for lineage, CD11c, MHCII, CD8, CD11b, and indicated markers or appropriate isotype controls and analyzed by flow cytometry. **A.** Mean fluorescent intensity of indicated marker (black bars) and appropriate isotype controls (white bar). Data are expressed as mean ± s.e.m. with n=4-5. **B.** Representative intensity data for Kb and isotype control staining in indicated splenic DC subpopulations of young and aged mice.
Supplemental figure 2. Comparable endosomal acidification rate in young and aged DCs. Young (white circles) and aged (black circles) 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). Data are expressed as mean ± s.e.m. with n=3.
Supplemental figure 3. Effect of FCCP and Oligomycin on viability and mitochondrial function.
Young DCs were cultured for 4 hrs in the presence of vehicle, FCCP, or oligomycin after which intracellular ATP levels, mitochondrial membrane potential ($\Delta\psi_m$), ROS production, and viability were determined. One (out of 3) experiments is shown. Data are expressed as mean ± s.e.m. with n=3.* p<0.05.