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Altered Innate Immune Functioning of Dendritic Cells in Elderly Humans: A Role of Phosphoinositide 3-Kinase-Signaling Pathway

Anshu Agrawal,* Sudhanshu Agrawal,* Jia-Ning Cao,* Houfen Su,* Kathryn Osann, † and Sudhir Gupta*

Aging represents a state of paradox where chronic inflammation is associated with declining immune responses. Dendritic cells (DCs) are the major APCs responsible for initiating an immune response. However, DC functions in aging have not been studied in detail. In this study, we have compared the innate immune functions of monocyte-derived myeloid DCs from elderly subjects with DCs from young individuals. We show that although phenotypically comparable, DCs from the aging are functionally different from DCs from the young. In contrast to DCs from the young, DCs from elderly individuals display 1) significantly reduced capacity to phagocytose Ags via macrophagocytosis and endocytosis as determined by flow cytometry; 2) impaired capacity to migrate in vitro in response to the chemokines MIP-3β and stromal cell-derived factor-1; and 3) significantly increased LPS and ssRNA-induced secretion of TNF-α and IL-6, as determined by ELISA. Investigations of intracellular signaling revealed reduced phosphorylation of AKT in DCs from the aging, indirectly suggesting decreased activation of the PI3K pathway. Because the PI3K-signaling pathway plays a positive regulatory role in phagocytosis and migration, and also functions as a negative regulator of TLR signaling by inducing activation of p38 MAPK, this may explain the aberrant innate immune functioning of DCs from elderly subjects. Results from real-time PCR and protein expression by flow cytometry demonstrated an increased expression of phosphatase and tensin homolog, a negative regulator of the PI3K-signaling pathway, in DCs from the aging. Increased phosphatase and tensin homolog may thus be responsible for the defect in AKT phosphorylation and, therefore, the altered innate immune response of DCs from elderly humans. The Journal of Immunology, 2007, 178: 6912–6922.

Decline in immune function is a hallmark of aging, which is associated with an increased susceptibility to infections and reduced response to vaccination (1–4). Paradoxically, aging is also associated with low-grade chronic inflammation coupled with elevated circulating levels of proinflammatory cytokines IL-6, TNF-α, IL-1β, and PGE2 (5–8). In aging, there is an increased incidence of diseases, which are characterized with a marked inflammatory component such as atherosclerosis, diabetes, Parkinson’s disease, Alzheimer’s disease, and arthritis (6, 7). The molecular mechanisms underlying this chronic inflammatory condition and reduced immunity during aging are not well-understood.

The decline in immune function has been mainly attributed to changes in the functioning of T and B lymphocytes with age. There is decreased response of T cells to specific Ags or mitogens, altered cytokine secretion patterns, involution of thymus causing changes in the ratio of naive to memory lymphocyte populations, decreased CTL responses, and defects in signal transduction (9–16). The situation is aggravated further by the failure of B cells to produce high-affinity Abs and to generate long-lasting memory responses (17, 18). In contrast to lymphocytes, very little is understood about the functioning of the APCs in aging. The most potent of APCs are the dendritic cells (DCs) which are critical mediators of both immunity and tolerance (19–23).

DCs are present at various portals of entry of pathogens: in the skin, and lining of nose, lungs, stomach, and intestines. DCs constantly sample the surroundings for pathogens through pattern recognition receptors, such as the TLRs. Upon Ag capture, DCs become activated and migrate to the lymphoid organs where they present Ags to lymphocytes, initiating an adaptive immune response. Thus, DCs are critical mediators of both innate and adaptive immune responses (19–23). Activation of DCs initiates an inflammatory response through secretion of a wide array of cytokines, which exert a broad influence on the immune system by paracrine and autocrine mechanisms (22, 24). The proinflammatory cytokines increased during aging can modulate the activation and functions of DCs thereby affecting the nature of the innate and the adaptive immune responses. Recent years have greatly expanded our knowledge of the functions and crucial role played by the DCs in the generation of both immune and inflammatory responses. Aberrant functioning of DCs has been identified as a major perpetuator in the pathogenesis of a number of immune and inflammatory disorders. Thus, it seems logical to focus strategies on understanding the age-associated alterations in the function of these cells with a view to boost innate immunity in elderly individuals.

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2 Address correspondence and reprint requests to Dr. Anshu Agrawal, Division of Basic and Clinical Immunology, Medical Sciences I, C-240A, University of California, Irvine, CA 92697. E-mail address: aagrawal@uci.edu

3 Abbreviations used in this paper: DC, dendritic cell; MDDC, monocyte-derived DC; SDF-1, stromal cell-derived factor-1; LU, Lucifer Yellow; FITC-DEX, FITC-conjugated dextran; MR, mannose receptor; PTEN, phosphatase and tensin homolog.
The present study investigates the alterations in the innate immune functions, in particular, Ag uptake, migration, and cytokine secretion of monocyte-derived DCs (MDDCs) in human elderly subjects. We have made significant progress toward understanding the signaling mechanisms responsible for the aberrant function of DCs in aging.

Materials and Methods

Blood donors

Blood was collected from healthy elderly and young volunteer donors. Young donors were between 20 and 35 years of age and elderly donors were between 65 and 90 years of age. Elderly subjects belong to middle-class socioeconomic status and are living independently. A week before the study, they were asked to discontinue any vitamins, minerals, and antioxidants that they may have been taking. This study was approved by the Institutional Review Board of the University of California (Irvine, CA).

Preparation of human MDDCs

MDDCs were prepared essentially as described (25). Briefly, PBMC were separated by Ficoll-Maqueaceous density gradient centrifugation. Monocytes were purified from the PBMCs by positive selection with anti-CD14 microbeads (Stromedix). The purity of the isolated CD14+ monocytes was >90% as determined by flow cytometry. For the induction of DC differentiation, purified CD14+ monocytes were cultured in a humidified atmosphere of 5% CO2 at 37°C in RPMI 1640 supplemented with 10% FBS, 1 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 ng/ml human rGM-CSF (PeproTech), and 10 ng/ml human rIL-4 (PeproTech). Half of the medium was replaced every 2 days and MDDCs (CD14+ HLA-DR+ CD11c+) cells were collected after 6 days. The purity of the MDDC was >95% as determined by the expression of CD14, CD11c, and HLA-DR. MDDCs were stimulated with 100 ng/ml LPS for 20–24 h at 37°C. The following day, the cells were harvested and stained for surface markers. Supernatants were collected and used for cytokine detection using ELISA.

DC surface staining

Control and LPS-stimulated DCs from both aged and young subjects were stained for the surface expression of CD40, CD80, CD86, CD83, HLA-DR, CCR-7, and TLR4 using directly conjugated Abs (BD Pharmingen). A total of 10,000 CD11c+ HLA-DR+ CD11c+ cells per condition were acquired using a FACSCalibur (BD Pharmingen). Analysis was performed using FlowJo software (Tree Star).

Cytokine and chemokine production by DCs

MDDCs were stimulated with LPS (100 ng/ml) for 20–24 h as described above. Supernatants were collected and stored at −70°C until analyzed. Cytokines TNF-α, IL-6, IL-12p40, IL-12p70, and IL-10 in the supernatants were measured by specific ELISA kits (BD Pharmingen) as per the manufacturer’s protocol. For experiments using the PI3K inhibitor, the procedure was similar except that DCs were pretreated for 1 h with 12.5 and 25 µM LY 294002 (EMD Biosciences) before stimulating with LPS. CCL19/Exodus-1, and stromal cell-derived factor-1 (SDF-1) levels from unstimulated DCs were determined using the searchlight assay from Pierce Biotechnology.

Evaluation of mannose receptor (MR)-mediated endocytosis and macrophagocytosis and phagocytosis

DCs (1 × 10⁶/tube) were suspended in serum-free RPMI 1640 medium with either FITC-conjugated dextran (FITC-DEX: Sigma-Aldrich) or Lucifer Yellow (LU; Sigma-Aldrich) at a final concentration of 1 mg/ml and incubated at 37°C for 10–60 min. The cells were washed three times with cold PBS before FACs analysis. The binding at 4°C was considered as background. CD11c-gated cells were analyzed for the uptake of FITC-DEX and LU. For experiments using the PI3K inhibitor, DCs were pretreated for 1 h with 50 µM LY 294002 before incubation with FITC-DEX.

To measure phagocytosis of apoptotic cells by DCs, Jurkat T cells were labeled with CFSE and induced to undergo apoptosis via incubation with actinomycin-D (200 ng/ml) for 14–15 h. MDDCs from the elderly and the young were cocultured with the apoptotic cells for 3 h at 37°C at a ratio of 1:2. During the last 10 min of the coculture, CD11c allophycocyanin was added to label the MDDCs. Cells were analyzed by FACSCalibur where double-positive cells indicate uptake of apoptotic cells.

DC migration assay

DC migration was measured in duplicate using a transwell system (24-well plate; 8.0-µm pore size; Costar). A total of 600 µl of RPMI 1640 medium with or without 200 ng/ml recombinant human CCL19/Exodus-1 or SDF-1 (PeproTech) was added to the lower chamber. Wells containing medium only were used as a control for spontaneous migration. A total of 5 × 10⁵ cells in 100 µl were added to the upper chamber and incubated at 37°C for 3 h. Cells that migrated into the lower chamber were harvested, concentrated to a volume of 200 µl, and counted by flow cytometry acquiring events for a fixed time of 30 s. The counts fell within a linear range of the control titration curves obtained by testing increasing concentrations of cells. Values were given as the mean number of migrated cells ± SEM. For experiments using the PI3K inhibitor, DCs were pretreated for 1 h with 12.5 and 25 µM LY 294002 (EMD Biosciences) before stimulating with LPS.

Flow cytometry staining of signaling molecules

The expression of phosphorylated AKT, phospho-p38, phospho-ERK, and phosphatase and tensin homolog (PTEN) in DCs was determined by FACS using Abs. On day 6, human MDDCs were stimulated for 0, 30, and 60 min with LPS. Cells were then fixed in Phosflow Buffer I (BD Biosciences) for 10 min at 37°C. After a washing, permeabilization was done with freshly prepared 90% ice-cold methanol for 30 min on ice. Then, the cells were washed in staining buffer (3% FCS in PBS) and stained with directly labeled Abs against phospho-p38-Alexa488 and phospho-AKT-Alexa647 (BD Pharmingen) for 30 min on ice. For experiments using the PI3K inhibitor, DCs were pretreated for 1 h with 50 µM LY 294002 before stimulation with LPS. Staining for PTEN was performed in essentially the same way as above except that unlabeled rabbit Ab (Cell Signaling Technology) was used for primary staining followed by secondary staining using F(ab')₂, anti-rabbit PE (Caltag Laboratories). Appropriate isotype controls were used for all staining. Cells were acquired on FACSCalibur and analysis was done using FlowJo software.

RNA extraction and purification

Total RNA was extracted from MDDCs of aging and young subjects using a TRI Reagent kit (Molecular Research Center), following the manufacturer’s protocol. The integrity of intact total RNA was verified with an Agilent 2100 Bioanalyzer (Agilent Technologies).

Real-time quantitative RT-PCR

Real-time PCR was performed with the Mx3005P QPCR System instrument (Stratagene) using the TaqMan Gene Expression Assay with a FAM dye-labeled TaqMan MGB probe and two PCR primers (Applied Biosystems). PTEN primers (Hs 00829813-s1) were purchased from Applied Biosystems. Briefly, 1 µg of total RNA was reverse-transcribed using the RT-PCR kit (Applied Biosystems), following the manufacturer’s instructions. Each sample was amplified in duplicate and the GAPDH gene was generated for normalization purposes. A 5-log standard curve dilution series was run using each primer pair at optimal concentration and amplification efficiencies were calculated. A correction factor was calculated by dividing the minimum values and averaging the adjusted control values for the sample. This value was used to correct each sample for differences in RNA content.

Statistical analysis

Statistical analysis was done by using the Mann-Whitney nonparametric tests for all figures except Fig. 8. The Wilcoxon signed rank test was used for Fig. 8. Values of p < 0.05 were considered significant.

Results

Numbers and morphology of DCs in blood from elderly and young human subjects are comparable

DCs are critical for the generation of innate and adaptive immune responses. We investigated whether the numbers and phenotype of DCs were altered with aging by examining them in the peripheral blood by flow cytometry. No significant difference was observed in the numbers of circulating myeloid and plasmacytoid DC in the blood between elderly and young subjects (Fig. 1A). The expression of the costimulatory molecules CD86 and HLA-DR was also similar on both aging and young circulating myeloid and plasmacytoid DCs (Fig. 1B), suggesting that the numbers and phenotype of circulating DCs between the elderly and young are comparable.
Aging does not affect MDDC differentiation and maturation

Next, we determined whether the differentiation of DCs from monocytes, in the presence of GM-CSF and IL-4, is affected with age. Purified monocytes were cultured with GM-CSF and IL-4 for 6 days and examined for differentiation into MDDCs by the expression of CD11c<sup>+</sup>, CD14<sup>-</sup>, and HLA-DR<sup>+</sup> using flow cytometry. No significant difference was observed in the phenotypic expression and numbers (1–2.5 × 10<sup>6</sup> MDDC/30–40 ml blood) of MDDC generation from the young and elderly (data not shown).

For activation and maturation, MDDCs (1 × 10<sup>6</sup>/ml) from young and elderly subjects were cultured with or without <i>Escherichia coli</i> LPS (100 ng/ml; List Biologicals) for 20–24 h as described above. Cells were collected and stained for CD40, CD80, CD86, CD83, CD11c, and HLA-DR. The CD11c<sup>+</sup>HLA-DR<sup>+</sup> gated population was analyzed for the expression of the above-mentioned markers (Fig. 2A). The basal level of CD86 tended to be higher, though not significantly (<i>p</i> > 0.05) different, in elderly MDDCs compared with young (Fig. 2, A and B). However, the up-regulation of CD86 on both elderly and young MDDCs was comparable after stimulation with LPS (Fig. 2C). There was no significant difference in the expression of CD40, CD80, CD83, and HLA-DR between the elderly and young, before and after activation with LPS. These data show that differentiation, activation, and maturation of MDDCs is not significantly altered in elderly subjects.

**FIGURE 1.** Numbers and morphology of DCs in blood from elderly and young human subjects are comparable. A, Graph represents the numbers of circulating myeloid (mDC) and plasmacytoid DCs (pDC) between the elderly and young subjects. B, Graph shows the cell surface expression of CD86 and HLA-DR on total circulating DCs in the elderly and the young. The number of dots corresponds to the number of separate individuals tested. Line represents the mean.

**FIGURE 2.** The expression of CD40, CD80, CD83, CD86, and HLA-DR on human MDDCs from young and elderly donors before and after LPS stimulation for 20–24 h. A, Expression of DC markers representative of 15 separate (different subjects) is shown in Fig. 1A. B, Mean MFI (mean fluorescence intensity) ± SD values of the MDDC markers at the basal level without stimulation. C, Mean MFI ± SD values of the markers after 24 h stimulation with LPS. B and C, Mean of 15 different subjects.
MDDCs from the elderly secrete significantly higher levels of proinflammatory cytokines, TNF-α and IL-6, in response to TLR stimulation compared with their young counterparts

Exposure of MDDCs to LPS also leads to secretion of proinflammatory and anti-inflammatory cytokines. Therefore, we examined the cytokine secretion from MDDCs from elderly and young subjects. Briefly, MDDCs from the elderly and young were exposed to LPS (100 ng/ml) for 24 h. Cytokines, TNF-α, IL-6, IL-12p70, IL-12p40, and IL-10 were assayed in the supernatant by ELISA. A significant (p < 0.015) increase in TNF-α secretion was observed by LPS-stimulated MDDCs from the elderly as compared with the young. IL-6 secretion was also significantly higher (p = 0.0018) in the elderly as compared with MDDCs from the young (Fig. 3). No significant difference was observed in the secretion of IL-12p40, IL-12p70, and IL-10 between the elderly and young subjects (Fig. 3A). Comparable levels of IL-10 in the elderly and young may be due to a defect in the negative regulation of inflammation in the elderly.

Because LPS signals through TLR-4 on DCs, we determined whether increased LPS-induced cytokine in the elderly is due to an increased expression of TLR4. MDDCs from elderly and young humans were analyzed for TLR4 receptor expression with specific Ab, using flow cytometry. The level of TLR4 was similar between the elderly and young MDDCs (Fig. 3B). Furthermore, the gene array analyses did not show any significant difference in TLR4 expression between elderly and young subjects (data not shown).

To confirm further whether MDDCs from elderly indeed secrete increased levels of proinflammatory cytokines, we stimulated the MDDCs from the elderly and young with ssRNA (4 μg/ml; Invivogen), which signals through TLR8. Significantly increased secretion of TNF-α (p = 0.03) and IL-6 (p = 0.04) was observed from the MDDCs of the elderly, on stimulating with ssRNA (Fig. 3C). Similar to LPS, IL-10 secretion was not significantly (p = 0.8) different. Thus, enhanced secretion of proinflammatory cytokines is not restricted to TLR4. These data show that MDDCs from the elderly preferentially produce higher levels of proinflammatory cytokines in response to TLR stimulation. This increased secretion of TNF and IL-6 by MDDC from elderly humans may be due to changes in the intracellular signaling downstream of TLRs.

FIGURE 3. Hypersecretion of proinflammatory cytokines by MDDCs from elderly compared with young after stimulation with TLR ligands. A, MDDCs from elderly and young were cocultured with LPS for 24 h. Supernatants were collected and assayed for IL-6, TNF-α, and IL-10 (top), IL-12p40 and IL-12p70 (middle) using the cytokine ELISA kits. The number of dots corresponds to the number of separate individuals tested. Line represents the mean. **, Highly significant. Significance was calculated by Mann-Whitney U test. B, The level of expression of TLR4 on elderly and young MDDCs, as determined by flow cytometry. Bar diagram represents the mean ± SE of the TLR4 MFI of six separate subjects. C, MDDCs from aged and young were cocultured with ssRNA for 24 h. Supernatants were collected and assayed for IL-6 and TNF-α (top) and IL-10 (bottom) using the cytokine ELISA kits. The number of dots corresponds to the number of individuals tested. Line represents the mean. **, Highly significant. Significance was calculated using the Mann-Whitney U test.
Phosphorylation of AKT is reduced in MDDCs from elderly subjects

In the next series of experiments, we investigated the mechanisms underlying the aberrant innate immune functioning of MDDCs from the elderly by examining changes in intracellular signaling in DCs from elderly and young subjects. We determined the functioning of the PI3K pathway, which has been implicated in the regulation of TLR activation, and shown to play an important role in cytokine secretion by MDDCs (26–28). PI3K, upon activation, phosphorylates AKT (a serine/threonine kinase) which then negatively regulates MAPK and NF-κB activation (28). MDDCs from elderly and young subjects were stimulated with LPS for different time points and AKT phosphorylation was measured by flow cytometry with anti-phospho-AKT Ab. The phosphorylation of AKT in MDDCs from the elderly was significantly reduced \( p < 0.05 \) (Fig. 4A) as compared with their young counterparts. Fig. 4B shows cumulative data from eight subjects. In this study also, MDDCs from the elderly showed a significant reduction \( p = 0.005 \) in phosphorylation of AKT. The total basal level of AKT was comparable in the elderly and young (Fig. 4C). Similar results were obtained with Western blot analysis (data not shown).

**FIGURE 4.** Phosphorylation of AKT is reduced in MDDCs from aging subjects. A, Histogram denotes the phosphorylation of AKT at 0, 30, and 60 min in elderly and young MDDCs after stimulation with LPS. \textit{Left:} elderly; \textit{right:} young. B, Graph depicts the MFI of phospho-AKT 1 h post-stimulation with LPS. The number of dots corresponds to the number of separate individuals tested. Line represents the mean. ***, Highly significant. Significance was calculated by Mann-Whitney \( U \) test. C, Bar graph depicts the MFI of nonphosphorylated, total level of AKT in MDDCs from elderly and young. Figure represents mean ± SD of six different subjects.

**FIGURE 5.** Phosphorylation of p38 and ERK in MDDCs from aging subjects. A, Histogram denotes the phosphorylation of p38 at 0, 30, and 60 min in elderly and young MDDCs after stimulation with LPS. \textit{Left:} elderly; \textit{right:} young. B, Graph depicts the MFI of phospho-p38 1 h post-stimulation with LPS. The number of dots corresponds to the number of separate individuals tested. Line represents the mean. ***, Highly significant. Significance was calculated by Mann-Whitney \( U \) test. C, Histogram denotes the phosphorylation of ERK at 0, 30, and 60 min in elderly and young MDDCs after stimulation with LPS. \textit{Left:} elderly; \textit{right:} young. Figure is representative of six such experiments. D, Bar graphs depict the MFI of nonphosphorylated, total levels of p38 and ERK in MDDCs from elderly and young. Figure represents mean ± SD of six different subjects.
LPS-stimulated MDCCs from the elderly show increased phosphorylation of p38 MAPK

P38 acts as a negative regulator of LPS-mediated TLR signaling (26–28). Therefore, reduced phosphorylation of AKT may cause overactivation of the MAPK pathway signaling components, resulting in increased secretion of proinflammatory cytokines. Using flow cytometry, we examined the phosphorylation of p38 and ERK at 0, 30 min, and 1 h poststimulation with LPS. A significant (p < 0.05) increase in phosphorylation of p38 was observed in MDCCs from the elderly, which remained elevated at 1 h, whereas in young MDCCs, phosphorylation of p38 returned to the basal level. Therefore, p38 showed prolonged and sustained phosphorylation in elderly MDCCs (Fig. 5, A and B). Increased activity of p38 with age would explain the increased secretion of proinflammatory cytokines TNF-α and IL-6 with age. ERK phosphorylation was not significantly different between the elderly and the young at all time points tested (Fig. 5C), which may explain the comparable levels of IL-10 secretion from elderly and young MDCCs. We have previously shown that sustained ERK activation in DCs leads to increased IL-10 secretion (25). Basal levels of p38 and ERK were comparable between the elderly and young (Fig. 5D). Therefore, inefficient negative regulation of TLR pathway due to reduced phosphorylation of AKT leads to increased p38 MAPK activation and subsequently increased secretion of proinflammatory cytokines in DCs from the elderly.

Phagocytic capacity of MDCCs is impaired with age

Innate immune functioning of DCs involves Ag uptake through various mechanisms such as phagocytosis, endocytosis, or macropinocytosis (29, 30). Because the PI3K/AKT pathway in aging appears to be impaired and PI3K plays an important role in phagocytic uptake by DCs (31, 32), we compared the capacity of DCs from elderly and young individuals, to phagocytose Ag via MRs, macropinocytosis, and phagocytosis by coincubating DCs with FITC-DEX, LU, or apoptotic cells, respectively. The phagocytosis of FITC-DEX and LU was found to be significantly reduced (p < 0.05) in the elderly subjects compared with young subjects (Fig. 6A and B). Similar results were obtained with the phagocytosis of apoptotic cells. MDCCs from elderly subjects were significantly less efficient (p < 0.05) than their young counterparts in their uptake (Fig. 6D). The expression of MR, one of the receptors involved in the uptake of FITC-DEX, was similar on DCs from the elderly and young (Fig. 6D). Furthermore, the expression of some of the receptors (CD36, αvβ3) involved in phagocytosis of apoptotic cells was also comparable between elderly and young MDCCs (data not shown). This suggests that the defect in phagocytosis could be receptor independent. This is further supported by the fact that macropinocytosis is a receptor-independent mechanism of phagocytosis. However, there are many other receptors involved in the uptake of FITC-DEX and apoptotic cells and we cannot rule out the possibility of them being involved at present. In summary, reduced functioning of PI3K in DCs from the elderly may be responsible for their impaired ability to phagocytose Ag.

Migration of MDCCs is reduced with age

Migration of DCs to lymph nodes is critical for inducing an effective immune response (33–35). Furthermore, PI3K is involved in migration of DCs (36–38). Therefore, we measured the capacity of MDCCs from the elderly and young to migrate...
in vitro in response to MIP-3β and SDF-1 using transwell chambers. LPS-stimulated MDDCs from the elderly were significantly impaired in their ability to migrate in response to MIP-3β ($p = 0.049$) and SDF-1 ($p = 0.045$) as compared with their young counterparts (Fig. 7A). The unstimulated MDDCs from the elderly were also impaired, albeit insignificantly ($p = 0.053$), to migrate in response to MIP-3β. Increased secretion of the chemokines by the MDDCs from the young may be responsible for increased migration of young unstimulated DCs. Therefore, we measured the basal levels of chemokines

![Figure 7](https://example.com/image7)

**FIGURE 7.** Migration of MDDCs is reduced with age. A, Bar diagram denotes the number of DCs which migrated in response to MIP-3β (□) and SDF-1 (■). Figure represents mean ± SE of eight different experiments. **Highly significant.** Mann-Whitney U test was used for determining significance. B, Bar diagram represents the basal level of chemokines secreted by elderly and young unstimulated DCs. C, Bar diagram on the right represents mean ± SE of the MFI of CCR-7 of six separate subjects. D, Bar diagram on the right represents mean ± SE of the MFI of CXCR4 of four separate subjects.

![Figure 8](https://example.com/image8)

**FIGURE 8.** PI3K inhibition in DCs from young renders them functionally similar to DCs from aging. A, Histogram denotes the phosphorylation of AKT, p38, and ERK at 0 and 60 min in LY-treated and untreated MDDCs after stimulation with LPS. Figure is representative of three similar experiments. B, Bar graphs represent cytokine secretion by LY-treated, LPS-stimulated MDDCs. 0, 12.5, 25 are μM concentrations of LY used. Figure represents mean ± SE of six separate individuals. Significance was determined using Wilcoxon signed rank test. **Highly significant.** C, Histogram represents the MFI of the uptake of FITC-DEX by LY-treated and untreated DCs. Figure is representative of four similar experiments. D, Bar graph depicts the number of LY-treated and untreated DCs which migrated in response to MIP-3β. Figure represents mean ± SE of six separate individuals. Significance was determined using the Wilcoxon signed rank test. **Highly significant.**
(MIP3-β, CCL21, and SDF-1) secreted by elderly and young subjects. There was no significant difference in the secretion of all chemokines tested between the elderly and young subjects (Fig. 7B). The young MDDC also did not display a difference in responsiveness to MIP-3β (data not shown). The reduced migration of the MDDCs from elderly could be a consequence of reduced expression of the receptors for these chemokines. Therefore, we measured the expression of CCR7 and CXCR4 (receptors for MIP-3β and SDF-1, respectively) on MDDCs from elderly and young before and after stimulation with LPS. There was no significant difference in the chemokine receptor expression between the elderly and young subjects (Fig. 7C and D). In summary, reduced migration of MDDCs of the elderly may be due to defects in the downstream signaling pathway.

**PI3K inhibition in DCs from the young renders them functionally defective, similar to DCs from the aging**

To further define a role of the reduced activity of the PI3K/AKT-signaling pathway in decreased Ag uptake, migration, and enhanced TNF-α and IL-6 secretion by DCs from aging, we treated the DCs from the young with a specific PI3K inhibitor LY294002 (LY) and examined its effect on the above functions. Initial experiments determined the dose of LY to be used for the experiments (data not shown). Addition of 50 μM LY to MDDCs from young subjects resulted in inhibition of LPS-induced phosphorylation of AKT along with a concomitant increase in p38 phosphorylation, while no significant effect was observed on ERK phosphorylation (Fig. 8A). Furthermore, inhibition of PI3K by LY in MDDCs resulted in increased secretion of TNF-α and IL-6 (p = 0.038, p = 0.039, respectively) in response to LPS (Fig. 8B). Finally, treatment of DCs with the PI3K inhibitor reduced the phagocytosis of FITC-DEX (Fig. 8C) and caused impairment in the capacity of LY-treated DCs to migrate in response to MIP-3β (Fig. 8D, p = 0.047).

These data demonstrate that the PI3K pathway in DCs plays a major role in cytokine secretion, phagocytosis, and migration aspects of DC function. This suggests that reduced functioning of the PI3K/AKT-signaling pathway might be responsible for the aberrant innate immune functioning of DCs in elderly subjects.

The expression of PTEN (negative regulator of PI3K) is increased in MDDCs from the elderly

We wanted to further determine the probable mechanisms underlying the defective phosphorylation of AKT observed with elderly MDDCs. Gene array of MDDCs from the elderly and young revealed (data not shown) an up-regulation of gene PTEN. PTEN is a tumor suppressor that reverses the action of PI3K by catalyzing the removal of the 3’ phosphate of phosphoinositides (39–41). It is a phosphatase with enzymatic activities toward both peptides and 3-phosphoinositides, phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P3) in particular (42–44). Accumulating evidence has shown that the tumor suppressor activity of PTEN relies on its 3-phosphoinositide phosphatase activity. This activity reduces the levels of PtdIns(3,4,5)P3 and other 3-phosphoinositides and thereby antagonizes the activity of PI3K that activates many downstream cellular processes including cell growth, apoptosis, and cell motility (41–44). Thus, PTEN expression could represent a potential mechanism that regulates phosphorylation of AKT. To further confirm our findings from gene array, we did real-time PCR on MDDCs from the elderly and young for the expression of PTEN. Indeed, we found a significant difference (p = 0.0003) in the expression of PTEN between elderly and young MDDCs (Fig. 9A). The expression of PTEN was higher on MDDCs from the elderly, which would explain the observed reduced phosphorylation of AKT in the elderly. To further confirm whether this difference at the genetic level is translated to the protein, we used intracellular flow cytometry to measure the expression of PTEN on elderly and young MDDCs (Fig. 9B). The MFI of PTEN was significantly higher (p = 0.04) at the basal level on elderly MDDCs compared with young (Fig. 9C). To summarize, the expression of PTEN is increased on MDDCs from the elderly and this may be one of the major mechanisms responsible for the reduced phosphorylation of AKT observed with age.

**Discussion**

Progressive decline in immune function and chronic inflammation have been the major causes of the mortality and morbidity associated with aging. Despite extensive investigation, we still do not understand the cellular and molecular mechanisms that are involved in aging-associated chronic inflammation and immune dysfunction. This lack of fundamental knowledge has made attempts at developing effective therapies for the same exceedingly difficult. In this study, we demonstrate that the innate immune function of DCs is greatly altered with age and may in part account for the reduced immunity and enhanced inflammation associated with aging.

Our results indicate that the numbers and phenotype of the circulating myeloid and plasmacytoid DC are comparable between the aging and young subjects of the population studied. MDDCs generated in vitro from the elderly were similar in numbers and phenotype to MDDCs from the young. Further
AGING ALTERS THE INNATE IMMUNE FUNCTIONS OF DCs

Peripheral self tolerance. The present study demonstrates that phagocytosis of self, which is critical for maintenance of infections. DCs present at the sites of pathogen entry “sample” from the elderly (54). A significant difference in TLR4 receptor expression on MDDCs has been reported in earlier studies by mononuclear cells from aging individuals (52). Very few studies have measured the cytokine secretion by DCs and have not reported any significant difference in cytokine secretion between the elderly and young (45). Grolleau-Julius et al. (53) report decreased secretion of cytokines from mouse bone marrow-derived DCs. The reasons for such a discrepancy are not clear. We and others have found no significant difference in TLR4 receptor expression on MDDCs from the elderly (54).

DCs play an important role in inflammation and clearing of the infections. DCs present at the sites of pathogen entry “sample” their environment by phagocytosis, initiating specific immune responses when they sense microbes or tissue damage (19–21). The efficient uptake of pathogens is thus essential for generation of immunity against an infectious agent. Phagocytosis by DCs can be classified in two ways: one is sensing and capture of foreign pathogens by TLRs or other pattern recognition receptors, and the other is phagocytosis of self, which is critical for maintenance of peripheral self tolerance. The present study demonstrates that MDDCs from the elderly are ~50% less efficient in phagocytic uptake through various mechanisms such as macroinocytosis (LY) and endocytosis (FITC-DEX) than their young counterparts. Thus reduction in the phagocytic capacity of DCs from the elderly may contribute to the increased frequency of infections during aging. This inefficiency is not restricted to foreign Ags and is also evident in removal of self, such as their ability to phagocytose apoptotic cells. Because phagocytosis of apoptotic cells triggers the anti-inflammatory response, inefficient removal of apoptotic cells by DCs during aging might result in increased inflammation (55, 56). The extracellular accumulation and subsequent lysis (secondary necrosis) of apoptotic cells due to their impaired uptake by DC would result in the maturation of DCs by endogenous danger signals, leading to proinflammatory cytokine secretion and presentation of self Ags (56). This results in loss of peripheral tolerance and autoimmunity, which are observed in aging.

The mechanisms underlying this defective phagocytosis in aging have not been investigated. Membrane remodeling and cytoskeleton reorganization play a major role in phagocytosis. Thus, alterations in these processes in DCs with age may be responsible for the observed defects. This is further supported by the observation that despite similar levels of expression of CCR-7 and CXCR4, there is impaired migration of DCs from the elderly in response to MIP-3β and SDF-1, compared with DCs from young. This is in agreement with studies in mice, suggesting impaired migration (57, 58). Another study in humans reports no change in transendothelial migration of DCs (51). This could be due to differences in the type of migration, such as migration in response to chemokine vs migration through an endothelial layer.

The PI3Ks are a conserved family of signal transduction enzymes that are involved in regulating cellular activation, inflammatory responses, chemotaxis, and apoptosis. Protein kinase B/AKT is a downstream target of PI3K, and plays a key role in cellular processes such as glucose metabolism, cell proliferation, apoptosis, transcription, and cell migration (59). In the present study, we have observed decreased phosphorylation of AKT in LPS-stimulated MDDCs from elderly subjects. AKT is known to play a major role in cytoskeleton motility and survival in DCs (60, 61). Defective functioning of this pathway is reported to result in decreased phagocytosis and migration of DCs (31, 36, 37). Recent evidence indicates that the PI3K/AKT-signaling pathway may also function as an endogenous negative feedback, or compensatory mechanism, that serves to limit proinflammatory cytokines in response to injurious stimuli. Reports indicate that there is cross-talk between the TLR and PI3K/AKT-signaling pathways. Guha and Mackman (26) have reported that the “PI3K-AKT pathway imposes a braking mechanism to limit the expression” of proinflammatory mediators in LPS-treated monocytes. Fukao et al. (28) have reported that p85 (α) knockout mice showed impaired clearance of enterobacteria injected into the peritoneal cavity. Fukao and Koyasu (27) have reviewed and concluded that PI3K may be an endogenous negative feedback regulator of TLR-mediated inflammatory responses. Martin et al. (61) have shown how the TLR and PI3K pathways serve to balance proinflammatory and anti-inflammatory responses. Our findings of reduced phosphorylation of AKT and concomitant increased phosphorylation of p38 MAPK in DCs from aging suggest this may be one of the mechanisms for increased proinflammatory cytokines in the elderly. A role of an impaired PI3K pathway in elderly MDDCs was further substantiated. We showed that the treatment of DCs from the young with the PI3K inhibitor resulted in functional defects similar to those in aging.

PI3K/AKT is regulated by a variety of intracellular and extracellular signals, including PTEN. PTEN appears to control the activity of protein kinase B/AKT by limiting the lipid substrate, PtdIns(3,4,5)P3, necessary for its activation (62). Increased PTEN expression may act as an inhibitor of AKT phosphorylation and may increase p38 MAPK activation in DCs with elderly subjects. Cells that lack PTEN have elevated levels of PtdIns(3,4,5)P3, phosphorylated AKT, and p70 S6 kinase (S6K) (63). These changes are associated with increased proliferation and reduced apoptosis in T cells, B cells, immature neurons, keratinocytes, and mammary epithelium. It has been shown that PTEN might exert effects on the cytoskeleton and has a role in controlling cell migration. Introducing PTEN into PTEN−/− human tumor cells alters actin fibers and inhibits cell migration (63). Increased expression of PTEN results in decreased phosphorylation of AKT and activation of p38 MAPK which in turn activates AP-1 and NF-κB transcription factors and target genes leading to increased production of TNF and IL-6. Furthermore, impaired AKT leads to defective cytoskeletal rearrangements and therefore impaired Ag capture and migration of MDDCs from the elderly. Thus, improper functioning
of a single pathway (the PI3K-signaling pathway) can affect multiple DC functions resulting in enhancement of proinflammatory cytokine secretion and simultaneous impairment of Ag uptake and migration. Because these results point to a defect in membrane and cytoskeletal dynamics of DCs from the elderly, it will be of interest to see the changes in Ag processing and presentation in future studies as well as the effect this has on immune synapse formation with T cells and its consequences on T cell priming.

Although PTEN and its implications in tumor genesis have been intensively studied, the present results reveal a previously unappreciated aspect of the multifunctional nature of PTEN in DC function. It remains to be seen whether this increased PTEN expression with age is a protective mechanism as the incidence of malignancies increases significantly with aging.

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References


