Flt3 Ligand and CpG ODN Abrogate Impaired Antigen Presenting Cell Function by Aged Dendritic Cells

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Abstract
The immunodeficiency and increased risk of infection seen in elderly patients may be attributable to an alteration in the immune system. Since dendritic cells (DCs) play a key role for the induction of antigen (Ag)-specific immune responses, we hypothesize that upregulation of aged DCs function by flt3 ligand and CpG ODN abrogate for the induction of Ag-specific adjuvant immune responses. Mononuclear cells were isolated from spleen of BALB/c mice (>24 months of age) and were stained with an array of fluorescence-conjugated mAbs in order to determine the frequencies of DCs by fluorescence activated cell sorter (FACS). The spleen from aged mice contained reduced frequencies CD8⁺ DCs and pDCs. Further, aged splenic DCs showed impaired APC function when compared with DCs from young adult mice. To compensate this age-associated alterations, flt3 ligand (FL) and CpG ODN were employed. When aged splenic DCs were cultured with recombinant (r) FL and CpG ODN, significant proliferative responses were induced, which is essentially identical to that of splenic DCs from young mice. Further, aged mice given nasal OVA plus FL plasmid (pFL) and CpG ODN showed high levels of OVA-specific IgG and IgA Abs in plasma which are comparable to those of young adult mice. Thus, the frequencies of CD8⁺ DCs and plasmacytoid DCs in spleen of aged mice were significantly increased after the immunization with pFL and CpG ODN. More importantly, aged splenic DCs expressed increased frequencies of the costimulatory molecules. These results showed that a combination of pFL and CpG ODN treatment was an effective strategy to enhance impaired immunity that is seen in aged mice.

Introduction
Immune functions are known to deteriorate with age in several species. In fact, the risk and severity of infections are higher and the susceptibility to certain types of autoimmune diseases and cancer are greater in the elderly (1, 2), while responses to vaccination are diminished (1, 3, 4). These studies provide evidence of disregulation and an overall decline in host immunity in the elderly. In systemic immune compartments, the age-associated alterations have been studied extensively. Dysfunctions occur in both B and T cells, though the latter are considered to be more susceptible to immunosenescence (1, 2, 5, 6).

It has been shown that dendritic cells (DCs) are central key player for the induction of innate and adoptive immunity. At least three to four subsets of DCs were identified and play distinct roles for the induction and regulation of immune responses. For example, Peyer’s patch DCs expressing CD11c⁺, CD11b⁺, CD8⁻ are immature-type DCs which show high endocytic activity and low levels of MHC and B7 molecule expression (7). Thus, these CD11c⁺,
CD11b+, CD8− immature DCs are myeloid-type and expressing CCR6 for directing their migration toward the SED (8). Further, myeloid-type CD11b+ DCs are known to migrate into the T cells zone upon Ag uptake and in process of maturation by expressing CCR7 (8). Mature interdigitating CD11c+, CD11b−, CD8+ DCs with low endocytic activity and high numbers of MHC class I and class II as well as B7 molecules were identified in the interfollicular T cell regions (7). Thus, this lymphoid-type CD11c+, CD11b−, CD8+ DC expresses high levels of CCR7 (8). One subset of DCs was recently identified as plasmacytoid DCs (pDCs) in humans due to their structural resemblance to Ig-secrating plasma cells (9). Murine pDCs were also identified as cells expressing CD11clow, B220high, Gr−1low and showing plasmacytoid morphology. These murine pDCs also produce IFN−α in response to viral stimulation (10–12) and can be differentiated in high numbers from bone marrow cells in the presence of FL (13–15).

Flt3 ligand (FL), which binds to the fms-like tyrosine kinase receptor Flt3/Flk2. FL mobilizes and stimulates myeloid and lymphoid progenitor cells (16), dendritic cells (DCs) (17), and natural killer (NK) cells (18). Although FL dramatically augments numbers of DCs in vivo, it fails to induce their activation (17). However, our previous study showed that pFL as nasal adjuvant expanded number of CD8+ DCs and up regulated their function for the induction of Ag-specific immune responses (19). It has been shown that bacterial DNA, pathogen-associated molecular patterns (PAMPs), contain a significantly higher frequency of unmethylated cytosine–guanine dinucleotides (CpG) motifs (20, 21). The innate immune system recognizes these unmethylated CpG motifs through the toll–like receptor 9 (TLR9) which is mainly expressed by B cells and pDCs (22). Thus, CpG DNA induces the maturation and stimulation of professional pDCs as well as the subsequent Ag-specific Th1-type and CTL responses (23, 24). Further, synthetic CpG ODN exhibit adjuvant activity through direct activation of TLR9 expressing pDCs (25–27).

Therefore, we hypothesize that impaired immune responses in aged mice is due to dysfunction of DCs and investigate whether a combination of FL and CpG ODN compensate altered DC function in aged mice.

**Materials and Methods**

**Mice**

Young adult (6–8 wks old) BALB/c mice were purchased from the Frederick Cancer Research Facility (National Cancer Institute, Frederick, MD). The retired BALB/c male breeders (8 month old) were obtained from the Jackson Laboratory (Bar Harbor, ME). Upon arrival, all mice were immediately transferred to microisolators and maintained in horizontal laminar flow cabinets and provided sterile food and water ad libitum. Experiments were performed using young adult BALB/c mice between 6 and 8 weeks of aged mice, those over two years of age. The health of the mice was tested semi-annually and mice of all ages used in these experiments were free of bacterial and viral pathogens.

**In Vitro APC Function Analysis**

To assess whether aged DCs maintain antigen presentation activity, DCs were isolated from spleen of young adult and aged mice. CD11c+ DCs from spleen and CLNs were purified by use of CD11c Microbeads (Miltenyi Biotec, Auburn, CA) and AutoMACS system. These purified DCs fractions was >96% CD11c+ and the cells were >99% viable. Splenic cells from DO11.10 Tg mice were stained with FITC conjugated anti-CD4 and PE labeled anti⁻KJ1.26 mAbs. OVA-specific naïve CD4+ T cells were then purified by FACSARia™. Splenic DCs (1 × 10^5 cells) were suspended in RPMI 1640 (Cellgro; Mediatech, Washington, DC) supplemented with HEPES buffer (10 mM), L-glutamine (2 mM), non-essential amino acid solution (10 ml/L), sodium pyruvate (10 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), gentamicin (80 μg/ml), and 10% FCS (complete RPMI 1640) and incubated with OVA (1 mg/ml) for 24 hr. Complete RPMI containing OVA was replaced with OVA–specific CD4+ T cells (1 × 10^5 cells) for 2 days. In some experiments, splenic DCs
from young adult and aged mice were cultured with recombinant FL (10 μg/ml) and CpG ODN 0.5 μg/ml) for 2 days in order to compare potent responsiveness of DCs to rFL and CpG ODN. To assess CD4⁺ T cell and DC proliferative responses, an aliquot of 0.5 μCi of tritiated [³H]TdR (Amersham Biosciences, Arlington Heights, IL) was added during the final 18 h of incubation, and the amount of [³H]TdR incorporation was determined by scintillation counting.

Nasal Adjuvants and Immunization
The plasmid pORF9-mFLt3L (pFL), consisting of the pORF9-mcs vector (pORF) plus the full-length mouse FL cDNA gene (InvivoGen, San Diego, CA) was used as nasal adjuvant (19). This plasmid DNA was purified using the Qiagen Plasmid Giga Kits (Qiagen, Valencia, CA). The Limulus amoebocyte lysate assay (BioWhittaker, Walkersville, MD) resulted in <0.1 endotoxin unit of LPS per 1 μg of plasmid. A synthetic oligodeoxynucleotides (ODN) containing CpG motifs 1826 (5’-TCC ATG ACG TTC CTG ACG TT-3’) (CpG ODN) was obtained from Coley Pharmaceutical Group (Wellesley MA) (27). Mice were immunized three times at weekly intervals nasally with 6.5 μl/nostril PBS containing 100 μg of OVA (fraction V; Sigma-Aldrich, St. Louis, MO) plus 50 μg of pFL and 10 μg of CpG ODN.

OVA-specific Ab assay
OVA-specific Ab levels in plasma was determined by ELISA (19, 28). Briefly, 96-well Falcon micro-test assay plates (BD Biosciences, Oxnard, CA) were coated with 1 mg/ml of OVA in PBS. After blocking with 1% BSA in PBS, 2-fold serial dilutions of the samples were added to each well. Following incubation overnight at 4°C, horseradish peroxidase (HRP) -labeled goat anti-mouse μ, γ, or α heavy chain-specific Abs [Southern Biotechnology Associates (SBA), Birmingham, AL] were added to wells. The color reaction was developed for 15 minutes at room temperature with 100 μl of 1.1 mM 2,2’-azo-ni-bis (3-ethylbenz-thiazoline-6-sulfonic acid) in 0.1 M citrate phosphate buffer (pH 4.2) containing 0.01% H₂O₂. Endpoint titers were expressed as the reciprocal log₂ of the last dilution that gave an OD₄₅₀ nm of 0.1 greater than the non-immunized samples.

Flow cytometry analysis
Aliquots of mononuclear cells were isolated from spleen and cervical lymph nodes (CLN) of mice immunized with OVA plus pFL and CpG ODN. Cells (0.2–1.0 x 10⁶ cells) were stained with FITC-conjugated anti-mouse CD8, CD11c, or B220 mAbs, PE-labeled anti-mouse CD40, CD80, or CD86 mAbs (BD PharMingen), and biotinylated anti-mouse CD11c mAbs (BD PharMingen), followed by PerCP-Cy5.5– streptavidin. The samples were then subjected to FACS analysis (FACS Calibur™; BD Bioscience).

Statistical analysis
The results are expressed as the mean±1 standard error of the mean (SEM). The experimental mouse groups were compared with control mice using an unpaired Mann–Whitney U test with Statview software (Abacus Concepts, Berkley, CA) designed for Macintosh computers. P values of <0.05 were considered significant.

Results
Impaired APC function by aged DCs
We initially examined whether splenic DCs from aged mice maintain normal APC function that is comparable to DCs from young adult mice. When OVA treated splenic DCs from aged mice were incubated with CD4⁺ T cells from naïve D011.10 mice, significant CD4⁺ T cell proliferative responses were noted. However, these responses were markedly lower than those with splenic DCs from young adult mice (Fig. 1). Similarly, when aged DCs from Peyer’s patches of aged mice were examined for their ability to stimulate CD4⁺ T cells, impaired APC function was also noted (data not shown). These results show that age-related alteration was clearly induced in DCs as the major APC.

Reduced numbers of DCs in spleen of aged mice
We next examined the frequency of DCs in spleen of two-yr old and young adult BALB/c mice. Splenic
mononuclear cells from both groups of mice were stained with a combination of anti–CD11c, anti–B220, anti–CD8 and anti–CD11b mAbs in order to identify the major three subsets of DCs: 1) lymphoid type DCs (CD11c+ CD8+); 2) pDCs (CD11c+ B220+); 3) myeloid type DCs (CD11c+ CD11b+). When samples were subjected to flow cytometric analysis, significantly decreased frequency of CD11c+ DCs were noted in spleen of aged mice in comparison to that of young adult mice (Table 1). When subsets of DCs from aged mice were analyzed, the frequency of CD8+ DCs and pDCs significantly decreased (Table 1), while the frequencies of splenic myeloid type DCs were increased in comparison to those in young adult mice. These results indicate that age related altered APC function of splenic DCs may be due to the decreased frequency of CD8+ DC and pDC subsets in aged spleen.

Table 1. Comparison of the frequency of DC subsets in Spleen

<table>
<thead>
<tr>
<th>Age of Mice</th>
<th>CD11c+</th>
<th>CD11c+CD8+</th>
<th>CD11c+B220+</th>
<th>CD11c+CD11b+</th>
</tr>
</thead>
<tbody>
<tr>
<td>8-12 weeks</td>
<td>4.35±0.15*</td>
<td>1.86±0.26</td>
<td>2.21±0.07</td>
<td>0.31±0.11</td>
</tr>
<tr>
<td>&gt;24 months</td>
<td>3.34±0.08*</td>
<td>0.45±0.04*</td>
<td>1.73±0.08*</td>
<td>1.16±0.17*</td>
</tr>
</tbody>
</table>

*p<0.05, when compared with young adult mice.

Aged DCs respond to FL and CpG ODN

Since the numbers of two major subsets of splenic DCs were diminished by aging, it is important to examine whether FL and CpG ODN compensate this age-related alteration. In this regard, we initially test whether aged splenic DCs are able to respond to FL and CpG ODN stimulation. Purified splenic DCs from aged and young adult mice were cultured with FL (10 μg/ml) and CpG ODN (0.5 μg/ml) for 2 days. Proliferative responses by DCs were determined by amount of [3H]-TdR incorporation. FL and CpG ODN stimulated DCs from spleen of aged mice resulted in high levels of proliferative responses which is comparable to those of young adult mice (Fig. 2).
These results show that aged DCs are capable to respond to the stimulation of FL and CpG ODN, which is equivalent to that seen in young adult mice.

**Nasal immunization with OVA plus pFL and CpG ODN induces Ag-specific antibody responses in plasma of aged mice**

Next, we examined whether in vivo application of FL and CpG ODN compensate impaired APC function of aged DCs. Thus, aged mice were nasally immunized with OVA only or OVA plus pFL and CpG ODN and Ag-specific Ab responses were examined. High level of anti-OVA IgG Ab response was seen in aged mice given nasal OVA plus pFL and CpG ODN which is comparable to that of young adult mice (Fig. 3). Further, OVA–specific IgA Ab responses were also detected in plasma of aged mice given nasal pFL and CpG ODN as mucosal adjuvant (Fig. 3). On the other hand, aged mice given OVA only failed to induce any OVA–specific Ab responses (Fig. 3). These results indicate that nasally administered pFL and CpG ODN effectively upregulate aged DC function for the subsequent induction of Ag–specific Ab responses.

**Nasal administration of pFL and CpG ODN expands DCs in aged mice**

Since significant levels of Ag-specific Ab responses were induced in aged mice, it is important to investigate the frequency of CD11c+ DCs. In this regard, mononuclear cells were isolated from CLN in addition to spleen since CLN is the main draining lymph nodes of nasal immunization. Significantly increased frequency of CD11c expressing DCs was noted in CLN and spleen of aged mice given nasal OVA plus pFL and CpG ODN when compared with mice give nasal OVA only (Fig. 4). It is interesting to note that the change in CLN was more significant than that in spleen. These results clearly show that nasal application of pFL and CpG ODN compensate decreased number of DC in aged mice for the induction of Ag–specific immune responses.

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**Fig. 3.** OVA-specific Ab responses in plasma of aged mice given nasal OVA with (□)/without (▲) pFL and CpG ODN. Each mouse group was nasally immunized weekly for 3 consecutive weeks with 100 μg of OVA plus 50 μg of pFL and 10 μg of CpG ODN as mucosal adjuvants. Seven days after the final immunization, the levels of OVA–specific IgG and IgA Ab responses in plasma were determined by an OVA–specific ELISA. Values are shown as the mean±SEM taken from 6 mice in each experimental group.

**Fig. 4.** The frequency of CD11c+ DCs in spleen of aged mice given nasal OVA with (□)/without (▲) pFL and CpG ODN. Mice were nasally immunized weekly for 3 consecutive weeks with 100 μg of OVA plus of pFL (50 μg) and CpG ODN (10 μg). Mononuclear cell from spleen were isolated 7 days after the last immunization and were stained with biotinylated anti-CD11c mAb followed by Cy5.5–streptavidin. The frequency of CD11c expressing DCs was determined by FACS Calibur™. Values are shown as the mean±SEM of 6 mice in each experimental group. *p<0.05, when compared with aged mice without pFL and CpG ODN.
**Nasal pFL and CpG ODN target CD8+ DCs and pDCs in aged mice**

Since increased frequencies of CD11c+ DCs were seen in the spleen and CLN of aged mice given nasal pFL and CpG ODN, it is important to examine that which subsets of DCs were increased and upregulated. In order to characterize the phenotype of these expanded DCs, we assessed cell surface expression by CD11c+ DCs [CD8, B220, MHC class II (MHC II), CD40, CD80, and CD86] in spleen and CLN. Interestingly, the distribution of CD11b+ DC subset was not changed after nasal pFL and CpG ODN. However, both spleen and CLN showed increased frequencies of the CD8+ DC (CD11c+ CD8+) and pDC (CD11c+ B220+ subsets (Table 2). Further, higher levels CD40, CD80 and CD86 molecules were expressed by CD8+ DCs and pDCs of mice given nasal pFL and CpG ODN when compared with those of mice given OVA only (Table 3). Taken together, these results indicate that nasal administration of pFL and CpG ODN preferentially expands and stimulates CD8+ DCs and pDCs subsets in CLN and spleen for the induction of Ag–specific Ab responses in aged mice.

**Table 2. The frequency of DCs in two-year old mice given nasal OVA plus pFL and CpG ODN**

<table>
<thead>
<tr>
<th>Nasal OVA plus</th>
<th>Percent of total lymphocyte</th>
<th>CD11c+ DCs</th>
<th>CD11b+ DCs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CD8+</td>
<td>B220+</td>
</tr>
<tr>
<td>Spleen</td>
<td>None</td>
<td>0.45±0.03</td>
<td>1.73±0.08</td>
</tr>
<tr>
<td></td>
<td>pFL/CpG ODN</td>
<td>1.00±0.06*</td>
<td>2.45±0.21*</td>
</tr>
<tr>
<td>CLN</td>
<td>None</td>
<td>0.41±0.05*</td>
<td>0.8±0.01</td>
</tr>
<tr>
<td></td>
<td>pFL/CpG ODN</td>
<td>1.50±0.10*</td>
<td>1.80±0.20*</td>
</tr>
</tbody>
</table>

*p < 0.05, when compared with mice given OVA only

**Table 3. The frequency of costimulatory molecule expressing DCs in two-year old mice given nasal OVA plus pFL and CpG ODN**

<table>
<thead>
<tr>
<th>Nasal OVA plus</th>
<th>CD11c+ DCs</th>
<th>CD80+ DCs</th>
<th>CD86+ DCs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD40+</td>
<td>CD80+</td>
<td>CD86+</td>
</tr>
<tr>
<td>Spleen</td>
<td>None</td>
<td>2.66±0.07</td>
<td>0.08±0.02</td>
</tr>
<tr>
<td></td>
<td>pFL/CpG ODN</td>
<td>2.90±0.01</td>
<td>0.30±0.02*</td>
</tr>
<tr>
<td>CLN</td>
<td>None</td>
<td>0.70±0.06</td>
<td>0.30±0.09</td>
</tr>
<tr>
<td></td>
<td>pFL/CpG ODN</td>
<td>2.50±0.20*</td>
<td>1.30±0.06*</td>
</tr>
</tbody>
</table>

*p < 0.05, when compared with mice given OVA only

**Discussion**

The present study shows that splenic DCs from aged mice possess lower APC activity than those of young adult mice. Thus, the number of DCs was decreased in spleen of aged mice. Flow cytometric analyses revealed that CD8+ DCs and pDC subsets were significantly affected by aging among different subsets of DCs. When DC targeted FL and CpG ODN were employed via the nasal route for the compensation of age–related alteration of DC function, pDC and CD8+ DC population were selectively up-regulated and their numbers were increased in CLN and spleen of aged mice. Further, co-administered Ag–specific Ab responses were also induced in aged mice given nasal pFL and CpG ODN. These results are first to show that impaired aged DC function can be converted by DC targeting FL and CpG ODN.

Recent studies have also shown that aged DCs exhibit less ability to trigger T and B cells (29, 30). On the other hand, others have shown intact APC functions in aged DCs (31–33). Thus, the quality of APC function by aged DCs is a controversial issue. However, our past study that lends the strongest support for impaired APC function by aged DCs showed that aged mice resulted in impaired Ag–specific Ab responses when orally immunized with OVA plus nCT as mucosal adjuvant. In this regard, it is logical to predict that DCs from aged mice would be less able to stimulate CD4+ T cells than those of young adult mice. Indeed, our study clearly showed that aged splenic DC exhibited lower APC function when co-cultured with OVA–specific CD4+ T cells. Thus, lower T cell proliferative responses were seen in CD4+ T cell cultures incubated with DCs from aged mice.

Our results showed that APC functions of splenic DCs altered in aged mice. Further, the frequencies of CD8+ DCs and pDCs were significantly reduced in spleen of aged mice. In this regard, we next examined the stimulatory potency of aged splenic DCs other than Ag. We employed FL and CpG ODN to stimulate aged DCs since FL are known to expand CD8+ DC population (19) and CpG ODN selectively to stimulate pDCs (22–24). Our results showed that
proliferative response by aged DCs is comparable to DCs of young adult mice. To confirm this finding, we are currently testing Flt3 and TLR9 expression by aged DCs since these receptors mediate the signaling pathways are essential for the upregulation of aged DCs. Flt3 expression by aged pDCs is determined by flow cytometry using PE conjugated anti–Flt3 (CD135) mAb together with FITC labeled anti–CD8, Allophycocyanin-conjugated anti–B220 and biotinylated anti–CD11c mAbs followed by PerCP–Cy5.5-streptavidin. Further, DCs are purified from spleen of both young adult and aged mice and then be subjected to Flt3– and TLR9–specific real–time quantitative RT–PCR analysis. Our preliminary results showed that both Flt3 and TLR9 expression by aged DCs are comparable to those of young adult mice (data not shown). These results show that a combination of FL and CpG ODN is potent stimulatory signals of aged DCs for the induction of Ag–specific immune responses. Although it would be logical to predict that diminished APC functions by aged DCs would indicate an impaired innate signaling by FL and CpG, we hypothesize that these responses are not correlated with each other. To support this, it has been suggested that age–associated deficiency can be corrected by the appropriate cytokine stimulation (30). Indeed, it has been shown that aged DCs derived by in vitro GM–CSF treatment showed intact APC activity (32, 33). Further, it was reported that aged DCs responded well to FL stimulation (34).

It has been shown that Ag–specific IgA B cell responses play a central role in the induction of mucosal immunity to infectious diseases (35). However, mucosal immunity is affected in aging and susceptibility to infectious diseases is increased in the elderly (4, 36). In fact, in elderly human pathogens which invade through mucosal surfaces, such as the influenza virus and the bacterial pathogen Streptococcus pneumoniae (S. pneumoniae), cause more severe and more frequently lethal infections (3, 37, 38). It has been shown that aged animals given oral nCT contained significantly lower anti–CT–B IgA Ab responses than did those from identically immunized young rats (39, 40) and rhesus macaques (41). When aged mice were orally immunized with nCT as mucosal adjuvant, Ag–specific S–IgA Ab responses were also lower than in young mice (42, 43). These results clearly indicate that Ag–specific mucosal IgA Ab responses are diminished in aged animals. Although the significant reductions in Ag–specific S–IgA Ab responses in the elderly is clear evidence for mucosal senescence, our previous study showed potency of nasal immunization regimen for the induction of Ag–specific IgA Ab responses in aged mice when compared with oral immunization strategy (28, 43). Even though the most potent mucosal adjuvant, such as nCT failed to induce Ag–specific IgA Ab responses in plasma as well as external secretions (28). These studies indicate that although nasal immunization is a potent immunization regimen to induce Ag–specific IgA Ab responses, it would be require another novel nasal adjuvant for the induction of mucosal immunity in aged mice. Our present study showed significant findings that nasal application of pFL and CpG ODN could induce Ag–specific IgA Ab responses in plasma of aged mice. These results also indicate that a combination of pFL and CpG ODN would be a potent DC targeting mucosal adjuvant for the induction of Ag–specific mucosal IgA Ab responses in addition to systemic immunity.

In summary, our studies show that impaired APC function could be corrected by FL and CpG ODN. Thus, nasal application of pFL and CpG ODN resulted in the increased frequency of DC subsets and the induction of Ag–specific IgG and IgA Ab responses in plasma of aged mice. To this end, our current finding would light on the shade in the development of effective mucosal vaccine for the elderly.

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