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TLR-Activated Dendritic Cells Enhance the Response of Aged Naive CD4 T Cells via an IL-6–Dependent Mechanism

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The most effective immunological adjuvants contain microbial products, such as TLR agonists, which bind to conserved pathogen recognition receptors. These activate dendritic cells (DCs) to become highly effective APCs. We assessed whether TLR ligand-treated DCs can enhance the otherwise defective response of aged naive CD4 T cells. In vivo administration of CpG, polyinosinic-polycytidylic acid, and Pam3CSK4 in combination with Ag resulted in the increased expression of costimulatory molecules and MHC class II by DCs, increased serum levels of the inflammatory cytokines IL-6 and RANTES, and increased cognate CD4 T cell responses in young and aged mice. We show that, in vitro, preactivation of DCs by TLR ligands makes them more efficient APCs for aged naive CD4 T cells. After T–DC interaction, there are enhanced production of inflammatory cytokines, particularly IL-6, and greater expansion of the aged T cells, resulting from increased proliferation and greater effector survival with increased levels of Bcl-2. TLR preactivation of both bone marrow-derived and ex vivo DCs improved responses. IL-6 produced by the activated DCs during cognate T cell interaction was necessary for enhanced aged CD4 T cell expansion and survival. These studies suggest that some age-associated immune defects may be overcome by targeted activation of APCs by TLR ligands. The Journal of Immunology, 2010, 185: 6783–6794.

The age-associated decrease in the function of the immune system contributes to the increased susceptibility to infectious diseases, such as influenza, seen in the elderly (1–7). With advanced age, responses to new pathogens and to vaccines containing new Ags are preferentially reduced. In the aged, efficacy of vaccination is limited by reduced Ag-specific B cell expansion and differentiation (8), and the Abs that are produced are less functional because of reduced class switching and somatic hypermutation (9). In mice, it has been shown that this is due, in large part, to the age-associated decline in cognate B cell help by aged naive CD4 T cells, which we have shown are profoundly defective in helping to generate germinal center B cells and high titers of Ag-specific Ab (10). The responses of aged naive CD4 T cells also display well-characterized defects in IL-2 production, expansion, and effector generation, and they become poorly responsive memory cells (11–17). Our more recent studies suggest that a limited complement of defects arise initially from intrinsic changes that are already present in the aged CD4 T cell recent thymic emigrant populations (14), and that more severe defects develop as the cells themselves age in the periphery (15–17).

Recent work by our laboratory and others has highlighted the valuable Ab-independent role that fully competent CD4 effectors and memory cells can play during viral infection, both as helpers of the CD8 T cell response (18) and as direct mediators of viral clearance (19, 20). Thus, defects in the aged CD4 T cell response are likely to cause both markedly diminished humoral and cell-mediated responses against infections with either new viruses or altered strains, as well as the failure to develop protective immunity.

The in vitro defect in the expansion of aged CD4 T cells and generation of effectors can be overcome by the addition of exogenous IL-2, but not other IL-2R-γc–binding cytokines (11, 14). However, effectors “rescued” by exogenous IL-2 did not become functional memory CD4 T cells (13, 21). Recently, we demonstrated that, in the presence of inflammatory cytokines (IL-6, TNF-α, IL-1) in vitro or when these cytokines, or a strong adjuvant such as CFA, were included as part of an in vivo immunization regimen, the response of aged CD4 T cells was greatly enhanced (12). Thus, we considered that the pretreatment of APCs with adjuvants that could enhance their production of inflammatory cytokines (22) could potentially stimulate stronger naive T cell responses against new Ags of emerging pathogens or new strains that have mutated to evade current immunity.

Aluminum hydroxide (alum) is the most commonly used adjuvant in human vaccines. However, its application is limited by its stimulation of Th2 responses, and thus by its limited ability to enhance protection against intracellular parasites such as Mycobacterium tuberculosis, HIV, and influenza (23, 24). TLRs are expressed by many immature immune cells, including dendritic cells (DCs) and other APCs, endothelial cells, fibroblasts, and lymphocytes. TLRs have recently garnered much attention for their role in initiating the innate immune response against infectious pathogens (reviewed in Ref. 25). Each of the 10 known TLRs recognizes a different “pathogen-associated molecular pattern” expressed by bacteria, vi-
ruses, and fungi. During an infection, triggering of TLRs on DCs regulates their migration to regional lymph nodes (LN s) via their downregulation of CCR5 and upregulation of CCR7 (26). In the LNs, the TLR-activated DCs promote strong T cell responses via their increased costimulatory molecule and MHC expression (27). TLR activation of DCs, as well as other APCs, induces production of a number of inflammatory cytokines such as IL-1, IL-6, and TNF-α (28, 29), as well as antiviral cytokines such as IFN-α and -β (27), and IL-12 (27, 30), which is critical for Th1 polarization.

The ability of TLR ligands to influence the subsequent adaptive immune response has stimulated great interest in their use as vaccine adjuvants (30). Many current vaccines that contain whole organisms also contain intrinsic microbial ligands for TLR; but in nonreplicating vaccines, the level of TLR ligand expression may be too low to act as an effective adjuvant. Thus, the level and role of TLR activation associated with current inactivated or subunit vaccines is unclear (27). Preclinical studies using TLR ligands such as viral dsRNA (e.g., polyinosinic-polycytidylic acid [PolyI:C; TLR3 ligand] and bacterial unmethylated CpG DNA [Cpg; TLR9 ligand]) have shown that the addition of exogenous TLR ligands during priming can enhance the humoral and/or cellular response to HIV-1 (31), hepatitis B (32), Leishmania major (33), influenza (34) and tumor model (35, 36) Ags.

There are conflicting data concerning the effect of age on the responsiveness of APCs to stimulation by TLR agonists. Studies with macrophages from aged mice have shown decreased TLR gene expression (37), reduced cytokine secretion (37, 38), and intracellular signaling (38) in response to TLR stimulation. Analysis performed on human monocytes demonstrated depressed cytokine secretion in the aged in response to Pam3CSK4, which signals through TLR1/2 (39, 40), and a more general defect in the upregulation of CD80 expression in response to a broad spectrum of TLR ligands (40). In contrast with these studies, data from a recent mouse study (41) suggests that activation and maturation of aged DC in response to a broad range of TLR agonists is intact. Most important in this study was the finding that both young and aged DCs effectively primed a T cell response (41). In addition, a study in humans demonstrated that aged DCs undergo normal phenotypic activation after LPS stimulation; however, these cells secreted increased amounts of inflammatory cytokines (42). Maue et al. (43) recently demonstrated that including a TLR ligand in an in vivo immunization regimen results in an increased T cell-dependent B cell response. However, because of the wide range of cells that express TLR in vivo, the mechanism by which this TLR agonist is enhancing the helper cell response is unknown. Thus, there remains relatively little data examining the extent to which TLR ligands acting on APCs can act as adjuvants to improve the response of aged naive CD4 T cells, and the mechanisms by which TLR stimulation of APCs can affect the outcome of aged T cell stimulation remain unclear. Moreover, the broad range of cells expressing TLRs means that systemic administration of TLR ligands would have pleiotropic effects on many targets, likely including some that could be deleterious, which would limit their use, especially in the elderly. If targeted activation of APCs is sufficient to overcome aged naive CD4 T cell defects, then new strategies could be developed to exploit this ability, thereby avoiding many potential problems of systemic administration.

In this study, we found that the TLR agonists Cpg, PolyI:C, and Pam3CSK4 are effective in activating the DCs of aged and young mice in vitro, as well as in vivo, and we show that the addition of Cpg to the immunization regimen enhances the in vivo Ag-specific response of aged and young naive CD4 T cells. To define the mechanisms responsible for this adjuvant activity, we have developed an in vitro model system to assess the hypothesis that direct stimulation of DCs by TLR ligands enhances their APC function and subsequent ability to prime aged naive CD4 T cells, and that this results in a reversal of key aspects of age-associated unresponsiveness. Our results indicate that TLR preactivated DCs used as APCs induce a substantial increase in the Ag-specific expansion of aged naive CD4 T cells, resulting from a combination of increased proliferation and enhanced survival with greater expression of Bcl-2 by activated CD4 T cells. The enhanced response depends on IL-6, whose production by DCs during cognate interaction with the T cell is greatly increased. These studies define key components responsible for enhancing defective responses of aged naive CD4 T cells and illustrate the potential of targeted vaccine approaches in the aged that take advantage of the natural adjuvant properties of TLR ligands.

Materials and Methods

Mice

HNT H-2b th TCR transgenic (Tg) mice express a Vβ8.3 (Vα unknown) transgene that recognizes the 126–138 fragment of the PR8 influenza hemagglutinin (HA) molecule in the context of I-Ab (44). HNT TCR Tg, C57BL/6 H-2b (B6) and BALB/c H-2b (BALB/c) were bred at the Trudeau Institute Animal Facility (Saranac Lake, NY). All mice were fed sterile standard diet ad libitum and housed in isolator cages under specific pathogen-free conditions. Mice referred to as young were 6–10 wk of age; aged B6 and BALB/c mice were 17–24 mo old; aged HNT TCR Tg mice were at least 15–17 mo old. Aged mice were inspected for gross pathology, and animals exhibiting pathology were excluded from experiments. The Trudeau Institute Institutional Animal Care and Use Committee approved all experimental animal procedures.

Flow cytometric analysis

For flow cytometric analysis, cells suspended in PBS supplemented with 2% BSA and 0.1% NaN3 were incubated with fluorochrome-conjugated Abs for 30 min on ice and in the dark. Cells were either analyzed immediately or fixed in 1% paraformaldehyde. mAbs used for these studies were specific for CD11c, CD11b, CD54, CD80, CD86, CD40, IAd, H-2b, IAb, CD4, CD8, Vβ8.3, CD25, CD44, CD26L, CD162, IL-6, and the appropriate irrelevant isotype controls. Flow cytometric data were acquired on a FACS Calibur (BD Biosciences, San Jose, CA) cytometer using CellQuest (BD Biosciences) software. Analysis of cytometric data was done using FlowJo version 6.1.1 software (TreeStar, Ashland, OR).

In vivo TLR administration and priming

Young and aged B6 mice were injected s.c. in the subcapsular region with the indicated TLR ligands (Cpg [60 μg], PolyI:C [100 μg], Pam3CSK4 [50 μg]). At the indicated time points, serum was harvested and cytokine levels were determined by either ELISA or the LumineX Cytokine Bead Array assay (Millipore, Billerica, MA), as per manufacturer’s instructions. When indicated, draining lymph nodes (DLNs) were harvested, teased apart, digested with collagenase D at 37°C for 1 h and stained, after which cells were prepared for FACs analysis as indicated. For priming experiments, young and aged B6 mice were primed i.p. with 50 μg OVA323–339 in PBS, with alum (Pierce, Rockford, IL), or with alum + 60 μg Cpg. Twenty-three days later, spleens and peripheral LN s were harvested from primed animals. CD4 T cells were positively selected using magnetic beads and restimulated with syngeneic spleen cells and relevant peptide in ELISPOT plates coated with anti-IL-2. ELISPOT analysis was performed as previously described (45).

Isolation of young and aged CD4 T cells

CD4 T cells were isolated from HNT TCR Tg mice as previously described (16). In brief, young and aged CD4 T cells were isolated with anti-CD4 beads according to the manufacturer’s protocol (Miltenyi Biotec, Auburn,
Bone marrow-derived DCs

Bone marrow-derived DCs (BMDCs) were generated as previously described (47), with minor changes. In brief, bone marrow was flushed from femurs and tibias of either young or aged BALB/c or B6 mice and plated at 5 × 10^5 cells/ml in complete RPMI 1640 plus 5 μg/ml recombinant murine GM-CSF (PeproTech, Rocky Hill, NJ). On days 5 and 5.5, half of the culture volume was replaced with fresh complete RPMI 1640 + GM-CSF. The nonadherent cells were harvested on day 7 and characterized to be CD4^+ CD8α^−, CD11b^+, CD11c^+, CD54^+, CD80^+, CD86^+, and MHC class I^+/. Harvested BMDCs were routinely enriched using directly conjugated CD11c magnetic beads and a MACS column, as per manufacturer’s instructions (Miltenyi Biotec). When indicated, CD11c-enriched BMDCs were incubated for 24 h with 5 μg/ml GM-CSF plus TLR adjuvants, such as CpG 1826 (1 μM; type B unmethylated CpG-oligodeoxynucleotides [ODNs]; Integrated DNA Technologies, Coralville, IA), PolyI:C (10 μg/ml; Sigma or Invivogen, San Diego, CA), or Pam3CSK4 (300 ng/ml; Invivogen). The working concentration of each TLR ligand was based on preliminary studies in which we determined the amount needed to consistently induce the activation of BMDCs as assessed by the up-regulation of CD80 and CD86. When indicated, BMDC culture supernatants were collected and analyzed for cytokine production using the Luminex Cytokine Bead Array assay (Millipore), as per manufacturer’s instructions. RBCs in the suspension were lysed with ammonium-chloride-potassium lysis buffer. The cells were then fixed with 4% paraformaldehyde for 20 min at room temperature. Cells were permeabilized with 0.1% NaN3, and 0.1% saponin) containing anti-mouse Bcl-2 (BD Pharmingen) or anti-mouse IL-6 (BD Pharmingen) or isotype control and incubated for 20–30 min at room temperature. Samples were then washed with PBS and analyzed immediately on a FACSCalibur flow cytometer.

Statistical analyses

Statistical analyses were performed by Prism 4.0 software (GraphPad, La Jolla, CA) using Student t test. The p values <0.05 were considered significant and are indicated by an asterisk.

Results

CpG enhances the aged CD4 T cell response in vivo

The use of adjuvants that contain TLR ligands has been proposed as a means to enhance the immune response of elderly individuals who respond poorly to current vaccination strategies (50). It is unclear whether TLR ligand activation of only APCs can enhance the Ag-specific naive CD4 T cell response, and if so, what mechanisms might be involved. To confirm the responsiveness of aged in vivo APCs, we tested whether TLR agonists could induce in situ activation of myeloid DCs in aged and young mice. After an initial test of agonists to TLR2-5, TLR7, and TLR9, we chose three TLR ligands for these in vivo studies: CpG (TLR9 ligand), PolyI:C (TLR3 ligand), and Pam3CSK4 (TLR2/1 ligand). We chose CpG and PolyI:C because both TLR3 and TLR9 are expressed in endosomes, and are capable of inducing DCs to produce inflammatory cytokines and promote the development of Th1 CD4 T cells (30). In contrast, TLR2 is expressed on the extra-cellular plasma membrane and is capable of promoting either a Th1 or a Th2 type of response (30).

Young and aged B6 mice were injected s.c. with either CpG, PolyI:C, or Pam3CSK4. Twenty-four hours later, the DLNs were removed and the cells were stained to assess CD86, CD40, and I-Ab expression on CD11c^+ CD11b^+ cells, which represent the predominant myeloid DCs. As seen in Fig. 1A and 1B, CD11c^+ CD11b^+ cells from young and aged mice treated with TLR agonists expressed markedly greater levels of these costimulatory molecules that are known to be key in activating naive CD4 T cells, compared with animals of the same age that received only PBS. In addition, CD11c^+CD11b^− lymphoid DCs from young and aged mice were found to be similarly activated after injection of CpG, PolyI:C, and Pam3CSK4 (data not shown). These data confirm, as previously suggested, that these key features of TLR activation of DCs are intact in aged mice treated with each ligand (41).

We have previously shown that the addition of alum by itself to the immunization regimen is not sufficient to enhance the response of aged CD4 T cells (12). To determine whether the addition of a representative TLR agonist, CpG, to the alum/Ag immunization regimen could enhance the response of aged CD4 T cells in vivo, we injected young and aged B6 mice with either OVA233–339 alone, OVA233–339 in alum, or OVA233–339 in alum + CpG. Twenty-one days after priming, CD4 T cells enriched from pooled spleen and LNs were restimulated with Ag, and the number of IL-2–producing cells was assessed by ELISPOT. We found that the inclusion of alum alone was effective in enhancing the young CD4 T cell response to peptide Ag. However, consistent with our previously published data (12), alum by itself was unable to
significantly boost the aged CD4 T cell response. In contrast, the inclusion of CpG in the vaccine formulation led to a significant increase in the number of primed aged CD4 T cells that produced IL-2 on restimulation ex vitro. These data illustrate that aged mice have an intact response to TLR agonists, which activate in situ DCs to upregulate costimulatory and MHC class II molecules, and enhance the aged naive CD4 T cell response.

This improved CD4 T cell response could be the result of TLR agonist action on DCs or on other APCs leading to enhanced Ag presentation or inflammatory cytokine production, or it could result from direct stimulation of the CD4 T cells by the TLR ligand (51, 52). Alternatively, inflammatory cytokines from other TLR-agonist–activated cell types, such as macrophages, B cells, neutrophils, and endothelial cells, could contribute to enhancing the aged CD4 T cell response. These possibilities would be difficult to evaluate in vivo, so we developed an in vitro model to isolate the effects of TLR agonists acting directly on APCs and to determine mechanisms by which the activated DCs might subsequently “rescue” aged responses of naive aged CD4 T cells.

**Ag presentation by TLR-activated DCs enhances the aged CD4 T cell response in vitro**

BMDCs were TLR-stimulated, washed, and then used as APCs in a cognate T cell response model to determine whether the direct activation of DCs by TLR agonists could generate APCs capable of overcoming defects in the aged CD4 T cell response to Ag. We generated “immature” BMDCs with GM-CSF and enriched for CD11c+ myeloid DCs (53). When these cells were stimulated for 24 h by either CpG, PolyI:C, or Pam3CSK4, they expressed markedly greater levels of CD80, CD86, and CD40, and secreted increased levels of TNF-α, RANTES, keratinocyte-derived chemokine (KC), IL-6, IL-12p70, and IL-12p40 than unstimulated BMDCs (Supplemental Fig. 2). Of most importance, BMDCs derived from bone marrow of aged BALB/c mice were as equally responsive as young BMDCs to TLR stimulation. This finding was indicated by the increased expression of CD80, CD86, and CD40 (Fig. 2A), as well as by the increased secretion of KC, RANTES, IL-6, IL-12p70, and IL-12p40 by both young and aged BMDCs after stimulation by CpG (Fig. 2B). The increased production of inflammatory cytokines in the young and aged BMDC cultures after TLR stimulation was mirrored by increased levels of these cytokines in the serum of young and aged BALB/c mice after the in vivo administration of CpG and PolyI:C, indicating similar effects of TLR-ligand administration in vitro and in vivo (Fig. 2C).

Purified naive (CD44loCD62Lhi) CD4 T cells from aged HNT TCR Tg mice that express a TCR specific for influenza HA and recognize A/PR8/34 influenza (54) exhibit a profound dysfunction relative to their young counterparts after encounter of cognate Ag (HA126–138) presented by B cell blasts in vitro, including diminished IL-2 production and CD25 expression, and reduced expansion (16). These in vitro defects correlate with defects in response to Ag in vivo, which included a diminished capability to produce IL-2 and a failure to provide efficient cognate help to a B cell response (16). In this study, we analyze the in vitro response of purified naive HNT Tg CD4 T cells from young and aged mice, sorted on the basis of their naive phenotype to be CD4+ Vb8.3+CD44lo. The naive CD4 T cells from aged mice mounted a significantly reduced proliferative response to their cognate Ag compared with identically treated young cells when unstimulated BMDCs were used as APCs (Fig. 3A). When the BMDCs were preactivated overnight with CpG, the proliferative response of the cocultured aged naive CD4 T cells was significantly enhanced. To better analyze the effects of CpG pre-
activation of BMDCs on the kinetics and magnitude of the aged naive CD4 T cell response, we determined the fold expansion over the input number of young and aged HNT CD4 T cells on each day of a 5-d culture. Presenting T cell numbers as “fold expansion” allows us to better analyze combined results of multiple experiments and compare effects of different experimental treatments. Expansion is a reflection of not only the division of the activated cells, but their survival, and is physiologically relevant. We noted that aged cells activated by Ag plus untreated BMDCs demonstrated a marked defect in expansion on days 4–6 compared with identically treated young cells (Fig. 3B), and that activated aged cells produced only about half of the amount of IL-2 produced by their young counterparts (Fig. 3C). In contrast, when Ag was presented by CpG-stimulated BMDCs, there was a significant increase in the expansion of both young and aged naive CD4 T cells on days 4–6 that correlated with a nearly 2-fold increase in the amount of IL-2 found in 48-h culture supernatants (Fig. 3B, 3C). Importantly, CpG-activated BMDCs derived from the bone marrow of aged BALB/c mice were equally capable of inducing the enhanced Ag-driven expansion of young and aged naive HNT CD4 T cells (Fig. 3D). Given that both young and aged BMDCs were functionally activated to the same extent by TLR stimulation and the relative greater availability of young mice, we used young BMDCs for the remainder of the studies.

We screened a number of other TLR agonists for their abilities to activate BMDCs to enhance the expansion of aged naive CD4T cells. We observed that, similar to CpG, activation of the immature BMDCs with either PolyI:C, Pam3CSK4, LPS, or loxoribine induced significantly greater Ag-driven expansion of aged naive CD4 T cells (Fig. 4A). At this time point, only BMDC
activated by PolyI:C and CpG induced a significantly greater level of expansion of young CD4 T cells than the already strong proliferative response of young cells activated by nonstimulated BMDCs (Fig. 4A). The differential effects of Ag presentation by TLR-stimulated APCs on young and aged responses may provide future clues as to what is defective in the aged naive T cell.

In further studies, we used CpG, which was often most effective, and PolyI:C and Pam3CSK4 because of the different T cell cytokine responses they have been shown to induce (30, 55) and because they work through distinct TLR pathways, giving us a greater opportunity to identify a pathway that may be shared with humans. We observed that BMDC activated by any of the three TLR ligands induced more pronounced Ag-stimulated division of aged CD4 T cells as monitored by the loss of the intracellular dye CFSE after 48 h of culture (Fig. 4B). Similar to aged cells, a slightly greater number of young naive CD4 T cells activated by TLR-activated BMDCs had undergone additional rounds of division compared with cells activated by nonstimulated BMDCs (Supplemental Fig. 3A).

Phenotypic analysis of aged CD4 T cells cultured for 4 d with TLR-activated BMDCs demonstrated increased expression of the IL-2R α-chain (CD25; Fig. 4C), an activation marker otherwise downregulated on effectors from aged naive CD4 T cells (11, 14, 16). This result was specific for responding aged CD4 T cells, as no increase in the expression of CD25 was noted on young CD4 T cells activated by TLR-activated BMDCs (Supplemental Fig. 3B). We observed no difference in the expression of CD62L and CD162 between aged effectors cultured with TLR-stimulated versus nonstimulated BMDCs (Fig. 4C). In comparison, young cells activated with TLR-stimulated BMDCs had slightly lower levels of CD62L compared with those activated with nonstimulated BMDCs (Supplemental Fig. 3B). Taken together, these data demonstrate that DCs directly stimulated by the TLR ligands CpG, PolyI:C, and Pam3CSK4 act as superior APCs to boost the cognate Ag-driven expansion of aged naive CD4 T cells.

**Aged CD4 T cells activated by TLR-stimulated BMDCs exhibit increased survival and Bcl-2 expression**

The level of expansion of responding Ag-specific CD4 T cells is determined both by the extent of division of responding cells and by the survival of the divided progeny. Thus, we asked whether aged versus young naive HNT CD4 T cells showed equivalent survival during the Ag-driven response and how this survival was influenced when TLR-stimulated BMDCs were used as APCs. We harvested the responding cells on days 4–6 of culture and examined both the number of recovered live cells and the fraction of dead cells by staining with 7-AAD, a dye that intercalates into double-stranded nucleic acids and is normally excluded by viable cells. We found that compared with young cells, the aged naive CD4 T cells expanded less in response to Ag presented by nonstimulated CD62L and had undergone additional rounds of division compared with those activated with nonstimulated BMDCs (Fig. 5A). Taken together, these data demonstrate that DCs directly stimulated by the TLR ligands CpG, PolyI:C, and Pam3CSK4 act as superior APCs to boost the cognate Ag-driven expansion of aged naive CD4 T cells.

**FIGURE 4.** Ag presentation by BMDCs activated by a panel of TLR agonists boosts the aged naive CD4 T cell response. Sorted young and aged CD4<sup>+</sup>V<sub>p8.3</sub>CD62L<sup>+</sup>CD44<sup>+</sup> HNT T<sub>g</sub> T cells were cultured with HA<sub>126-138</sub> plus either nonstimulated BMDCs or BMDCs stimulated with the indicated TLR agonist. A, Fold expansion of young and aged CD4 T cells over input number of T cells by day 5 of culture. Data are combined from three separate experiments. B, CFSE-monitored division of aged CD4 T cells on day 2 of culture; (C) expression of CD25, CD62L, and CD162 by aged T cells on day 4 of culture with HA<sub>126-138</sub> plus nonstimulated (shaded) versus either CpG, PolyI:C, or Pam3CSK4-stimulated BMDCs (heavy line). Data are representative of two similar experiments. *p < 0.05 for TLR-treated group compared with appropriate young or aged nonstimulated control.

**FIGURE 5.** Enhanced survival of aged CD4 T cells activated by TLR-stimulated DCs correlates with increased Bcl-2 expression. Sorted young and aged CD4<sup>+</sup>V<sub>p8.3</sub>CD62L<sup>+</sup>CD44<sup>+</sup> HNT T<sub>g</sub> T cells were cultured for with HA<sub>126-138</sub> plus either nonstimulated or BMDCs stimulated with the indicated TLR. Fold expansion over input number of T cells (A), and percentage of dead cells in culture as determined by 7-AAD staining (B) on days 4–6 of culture. C, Mean fluorescence intensity (MFI) of intracellular Bcl-2 staining on indicated CD4 T cells on day 5 of culture. Data are representative of two experiments. *p < 0.05 for aged-no stimulation versus young-no stimulation; **p < 0.05 for aged TLR-treated groups versus aged-no stimulation control.
Importantly, by day 5, the 7-AAD<sup>+</sup> cells from all groups were between 91 and 94% CFSE<sup>low</sup> (Supplemental Fig. 4A), indicating that the greater proportion of dead cells in the aged culture was not due to a larger fraction of cells that failed to respond to Ag, and thus might be dying of neglect, but instead was a result of a greater number of cells that divided and then became apoptotic. When Ag was presented by TLR-activated BMDCs, the enhanced expansion of aged CD4<sup>+</sup> T cells (Fig. 5A) was accompanied by a significant decrease in the percentage of dead cells in the aged CD4<sup>+</sup> T cell cultures (Fig. 5B). Similar overall trends were noted with young CD4<sup>+</sup> T cells as well, in that Ag presentation by TLR-activated BMDCs enhanced the expansion of young cells and decreased the percentage of dead cells found in the culture (Supplemental Fig. 4B, 4C).

We also examined expression of Bcl-2, an antiapoptotic molecule that can promote the viability of T cells. The level of Bcl-2 in aged cells was greater when TLR-activated versus nontreated BMDCs had been used as APCs, suggesting that the enhanced survival of the aged CD4<sup>+</sup> T cell effectors might be the result of factors that induce antiapoptotic proteins that actively protect the responding cells (Fig. 5C). Bcl-2 staining of young cells activated by TLR-stimulated versus nonstimulated BMDCs showed an overall increase in the TLR-treated groups; however, this increase was not statistically significant (Supplemental Fig. 4D).

**IL-6 production by TLR-activated BMDCs is specifically increased on cognate CD4<sup>+</sup> T cell interaction**

TLR stimulation is known to induce DCs to produce greater levels of multiple cytokines that directly impact T cell function. We hypothesized that preactivated DCs might retain their ability to produce greater levels of cytokines, and that they might be reactivated to do so during cognate interaction with the CD4<sup>+</sup> T cells. We measured the accumulation of cytokine in the BMDC/aged CD4<sup>+</sup> T cell cultures when the BMDCs had been preactivated or not with TLR agonists. The results from an initial screen of day 2 supernatant for a wide range of cytokines demonstrated that the levels of IL-12p70, MIP1β, TNF-α, and RANTES were only slightly or not significantly altered by CpG preactivation of the BMDCs (Fig. 6A). In contrast, the low levels of IL-10 and IL-17 were increased several fold, and levels of KC and IL-6 were dramatically greater in cocultures with CpG-activated BMDCs. Interestingly, the amount of IFN-γ in the aged CD4<sup>+</sup> T cell cultures was significantly less when Ag was presented by TLR-activated BMDCs, a finding that may reflect the increase in IL-6, which actively reduces IFN-γ production by CD4<sup>+</sup> T cells via the upregulation of the suppressor of cytokine signaling-1 (SOCS-1) protein (56). This finding is in contrast with the in vivo data presented in Fig. 2C, which illustrates increased serum levels of IFN-γ 4 hours after TLR agonist injection. This difference is likely because of the fact that the main source of IFN-γ in Fig. 6A is Ag-activated CD4<sup>+</sup> T cells, whereas in vivo, IFN-γ secreted in response to TLR stimulation is likely to be derived from multiple cells including activated NK or other innate cells. Overall, similar observations in cytokine levels were found in the young T cell cultures with CpG-activated BMDCs at this time point (Supplemental Fig. 5A).

The increase in IL-6 was of particular interest because it is known to increase the survival of primed CD4<sup>+</sup> T cells (57), and it is also one of a trio of cytokines (including IL-1 and TNF-α) previously shown to enhance the aged CD4<sup>+</sup> T cell response in vivo (12). Fig. 6B shows that supernatant levels of IL-6 remained at least 5- to 20-fold greater from days 2-5 in those cultures containing aged naive CD4<sup>+</sup> T cells and CpG-activated BMDCs versus BMDCs with no preactivation. In addition, greater than 10-fold increases in levels of IL-6 were also found in day 2 (Fig. 6C) and 5 (data not shown) supernatants from aged CD4<sup>+</sup> T cells cultured with either PAM<sub>3</sub>CSK<sub>4</sub> and PolyI:C-activated BMDCs. Supernatant from 2-day cultures that contained the equivalent number of preactivated BMDCs without naive CD4<sup>+</sup> T cells did not contain significant IL-6 (Fig. 6C). TNF-α production was also seen in the cocultures and required CD4<sup>+</sup> T cells, but it was not enhanced by TLR agonist preactivation of the BMDCs. Levels of IL-6 were also increased in the day 2–5 supernatant of young cells cultured with CpG-activated BMDCs, and they were similarly increased in the cocultures with PolyI:C- and PAM<sub>3</sub>CSK<sub>4</sub>-activated BMDCs (Supplemental Fig. 5B, 5C).

Naive HNT Tg CD4<sup>+</sup> T cells were cocultured BMDCs prelabeled with CellTrace Violet-plus Ag for 24 h, with brefeldin added during the final 5 h, to determine whether the BMDCs or the CD4<sup>+</sup> T cells were the principal producers of IL-6 in culture. Intracellular cytokine staining for IL-6 was then performed and BMDCs, and CD4<sup>+</sup> T cells were differentiated based on the presence of the CellTrace label and expression of CD11c and CD11b (Supplemental Fig. 6). We found that BMDCs that had been preactivated by CpG and PolyI:C demonstrated a significant increase in intracellular IL-6 staining (Fig. 6D). In comparison, there was no difference in IL-6 staining of CD4<sup>+</sup> T cells cultured with either nonstimulated versus TLR-stimulated BMDCs (Fig. 6E). The specific increase in the production of IL-6 by TLR-stimulated BMDCs in culture was seen with either young or aged naive CD4<sup>+</sup> T cells and is quantified in Figs. 6F and 6G, respectively. These data suggest that the TLR-activated BMDCs are the principal initial source of IL-6 during CD4<sup>+</sup> T cell–BMDC cognate interaction. Moreover, the data in Fig. 6C illustrating little IL-6 production in the absence of T cells suggest that IL-6 production by BMDCs in the absence of further TLR agonist stimulation requires induction by the cognate CD4<sup>+</sup> T cell–BMDC interaction.

**Ag presentation by TLR-activated splenic DCs enhances the aged naive CD4<sup>+</sup> T cell response**

BMDCs are thought to represent immature myeloid DCs, and their immaturity could potentially make them more dependent on TLR signaling than in situ DCs. To evaluate whether splenic DC would also become improved APCs for naive aged CD4<sup>+</sup> T cells, we determined whether Ag presentation by TLR-activated splenic DCs was similarly capable of enhancing the aged CD4<sup>+</sup> T cell response. As illustrated in Fig. 7A, both young and aged naive HNT CD4<sup>+</sup> T cells underwent a significantly greater level of expansion when placed in culture with cognate Ag and splenic DCs activated by CpG and PolyI:C versus nonstimulated splenic DCs. In addition, similar to the experiments with BMDCs, the splenic DCs activated by TLR ligands produced increased levels of IL-6 shortly after the initiation of coculture with the young or aged naive CD4<sup>+</sup> T cells (Fig. 7B). These data demonstrate a similar capacity of the BMDC and ex vivo splenic DC to enhance the aged CD4<sup>+</sup> T cell response when preactivated by TLR ligands.

**IL-6 blockade diminishes the adjuvant activity of TLR ligands**

The high levels of IL-6 produced by the TLR-activated BMDCs suggest that this cytokine might play a key role in enhancing the naive CD4<sup>+</sup> T cell response. To test whether IL-6 was necessary for the enhanced response, we cultured aged naive HNT CD4<sup>+</sup> T cells with either CpG-, PolyI:C-, or PAM<sub>3</sub>CSK<sub>4</sub>-treated BMDCs in the presence of anti–IL-6 blocking Ab, and for comparison, anti–TNF-α blocking Ab. We found that addition of blocking Ab to IL-6, but not to TNF-α, at the initiation of culture significantly diminished the enhancing effect of TLR activation of BMDCs on T cell expansion and survival (Fig. 8A, 8B). The enhanced expansion and survival of young naive CD4<sup>+</sup> T cells activated by CpG-stimulated BMDCs were also blocked by the addition of...
blocking Ab to IL-6, but not to TNF-α (Supplemental Fig. 7). Importantly, including IL-6 and TNF-α blocking Abs together showed no synergistic effects compared with blocking IL-6 alone (data not shown).

Based on the earlier blocking experiments, we tested whether the addition of IL-6 alone during the activation of naive aged CD4 T cells was sufficient to enhance their expansion, and because multiple inflammatory cytokines were made, we also tested whether supernatant from (day 2) aged HNT CD4 T cell–TLR-activated BMDC cultures was able to enhance aged CD4 T cell expansion. In this experiment, aged naive HNT CD4 T cells were stimulated with plate-bound anti-CD3 and anti-CD28. We had previously shown that stimulation using plate-bound anti-CD3 and anti-CD28 revealed similar defects in the aged naive CD4 T cell response equivalent to those seen with Ags and APCs (58). This mode of stimulation allowed us to assess the effects of IL-6 without the complication of additional cytokines that would be elaborated by coculture with BMDCs. As seen in Fig. 8C, the addition of titrated amounts of recombinant IL-6 (from 10–90 ng/ml) (24) did not enhance the expansion of aged CD4 T cells. In contrast, the addition of the day 2 supernatant significantly enhanced the expansion of the aged CD4 T cells, suggesting that IL-6 is necessary but not sufficient, and that other inflammatory cytokines produced during naive CD4 T cell–TLR activated DC coculture might also play key roles in enhancing expansion of Ag-specific CD4 T cells.

**FIGURE 6.** Enhanced IL-6 production by TLR-activated BMDCs during coculture with CD4 T cells. Sorted young and aged CD4+Vβ8.3+CD62Lhi CD44lo HNT Tg T cells were cultured with HA126–138 plus either nonstimulated or CpG-stimulated BMDCs. Supernatant levels of the indicated cytokines on day 2 of culture (A) and of IL-6 on days 2–5 of culture (B) were determined. C. Amount of IL-6 and TNF-α on day 2 of culture of aged naive CD4+ HNT Tg T cells cultured with HA126-138 plus either nonstimulated BMDCs or BMDCs stimulated with the indicated TLR ligands. D–G. Naive HNT Tg CD4 T cells were cultured with HA126-138 plus either nonstimulated or CpG- and PolyI:C-stimulated BMDCs labeled with CellTrace violet for 24 h with brefeldin added during the last 5 h. D and E. Representative histograms of IL-6 intracellular staining of gated (D) CellTrace violet-labeled BMDCs or (E) young CD4 T cells. F and G. Percentage of indicated cells positive for intracellular IL-6 in cultures containing either nonstimulated or CpG- and PolyI:C-stimulated BMDCs and either young (F) or aged (G) T cells. All culture supernatant cytokine analyses performed by cytokine bead array. The cytokine screen in A was performed on supernatant from a single experiment, and the cytokines IL-1, IL-4, IL-5, IL-13, and MCP-1 were not detected above background at this time point (data not shown). B and C. Data are representative of two to three similar experiments. *p < 0.0001 for TLR-treated group compared with nonstimulated control.
Together, these findings indicate that preactivation of BMDCs with TLR agonists results in the production of IL-6 when the BMDCs act as APCs for aged naive CD4 T cells, and that this IL-6, likely working in concert with some other soluble factor(s), is necessary for the enhanced expansion and survival of the responding cells as they become effectors.

**Discussion**

In this paper, we report that preactivation of DC through multiple TLR endows them with an enhanced ability to promote naive CD4 T cells responses. In particular, the otherwise poor responses of aged naive CD4 T cells are improved, resulting in greater expansion of the aged T cells, in part because of the reduction of apoptosis that otherwise occurs preferentially in the aged T cells and limits their expansion. The improved aged CD4 T cell response is dependent on IL-6, whose production in cognate cultures is much enhanced by the TLR preactivation. The DCs are a major source of IL-6 in the coculture. Addition of IL-6 alone cannot replace the TLR preactivation, but supernatants from cocultures have some enhancing ability, suggesting synergy among the factors produced. These results suggest that strategies to specifically target DC APCs with TLR adjuvants might improve the efficacy of vaccines without the need for systemic TLR administration.

Although naive CD4 T cells show severe age-associated defects in vitro and in vivo in response to peptide and protein Ags, the fact that addition of proinflammatory cytokines can improve the response of such T cells and their ability to provide help for B cells suggested that TLR-agonists might act to improve aged naive CD4 T cell responses by inducing production of such cytokines by APCs. A recent paper by Maue et al. (43) demonstrated that PolyI:C was an effective adjuvant in vivo, and that it increased the helper activity of aged CD4 T cells. However, studies of the mechanism of TLR ligand adjuvant activity in vivo are limited in their ability to pinpoint cell types activated by TLR administration and the mechanisms by which they contribute to the enhanced T cell-dependent B cell response. In the in vitro model presented in this study, DCs are first preactivated with the TLR ligand before being extensively washed, so that TLR activation is restricted to the APCs and we can avoid the influence of direct engagement of TLRs expressed by T cells themselves. The preactivated DCs are cocultured with Ag plus young versus aged sorted naive HNT Tg T cells. In this model, the untreated BMDCs represent those functionally immature DCs found underneath the epithelial surfaces of the body. In vivo, on stimulation by cytokines or conserved pathogen motifs, these cells migrate to the DLNs where

**FIGURE 7.** Ag presentation by splenic DCs activated by TLR agonists boosts the aged naive CD4 T cell response. Sorted young and aged CD4+ Vβ8.3+CD62LhiCD44lo HNT Tg T cells were cultured with HA126-138 plus either nonstimulated or Cpg- and PolyI:C-stimulated splenic DCs. A, Fold expansion of young and aged CD4 T cells over input number of T cells by day 5 of culture. B, Percentage positive IL-6–producing splenic DCs after 24 h of coculture with aged or young CD4 T cells where splenic DCs were treated with or without TLR ligands. *p < 0.05 for TLR-treated group compared with young or aged nonstimulated control.

**FIGURE 8.** IL-6 is required for the TLR-mediated enhancement of the aged naive CD4 T cell response. A and B, Sorted young and aged CD4+ Vβ8.3+CD62LhiCD44lo HNT Tg T cells were cultured with HA126-138 plus either nonstimulated or TLR-stimulated BMDCs with or without either anti–IL-6, anti–TNF-α, or irrelevant isotype control. A, Fold expansion over input number of T cells; (B) percentage of dead cells on day 5 of culture as determined by 7-AAD staining. C, Fold expansion of sorted young and aged CD4+ Vβ8.3+CD62LhiCD44lo HNT Tg T cells stimulated by plate-bound anti-CD3 and anti-CD28 with or without the addition of recombinant IL-6 at the indicated concentrations or 48-h supernatant (1:7 final dilution) from aged HNT Tg T cell + HA126-138 + TLR-activated BMDC cultures. A and B. Data are representative of two similar experiments. *p < 0.05 for blocking Ab-treated samples compared with isotype Ab-treated control of the same TLR treatment. C. Data are from a single experiment. *p < 0.05 for aged plus supernatant compared with aged alone.
they present Ag to naive CD4 T cells. This transformation includes the downregulation of pathogen receptors and the upregulation of molecules involved in T cell activation (MHC class II, CD80, CD86, IL-12). Our results suggest that immature DCs activated this way, via either TLR2, TLR3, or TLR9, are highly potent APCs with the capacity to provide strong enough activation and costimulation signals such that the defects in expansion normally seen in aged naive CD4 T cells are largely overcome. We note this does not imply that the specific defective pathways are corrected, merely that enhancement is sufficient that function is improved.

Because the addition of supernatant from CD4 T cell/TLR-stimulated BMDC cocultures was sufficient to significantly enhance the expansion of aged CD4 T cells, we suggest that many of the effects of TLR preactivation are likely to be due to increased cytokines. IL-6 was a likely candidate because it was one of a small number of cytokines that was dramatically upregulated in culture, and it is known to impact naive CD4 T cell responses. This hypothesis was strengthened by the fact that the addition of anti-IL-6 blocking Abs (but not anti-TNF-α) prevented the TLR-activated BMDCs from boosting the aged HNT CD4 T cell response.

Comparison of the responses of young versus aged naive cells stimulated with untreated DC suggests that increased apoptosis of dividing aged T cells is at least partly responsible for their poor expansion. In addition to enhancing the proliferation of aged effectors, the TLR ligand treatment of APCs promoted activated T cell survival and slightly increased expression of Bcl-2, as in previous studies of young CD4 T cells (59). We found that the enhanced survival is also dependent on IL-6 and is thus likely to be due to the increased IL-6 production that results from the TLR ligand stimulation of the DC, consistent with previous studies demonstrating that the addition of IL-6 increases the survival of primed CD4 T cells (57). The results from the current study suggest an additional role for IL-6, as a key factor produced during the cognate interaction between a CD4 T cell and a TLR-activated DC that is capable of enhancing the response of aged naive CD4 T cells. The intracellular staining presented in Fig. 6 suggests that the TLR-activated BMDCs were the principal initial producers of IL-6 in the CD4 T cell-DC cocultures. In this regard, it is significant to note that supernatant from 2-d cultures that contained TLR-activated BMDCs alone in numbers equivalent to those in cocultures did not contain significant IL-6 (Fig. 6). This suggests that IL-6 production by BMDCs in the absence of further TLR agonist stimulation requires the CD4 T cell–BMDC interaction. Such an induction of APC function could potentially be mediated through CD40–CD40L interactions (60).

The study of TLR and their natural agonists has revealed that ligation of different TLRs may trigger differential cytokine production in the same cells, or result in different cytokines produced by different types of APCs (61). In addition, several reports suggest that response to TLR stimulation and the patterns of TLR expression are different on human versus mouse APCs (62, 63). We were impressed by the consistency of response we saw to the different TLR agonists used in these studies. Thus, despite the fact that CpG, PolyI:C, and PAM3CSK4 mimic both viral and bacterial components and interact with different TLRs, including those expressed on the cell surface and within endosomes, we found that all three agonists induced comparable improvements in aged naive CD4 T cell expansion. In each case, this was due, in part, to less apoptosis and was dependent on IL-6. NF-κB, a transcription factor responsible for IL-6 production, is activated by most TLRs downstream of the MyD88 adaptor protein. Activation via TLR3, which does not recruit MyD88, can still result in the activation of NF-κB via the recruitment of TNFR-associated factor 6. Therefore, it is likely that the activation of BMDCs to enhance aged naive responses is part of a shared pathway induced by many TLR agonists, thus making it more likely that a similar pathway will occur in the human.

It does not appear that the results from this study were influenced by contaminating endotoxin, because a number of steps were taken to rule out contamination of the different TLR agonist preparations, including use of control non-CpG ODNs, LAL testing of endotoxin levels, and stimulation of TLR4 KO BMDCs with the panel of TLR ligands (see Materials and Methods).

Future experiments are planned to further dissect the mechanism by which IL-6 may help to rescue the aged response. Like IL-12, IL-6 has been shown to be important for the clonal expansion and differentiation of primed cells (57, 64). However, whereas IL-12 promotes Th1 responses, particularly through the induction of IFNγ (64), IL-6 promotes Th2 responses via the induction of IL-4, and the active suppression of IFN-γ via the upregulation of SOCS-1 (56). Furthermore, IL-6 is necessary for polarization of naive CD4 T cells in mice and humans to become Th17 polarized cells. IL-6 has also recently been shown to induce the production of IL-21 by activated CD4 T cells (65). This finding is especially important because the production of IL-21 by CD4 T cells is now understood to be essential for B cell activation, expansion, and PC differentiation (66), as well as playing a role in polarization of T cells. Thus, we are especially interested in whether augmentation of IL-21 production by IL-6 produced by TLR-activated BMDCs will help to overcome the observed defects in aged CD4 T cell cognate B cell help.

It will be interesting to directly compare the efficacy and to determine whether shared mechanisms are responsible for alternate protocols that enhance aged naive CD4 responses including the addition of either IL-2 (11) or a combination of inflammatory cytokines (IL-6, TNF-α, and IL-1), which have also been shown to enhance the expansion of aged naive CD4 T cells in vitro and in vivo (12). The effect of these treatments appeared to be primarily caused by the increased proliferation of Ag-activated T cells. Systemic cytokine treatments are probably not appropriate for elderly patients because they may cause unwanted nonspecific, systemic effects. Localized administration of TLR ligands, potentially in conjugation with Ag, has the potential to limit such nonspecific effects. Ex vivo treatment of autologous APCs would further limit bystander exposure, and the requirement for cognate CD4 T cell–APC interaction for the production of IL-6 by the preactivated BMDCs, as shown in this study, may help to direct cytokines primarily to Ag-specific T cells, reducing unwanted systemic effects.

In conclusion, the wide range of functional defects that accumulates with age in the naive CD4 T cell compartment results in the reduced immune response to vaccines in the aged, thus limiting vaccine efficacy in an already highly susceptible population. The data presented here suggest that the use of TLR ligands as vaccine adjuvants acting directly on aged APC is effective in vitro and probably in vivo, where it is seen in intact aged mice. Through definitive in vitro models, we have identified a likely mechanism by which TLR stimulation acts to enhance aged CD4 T cell proliferation and survival. Central to this mechanism is the increased production of the inflammatory cytokine IL-6 by TLR-activated APCs during their cognate interaction with Ag-specific naive CD4 T cells, which, perhaps together with additional factors, enhances the expansion and survival of responding aged T cells. This effect is remarkable in that it restores the magnitude of the response of aged naive CD4 T cells to levels comparable with those seen with young cells.
Disclosures

The authors have no financial conflicts of interest.

References


