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Early-onset age-related changes in dendritic cell subsets can impair antigen-specific T helper 1 (Th1) CD4 T cell priming

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ABSTRACT

Decline in CD4 T cell immune responses is associated with aging. Although a number of immunological defects have been identified in elderly mice (>18 months old), a key early-onset immune defect at middle age could be a driver or contributor to defective CD4 T cell responses. Our studies demonstrate that age-related alterations in DC subsets within the priming environment of middle-aged mice (12 months old) correlate with and can directly contribute to decreases in antigen-specific CD4 T cell Th1 differentiation, which measured by T-bet and IFN-γ expression, was decreased significantly in T cells following VSV infection or s.c. immunization with a protein antigen in the context of immune stimulation via OX40. The deficient Th1 phenotype, observed following protein antigen challenge, was found to be the result of an age-related decrease in an inflammatory DC subset (CD11b+ Gr-1/Ly6C+) in the dLN that corresponded with T cell dysfunction. In the virus model, we observed significant changes in two DC subsets: mDCs and pDCs. Thus, different, early age-related changes in the DC profile in the priming environment can significantly contribute to impaired Th1 differentiation, depending on the type of immunological challenge. *J. Leukoc. Biol.* 96: 245–254; 2014.

Introduction

A hallmark of aging is a decrease in immune responses that can result in an increase in infections and tumors. Indeed, our previous findings demonstrated that old mice (20 months of age) experienced impaired tumor immunity, which resulted in a dramatic decrease in tumor-free survival [1]. Interestingly, younger, middle-aged mice (12 months old) also experienced the same abrogation of tumor immunity. This effect in the younger 12-month-old mice was initially unexpected, as other immune responses in mice this age have been observed to be intermediate to the effects in young and elderly mice [2, 3]; however, more recent findings point to significant immunological deficiencies occurring around this age [4, 5]. These findings support the concept that certain age-related immune deficiencies become established early in the aging process and may represent a "driver" of age-related immunological defects, which then facilitate the accumulation of other secondary defects.

CD4 Th cells occupy an important position in the generation of adaptive immune responses; unfortunately, their function becomes impaired significantly with increasing age. Both intrinsic age-associated alterations in CD4 T cells and extrinsic deficiencies in the host environment contribute to dysfunctional helper cell responses. Intrinsic, age-related CD4 T cell alterations, such as decreased TCR signaling that is associated with less rigorous proliferation and decreased IL-2 production, have been well-described in 18+ month-old mice [6, 7]. In addition, the contribution of the T cell-extrinsic host environment in inducing CD4 T cell dysfunction has been assessed. CD4 T cell proliferation and differentiation of IFN-γ^+ Th1 cells have been observed to be delayed or decreased significantly following priming in an aged host [1, 8, 9]. In fact, several studies, including our own, suggest that the contribution of the host environment on activation and differentiation of CD4 helper cells has a greater role than the T cell-intrinsic, age-related deficiencies [1, 9].

In the studies presented here, we sought to understand better the effect of the middle-aged host environment on T cell priming and identify early-onset age-related cellular and/or molecular deficiencies in the host environment. The priming of antigen-specific CD4 T cells was measured in two immune models: one using VSV and the other a s.c. protein immunization that mimics vaccination. We observed significant age-related changes in antigen-specific CD4 T cell differentiation, evaluated by decreased expression of T-bet and production of

Abbreviations: αOX40=OX40-activating antibody, ACK=ammonium-chloride-potassium, CD62L=CD62 ligand, cDC=conventional DC, dLN=draining lymph node, LN=lymph node, mDC=myeloid DC, OVA=ovalbumin, pDC=plasmacytoid DC, VSV=vesicular stomatitis virus

The online version of this paper, found at www.jleukbio.org, includes supplemental information.

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IFN-γ, in both models. These results led to a detailed assessment of several DC subsets in the host environment involved in priming CD4 T cells. It has been established that distinct DC subsets within the LN and in the periphery effectively promote proliferation and differentiation of antigen-specific T cells [10–12]. Overall, there was no difference in the number of bulk CD11c+ DCs during early priming; however, we observed significant age-related changes in different DC subsets depending on the type of immune challenge. There was a decrease in the numbers of an inflammatory DC subset (CD11c+ CD11b+ Gr-1/Ly6C+) in the dLN before the changes in T cell differentiation following s.c. protein immunization. In contrast, there was an age-related increase in the frequency of the pDC subset (CD11c+ CD11b^{neg} B220+) and a corresponding decrease in the mDC subset (CD11c+ CD11b+ B220^{neg}) after VSV infection. These findings further expand the understanding of immunological dysfunctions that are established early in the process of aging. More importantly, they suggest tailoring immune therapies and vaccination, based, in part, on the age-related deficiencies of specific DC subsets involved in the immune response.

MATERIALS AND METHODS

Mice and infections

Young, 6- to 8-week-old female BALB/c mice were purchased from Harlan Laboratories (Indianapolis, IN, USA) and housed until 8–9 weeks of age. Ten-month-old BALB/c females were purchased from Harlan Laboratories and used at ~12 months of age. D011.10 mice were bred and housed until 2 or 12 months of age. BALB/c mice were infected with 10^6 PFU of VSV-OVA Indiana by s.c. footpad injection. At the time of tissue harvesting, blood samples were collected, and serum was isolated and pooled for young and old host groups. Titers were determined by a nonagar titration method, where baby hamster kidney cells were plated at 1.5×10^3 cells in 150 μl DMEM/well in a 96-well plate, and varying dilutions of serum were added across the plates, which were incubated and monitored for signs of cell death, indicating that viral infection and viral titers were calculated. All animals were housed at Rush University's Comparative Research Center, and all studies were approved by the Institutional Animal Care and Use Committee.

DO11.10 transgenic TCR adoptive transfer and immunization schedule

DO11.10 CD4$^+$ T cells have a transgenic TCR specific for chicken OVA$_{323–339}$ peptide. Spleens and LNs of DO11.10 mice were harvested, and tissues were processed by mechanical disruption between two frosted glass slides, followed by lysis of red blood cells using ACK buffer (Lonza, Allendale, NJ, USA). Cells were stained with CFSE, where indicated, before adoptive transfer by incubation at 37°C with 1 μl CFSE (2.5 mg/ml)/5.0×10^7 cells. DO11.10 (1×10^6) cells were adoptively transferred i.v. into recipient mice. The following day, mice were challenged with virus or immunized with whole OVA. Mice were immunized s.c. with 500 μg OVA (Sigma Life Science, St. Louis, MO, USA) or AF-647-conjugated OVA (Invitrogen, Carlsbad, CA, USA) and 50 μg αOX40 (OX86) or IgG control (Jackson ImmunoResearch Laboratories, West Grove, PA, USA), 1 day after adoptive transfer or as an alternative, 500 μg OVA in Inject Alum (Thermo Fisher Scientific, Waltham, MA, USA).

FACS analysis of CD4 T cells

Lymphocytes were released from lymphoid tissues by dispersing between two frosted glass slides, and red blood cells were lysed using ACK buffer. Staining controls were prepared, and samples were stained using the following antibodies: PerCP-Cy5.5-CD4, allophycocyanin-KJ-126, PE-CD25, PB-CD62L, and biotinylated CD44 (eBioscience/Affymetrics, Santa Clara, CA, USA). Cells were fixed and permeabilized using fixation/permeabilization buffers (eBioscience/Affymetrics). Samples were stained additionally with PE-Cy7-T-bet (BD PharMingen, San Diego, CA, USA). Samples were then analyzed using a FACSCanto II flow cytometer and FACSDiva software (BD Biosciences, San Jose, CA, USA).

Intracellular cytokine staining

T cells were acquired from dLNs, as described previously, plated in a 24-well plate at 2.5×10^6 cells in 1 ml complete media/well and stimulated with 1 μg/ml OVA$_{323–339}$ peptide overnight at 37°C in vitro. The following day, 0.5 μg/ml Golgi Stop (BD PharMingen) was added to each well and allowed to sit for 6 h. Cells were then surface-stained with PerCP-Cy5.5-CD4 and allophycocyanin-KJ-126 and were fixed using fixation/permeabilization buffer from BD PharMingen. Subsequent to fixing, cells were made permeable using CytoPerm Perm/Wash buffers and stained with allophycocyanin-Cy7-TNF-α, PE-Cy7-IFN-γ, and PE-IL-2 (BD PharMingen). Samples were then acquired on a FACSCanto II flow cytometer, and adoptively transferred transgenic DO11.10 CD4 T cells were analyzed using FACSDiva software (BD Biosciences).

DC isolation and assessment

At various times after immune challenge, single-cell suspension was made from the dLNs by disruptions between frosted glass slides. Cells were incubated with collagenase (Sigma Life Science) for 30 min at room temperature and enumerated. Cells were incubated with a combination of the fluorescently labeled antibody stains FITC-B220, PE-CD40, biotin/streptavidin-PE-MHC class II, allophycocyanin-CD11c Cy7, PE-CD205 Cy7, FITC-Gr-1, AmCyan-B220, allophycocyanin-CD11c, eFluor450-CD11b, PE-Cy7-CD11c, and PerCP-Cy5.5-Ly6c and analyzed by FACS. In some experiments, CD11c+ cells that had taken up FITC-conjugated OVA were enriched by multicolor cell sorting using a FACSAria (95% purity; BD Biosciences). These cells were then mixed with CFSE-labeled DO11.10 (1:10 DC:T cell) and incubated in 96-well plates for 3 days. DO11.10 T cell proliferation was then measured by CFSE dilution. In addition, antibodies known to deplete DCs were administered to mice after immunization or infection. Specifically, 500 μg anti-Gr-1 (RB6-8C5) or anti-Ly6G (1A8) or control IgG was injected i.p., 24 h following immunization with OVA in the context of αOX40 or Alum. Mice infected with VSV-OVA received 500 μg anti-Gr-1 (BioXcell, West Lebanon, NH, USA) or PDCA-1 (Miltenyi Biotec, Bergisch Gladbach, Germany) or control IgG i.p., 24 h after infection.

Statistical analysis

For each experiment, significance was evaluated in GraphPad Prism by carrying out a two-tailed Student's *t*-test comparing the means of different experimental groups. $P \leq 0.05$ were deemed to be significant and designated as follows: $^*P \leq 0.05$, and $^{**}P \leq 0.01$.

RESULTS

Early Th1 differentiation following virus infection is impaired in 12-month-old hosts

To understand better the potential immunological impact of early-onset age-related deficiencies in the host environment on priming virus-specific CD4 T cells, we used a VSV model that expresses the antigen OVA (VSV-OVA). VSV is a negative-stranded virus that replicates in the cytoplasm of infected cells and is capable of infecting a variety of cells. It exists in two major serotypes that are extremely sensitive to IFN-γ [13, 14]. This feature is important, as our previous work suggested a significant age-related deficiency in the IFN-γ/Th1 CD4 T cell

response [1]. Furthermore, VSV is an understudied virus model in the context of aging.

We first sought to determine when critical CD4 T cell activation and differentiation events occur during the course of VSV-OVA infection. Naïve, 2-month-old DO11.10 CD4 T cells, specific for OVA, were transferred to recipient mice (2 months old) and 24 h later, infected with VSV-OVA (via the footpad). The dLNs (popliteal) were then harvested daily, starting at 2 days after infection and ending 7 days after infection (Days 2–7). Total numbers of DO11.10 T cells were the highest at Day 5 postinfection (Supplemental Fig. 1), and peak T cell activation, measured by the expression of CD25 and down-regulation of L-selectin (CD62L), was observed to occur at Day 3 in the dLN (Supplemental Fig. 1). Thus, Days 3 and 5 following VSV-OVA infection appear to represent key times in antigen-specific T cell activation.

The priming of CD4 T cell effector cells, differentiation and accumulation, was analyzed in 12-month-old mice, 3 and 5 days after VSV-OVA. In these experiments, young, 2-month-old DO11.10 CD4 T cells were adoptively transferred into young (2 months old) or middle-aged (12 months old) recipients. This approach allowed for the direct assessment of the aged host environment (T cell-extrinsic) effects on the activation and differentiation of CD4 Th cells. To analyze early proliferation, CFSE-labeled donor DO11.10 T cells were transferred into recipient mice and infected. Three days after infection, we observed a significant difference in the dilution of CFSE between the 2- and 12-month-old groups in the dLNs (**Fig. 1A**). Interestingly, we did not observe a significant difference in the number of DO11.10 T cells in the LNs at this same time (Fig. 1B); however, 5 days after infection, young recipients had a significantly greater number of DO11.10 T cells in the dLN (Fig. 1B). T cell activation, measured as an increase in the expression of CD44 and CD25 and decreased expression of CD62L on the cell surface, was not significantly different between the two age groups at both time points (Days 3 and 5; Fig. 1C, and data not shown).

Although we did not observe a significant alteration in viral antigen-specific T cell activation, viral infection generates the development of IFN-γ-producing Th1-differentiated effector cells, which is associated with the expression of the transcription factor T-bet [15]. On Day 3, both frequency and number of T-bet-expressing cells were found to be significantly higher in the young host mice compared with the older host environment (Fig. 1D). Additionally, mean fluorescence intensity of T-bet was also determined to be significantly greater in younger recipients (Fig. 1D). Induction of T-bet is critical for the expression of the Th1 cytokine IFN-γ [16], and upon antigen restimulation, the frequency and number of IFN-γ-producing DO11.10 T cells were found to be significantly greater for cells adoptively transferred into young hosts on both days (Fig. 1E). In contrast, the cytokine IL-2 did not differ between old and young recipients on Day 3 (Fig. 1F). Thus, in a viral infection, the differentiation of effector Th1 cells is impaired in middle-aged hosts in the early stages of the anti-VSV immune response (Days 3–5).

Early Th_1 effector responses following protein immunization in the context of a T cell stimulant (αOX40) are impaired in 12-month-old hosts

We next sought to investigate if the defects in the early effector differentiation of Th1 cells, observed in the VSV viral model, also occurred after the combination of a protein immunization and administration of an immune enhancer. In these experiments, the model antigen, OVA, is injected s.c. along with an αOX40. OX40, a member of the TNFR superfamily, is transiently expressed on T cells after TCR stimulation, and when engaged, OX40 significantly enhances antigen-specific T cell responses [17, 18]. Because of its ability to boost T cell responses, stimulating OX40 on T cells has been proposed to be used as a potent vaccine enhancer/adjuvant [19, 20].

Our previous investigations in older mice (12 and 20 months old) revealed a significant decrease in the number of differentiated CD4 T cells within the antigen dLNs compared with young mice (2, 6, and 8 months old) and a dramatic loss of tumor immunity [1] (data not shown). To determine the early differentiation and proliferation of antigen-specific cells in the context of OX40 stimulation, numbers of DO11.10 T cells and the dilution of CFSE were measured, 48 and 72 h after immunization. Specifically, 2-month-old DO11.10 T cells were transferred into 2- or 12-month-old hosts, followed by immunization (s.c.) with whole OVA and agonist OX40 antibody [21]. The accumulation of DO11.10 T cells in the dLNs (axillary) in the young and middle-aged hosts was not significantly different, and CFSE dilution was also similar between the two groups (**Fig. 2A** and **B**). To assess the activation of these antigen-specific T cells, the expression of CD25 and the down-regulation of CD62L on the surface were measured. There was significantly greater activation (CD25+ $CD62L^{low}$) of the DO11.10 T cells in the younger hosts compared with the older hosts (Fig. 2B and C). Th1 differentiation, measured in part by the expression of the master regulatory transcription factor T-bet, was also significantly greater in DO11.10 T cells primed in the younger, 2-month-old host (Fig. 2D). These results demonstrate that the activation and differentiation of antigen-specific T cells are impaired in middle-aged hosts.

The impaired differentiation of antigen-specific T cells in middle-aged hosts was further evident in the expression of Th1 cytokine IFN-γ. DO11.10 T cells, isolated from the dLN, 72 h after immunization, were restimulated with antigen and assessed for the production of IFN-γ, IL-2, and TNF-α by intracellular staining. The production of IFN-γ, but not IL-2 and TNF-α at this time-point, was reduced significantly in DO11.10 T cells primed in a 12-month-old host compared with those primed in younger, 2-month-old mice (Fig. 2E).

Age-related changes in DC subsets after OVA immunization and VSV-OVA infection

The significant age-related changes in CD4 T cell differentiation observed previously suggested that the host environment, which includes DCs, was insufficient to prime the antigen-specific T cells properly. DCs are critical for presenting antigen to T cells and it is well-understood that aging can affect DCs [22]. Thus, we assessed DCs from 2- and 12-month-old mice that had recently taken up antigen after injection with FITC-

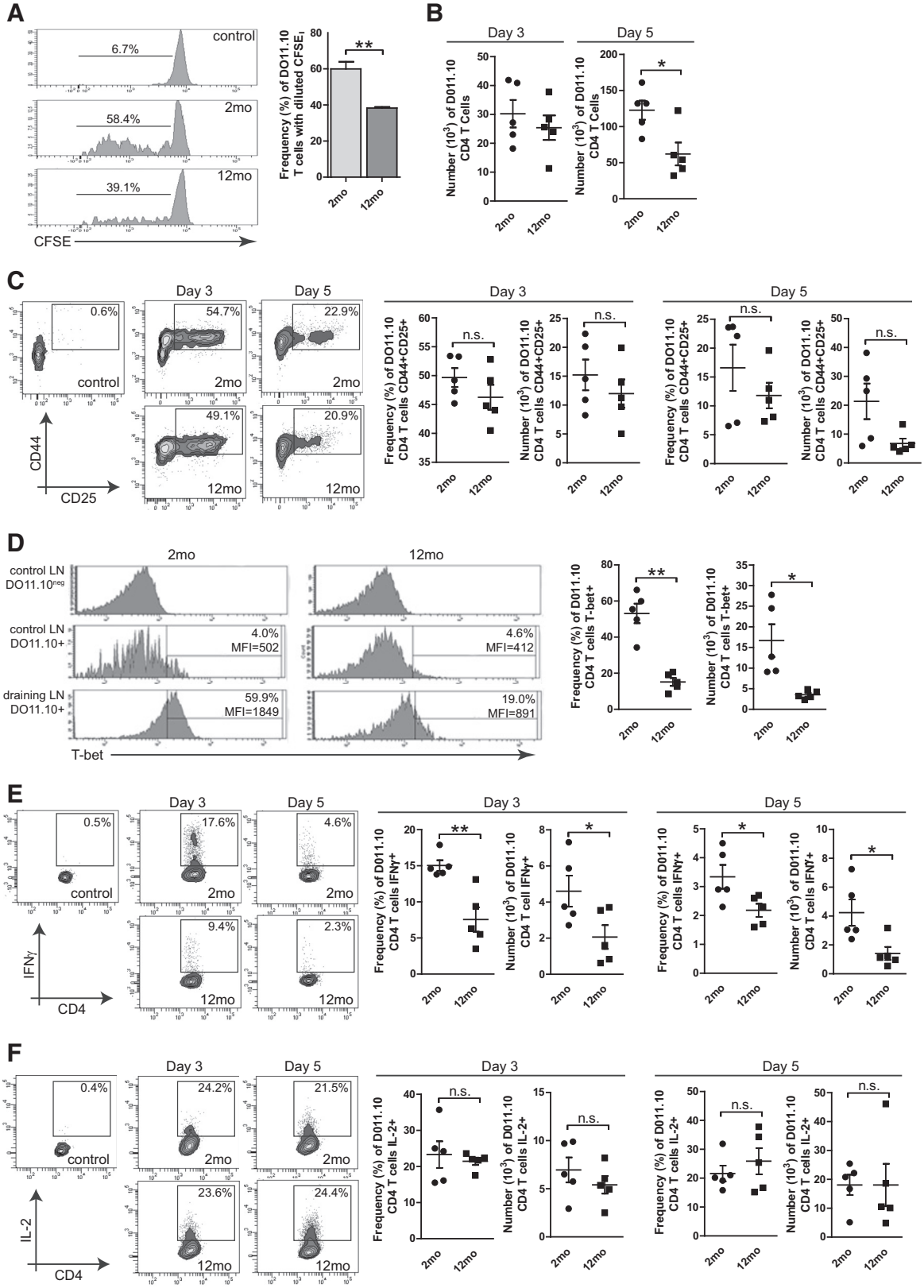

Figure 1. Antigen-specific CD4 T cell proliferation, differentiation, and function are reduced in middle-aged hosts after VSV infection. Two-month-old DO11.10 CD4 T cells (CD4+KJ126+) were adoptively transferred into 2- or 12-month-old BALB/c recipients, 1 day before infection with VSV-OVA (10^6 PFU). (A) CFSE-labeled DO11.10 T cells were transferred 1 day before VSV-OVA infection. Three days after infection, dLNs

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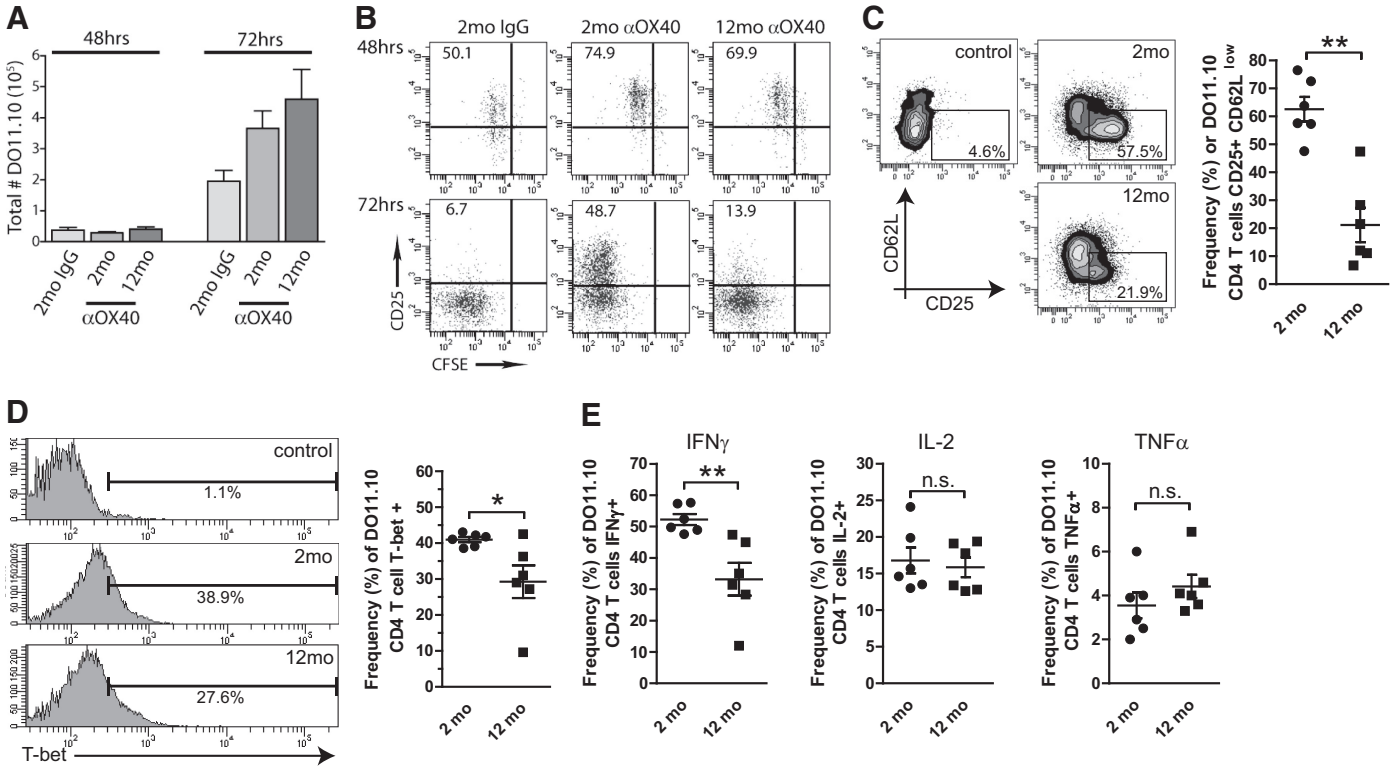

Figure 2. CD4 Th cell priming in a middle-aged-old host results in incomplete early differentiation in the context of immunization with protein antigen and αOX40. Adoptive transfer of 2-month-old CFSE-stained DO11.10 CD4 T cells, into 2- or 12-month-old BALB/c recipients, was followed by immunization with 500 μg OVA and 50 μg αOX40 or rat IgG (s.c.), 24 h later. The dLNs and non-dLNs (control) were harvested at various times after immunization. (A) Enumeration of the antigen-specific DO11.10 T cells from the dLNs, 48 and 72 h after immunization. Data are the mean ± SEM of four mice/group from one representative experiment from two independent experiments. (B) Representative FACS plots of DO11.10 T cell CFSE dilution and CD25 expression, 48 and 72 h after immunization. Data are the mean ± SEM of four mice/group from one representative experiment from two independent experiments. (C) Frequency of DO11.10 T cell expression of the activation markers $CD62L^{low}$ and CD25, 72 h after immunization in the context of αOX40. Data are the mean ± SEM of one representative experiment from two independent experiments. (D) Assessment of T-bet expression in antigen-specific DO11.10 CD4 T cells, 72 h after immunization in the context of αOX40. Data are the mean ± SEM of one representative experiment from two independent experiments. Three days (72 h) after immunization, the dLNs were harvested and antigen-specific DO11.10 CD4 T cells assessed for IFN-γ, IL-2, and TNF-α. Data represent two independent experiments.

conjugated OVA (OVA-FITC). The number and maturation of CD11+ DCs that had taken up the OVA-FITC (CD11c+ FITC+) in the dLNs, 18 h after antigen challenge, were shown not to be overtly affected by age (Supplemental Fig. 2). Furthermore, the function of the DCs (CD11c+ OVA-FITC+) from 12-month-old mice was intact (Supplemental Fig. 2). It appeared that the DCs in the LNs of the older host at this early time were functional, but these experiments did not dissect the specific DC subsets involved in priming in vivo. The network of DCs that prime T cells is complex. A number of distinct subsets, including resident DCs found in lymphoid tissue and migratory DCs that traffic from the periphery, are integrated to prime T cell-mediated immune responses effectively [10–12, 23]. Therefore, a detailed analysis of the specific DC subsets in the antigen dLN after immunological challenge was required to identify the potential cellular and/or molecular deficiencies that result in the observed impairment of Th1 priming in 12-month-old hosts.

We measured in vivo numbers of several DC subsets found in LNs after immunization and OX40 stimulation. In these

Figure 1 *(continued)* and non-dLNs (control) were harvested, and dilution of CFSE was measured. Histograms are representative of CFSE dilution. Data are the mean ± SEM of four mice/group from one representative experiment from two independent experiments. (B) Numbers of DO11.10 T cell in the dLNs were enumerated, 3 and 5 days after infection. Data are the mean ± SEM of one representative experiment from at least three independent experiments. (C) Assessment of antigen-specific DO11.10 CD4 T cell activation (CD25+ and CD44+). Data are the mean ± SEM of one representative experiment from at least three independent experiments. (D) Assessment of T-bet expression in antigen-specific DO11.10 CD4 T cells. Data are the mean ± SEM of one representative experiment from at least three independent experiments. Three or 5 days after infection, the dLNs and non-dLNs (control) were harvested and antigen-specific DO11.10 CD4 T cells assessed for cytokine production. MFI, Mean fluorescence intensity. (E) IFN-γ. (F) IL-2. Data are the mean ± SEM of five mice/group from one of two independent experiments.

experiments, 2-month-old DO11.10 T cells were adoptively transferred into 2- or 12-month-old hosts and injected s.c. with OVA and αOX40. dLNs were harvested, 48 and 56 h later, time-points just before the age-related T cell changes observed previously at Days 3 and 5. The following specific DC subsets were then assessed: pDC (CD11c+B220+), CD8-positive DC (CD11c+ $B220^{neg}$ CD8+ $CD11b^{neg}$), mDC (CD11c+ $B220^{neg}$ $CD8^{neg}$ CD11b+), dermal DC (CD11c+ $B220^{neg}$ $CD8^{neg}$ CD11b+ CD205+ Gr-1/$Ly6C^{neg}$), and inflammatory DC (CD11c+ $B220^{neg}$ $CD8^{neg}$ CD11b+ $CD205^{neg}$ Gr-1/Ly6C+; **Fig. 3A**). These phenotypes have been characterized previously and represent several of the primary DC populations found in the LNs [24, 25], and we observed no age-related changes in these subsets in non-immune mice (data not shown). There was no significant age-related change in the numbers of all of these subsets, except for the inflammatory DC subset, which was significantly more abundant in the young mice at both time-points (Fig. 3B). To verify further this age-related change in inflammatory DCs, we measured additional time-points. A significant decrease in inflammatory DC numbers in 12-month-old hosts was first observed at 24 h and was greatest at 64 h after immunization (**Fig. 4A**). To more accurately determine if there was also a decrease in the inflammatory DCs that had taken up antigen, we injected OVA conjugated to the fluorescent molecule AF647 (OVA-AF647). This fluorescent molecule is more stable at the low pH conditions found in intracellular vesicles than FITC and extended the amount of time we could assess the DCs that had taken up antigen. At 48 h after immunization with OVA-AF647, the number of CD11c+ DCs in the dLNs of young and middle-aged mice that had taken up OVA-AF647 was similar (Fig. 4B). However, there was a significant decrease in the frequency and number of inflammatory DCs that had taken up OVA-AF647 in the middle-aged mice compared with young control mice (Fig. 4B). These results reveal an early-onset age-related deficiency in the accumulation of an inflammatory DC subset after injection of antigen and αOX40.

Measurement of the in vivo DC subsets found in the dLNs after VSV-OVA infection revealed an age-related change in a different DC subset compared with s.c. OVA immunization. In these experiments, 2-month-old DO11.10 T cells were adoptively transferred into 2- or 12-month-old hosts and infected with VSV-OVA. dLNs were harvested 48 h later, and the pDC, CD8-positive DC, mDC, and dermal and inflammatory DC subsets were analyzed, as described previously (Fig. 3A). Overall numbers of DCs (CD11c+) were not significantly different, but there was a significant age-related increase in the frequency of pDCs and a concomitant decrease in mDCs (**Fig. 5A** and **B**).

Depletion of inflammatory DCs decreases antigen-specific T cell differentiation following s.c. immunization

The inflammatory DC population, described by the expression of CD11c+ CD11b+ Gr-1/Ly6C+, has been found to be criti-

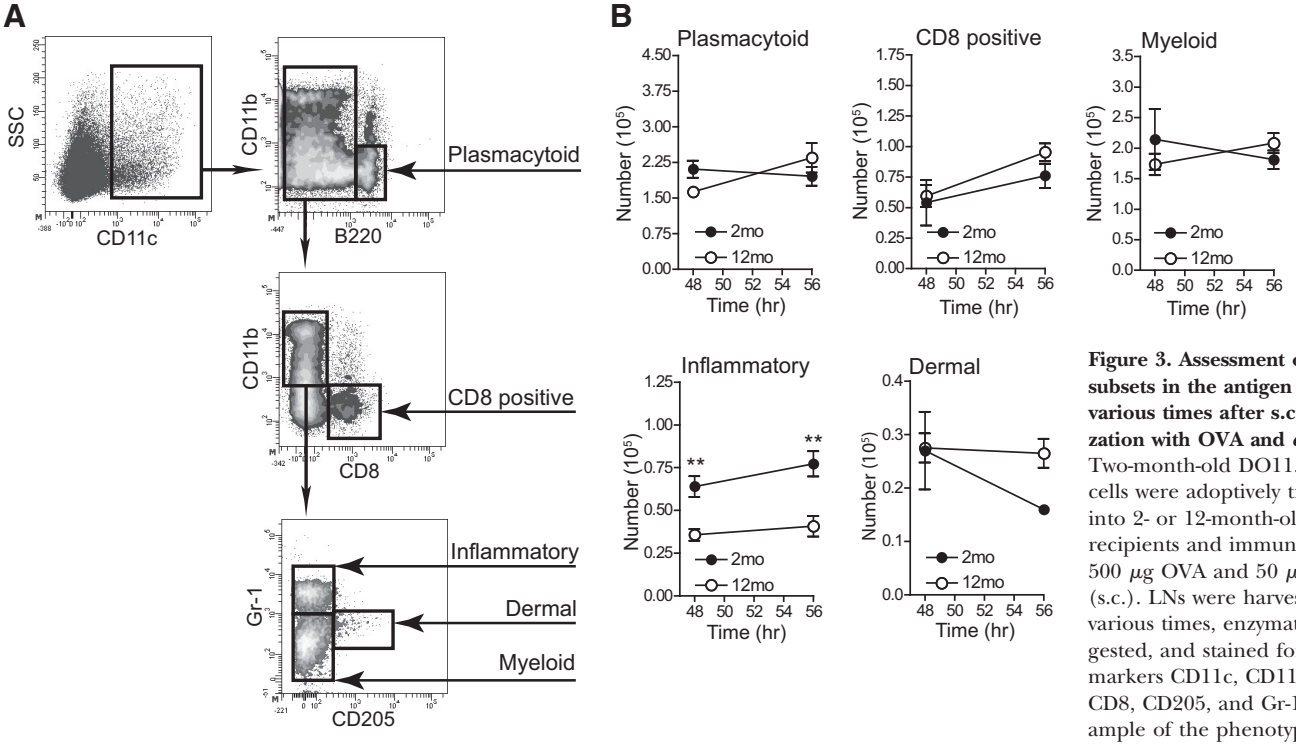

Figure 3. Assessment of DC subsets in the antigen dLNs, various times after s.c. immunization with OVA and αOX40. Two-month-old DO11.10 CD4 T cells were adoptively transferred into 2- or 12-month-old BALB/c recipients and immunized with 500 μg OVA and 50 μg αOX40 (s.c.). LNs were harvested at various times, enzymatically digested, and stained for the DC markers CD11c, CD11b, B220, CD8, CD205, and Gr-1. (A) Example of the phenotypic analysis of CD11c+ DCs from the antigen dLNs of immunized mice (48 h). Specific DC subsets were interrogated based on the following phenotypes: pDC, CD11c+ $CD11b^{neg}$ B220+; CD8-positive DC, CD11c+ $B220^{neg}$ CD8+ $CD11b^{neg}$; mDC, CD11c+ $B220^{neg}$ $CD8^{neg}$ CD11b+; dermal DC, CD11c+ $B220^{neg}$ $CD8^{neg}$ CD11b+ CD205+ Gr-1^{neg}; inflammatory DC, CD11c+ $B220^{neg}$ $CD8^{neg}$ CD11b+ $CD205^{neg}$ Gr-1+. SSC, Side-scatter. (B) Enumeration of DC populations from 2- and 12-month-old mice at various times after immunization with OVA and αOX40. Data represent one experiment (n=4) of two independent experiments.

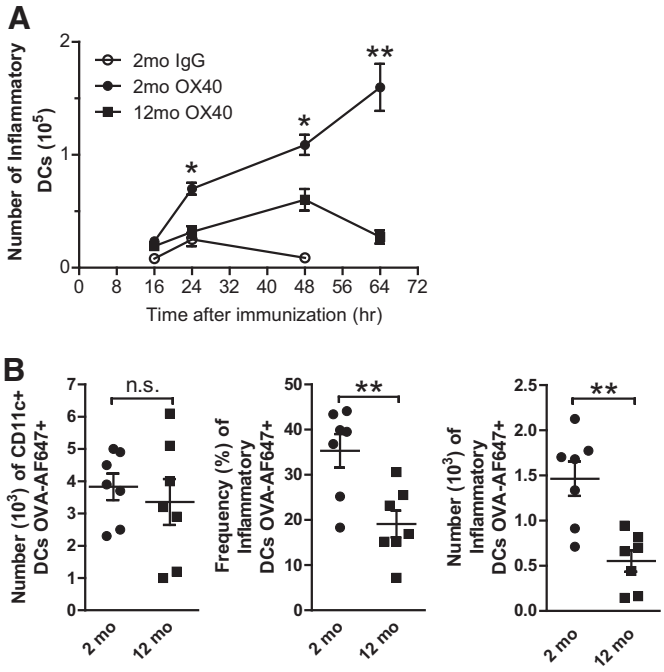

Figure 4. Inflammatory DCs are deficient in middle-aged hosts. Adoptive transfer of 2-month-old DO11.10 CD4 T cells into 2- or 12-month-old BALB/c recipients was followed by immunization with 500 μg OVA or 500 μg fluorescent AF647 OVA and 50 μg αOX40 or rat IgG s.c., 24 h later. (A) Enumeration of the inflammatory DCs (CD11c+ $B220^{neg}$ $CD8^{neg}$ CD11b+ Gr-1/Ly6c+ $CD205^{neg}$) from the dLNs, various times after antigen challenge. Data are the mean ± SEM of four mice/group from one representative experiment from two independent experiments. (B) Assessment of inflammatory DCs, which had taken up fluorescent AF647 OVA, 48 h after immunization (s.c.). Data are the mean ± SEM of the combination of two independent experiments.

cal in the optimal activation of T cells and clearance of pathogens [25, 26]. Inflammatory DCs move from the blood and into the LNs and can drive Th1 differentiation, via the production of IL-12 [25, 26]. To determine the role of this subset in activating antigen-specific T cells in our model, we depleted inflammatory DCs in young mice, 24 h after a systemic injection with an anti-Gr-1 antibody (RB6-8C5), which has been shown to deplete inflammatory DCs, monocytes, and neutrophils, but not pDCs (Supplemental Fig. 3) [27]. In addition, we injected a control antibody (IgG) or an antibody that specifically depletes neutrophils (1A8). When antigen-specific T cells were assessed, 72 h after immunization, the depletion of Gr-1 cells resulted in the dramatic decrease in differentiated and activated T cells, which was similar to intact, middle-aged mice (**Fig. 6A–C**). Depletion of neutrophils did not affect the responses (Fig. 6A–C). Interestingly, when young VSV-OVA-infected mice were depleted of their Gr-1+ cells, T-bet expression, 3 days later, was unaffected when compared with control IgG mice and was significantly greater than 12-month-old mice (Fig. 6D). These findings suggested that inflammatory DCs could be dispensable for viral priming but not priming after s.c. protein immunization. Indeed, Gr-1 depletion of young mice, immunized s.c. with OVA in Alum, exhibited a significant decrease in the accumulation of activated, antigen-specific CD4 T cells (Fig. 6E). Thus, it appears that Gr-1/Ly6C+ DCs and monocytes significantly contribute to T cell activation and differentiation following s.c. immunization.

DISCUSSION

The data presented here establish that early-onset age-related alterations in different DC subsets can decrease Th1 differentiation of antigen-specific T cells. Previous findings have implicated the host environment in mediating impaired T cell immune responses in mice 18+ months old [8, 9]. Our results expand these findings to include younger, middle-aged mice (12 months old), which could have far-reaching implications. Th1 differentiation of intact, 2-month-old, antigen-specific CD4 T cells, measured by the expression of the master lineage transcription factor T-bet and production of IFN-γ, was decreased shortly after VSV infection or s.c. protein immunization in 12-month-old hosts compared with 2-month-old hosts. Although antigen-specific CD4 T cells primed in older hosts in both models displayed a similar phenotypic impairment in Th1 differentiation, the underlying cellular mechanisms appeared to be dissimilar. Examination of DC subsets in the dLNs revealed an age-related deficiency in the accumulation

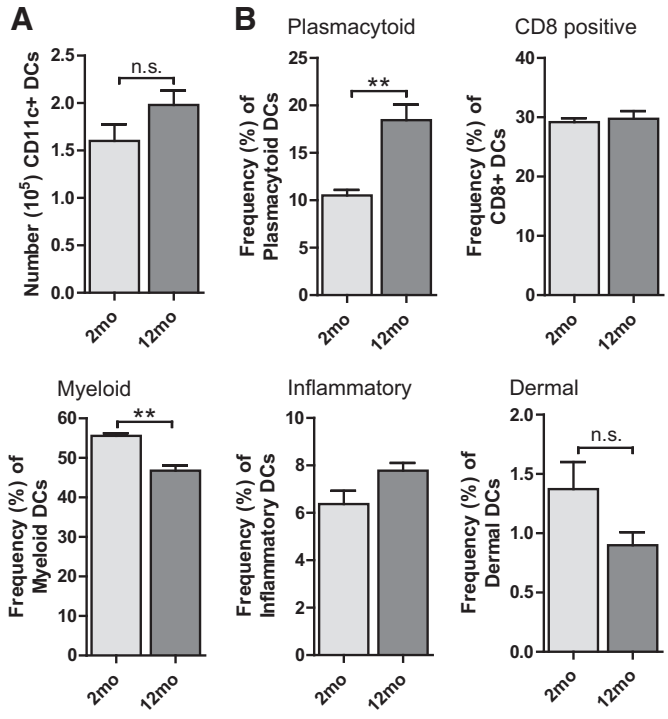

Figure 5. Age-related change in the DC composition in the dLNs following VSV-OVA infection. Two-month-old DO11.10 CD4 T cells were adoptively transferred into 2- or 12-month-old BALB/c recipients and infected with VSV-OVA (10^6 PFU) via the footpad. dLNs were harvested at 48 h, enzymatically digested, and stained for the DC markers CD11c, CD11b, B220, CD8, CD205, and Ly6C. DC subsets were interrogated, as described previously. (A) Enumeration of CD11c+ DCs. (B) Frequency of the DC subsets. Data are the mean ± SEM of one experiment (n=5) of three independent experiments.

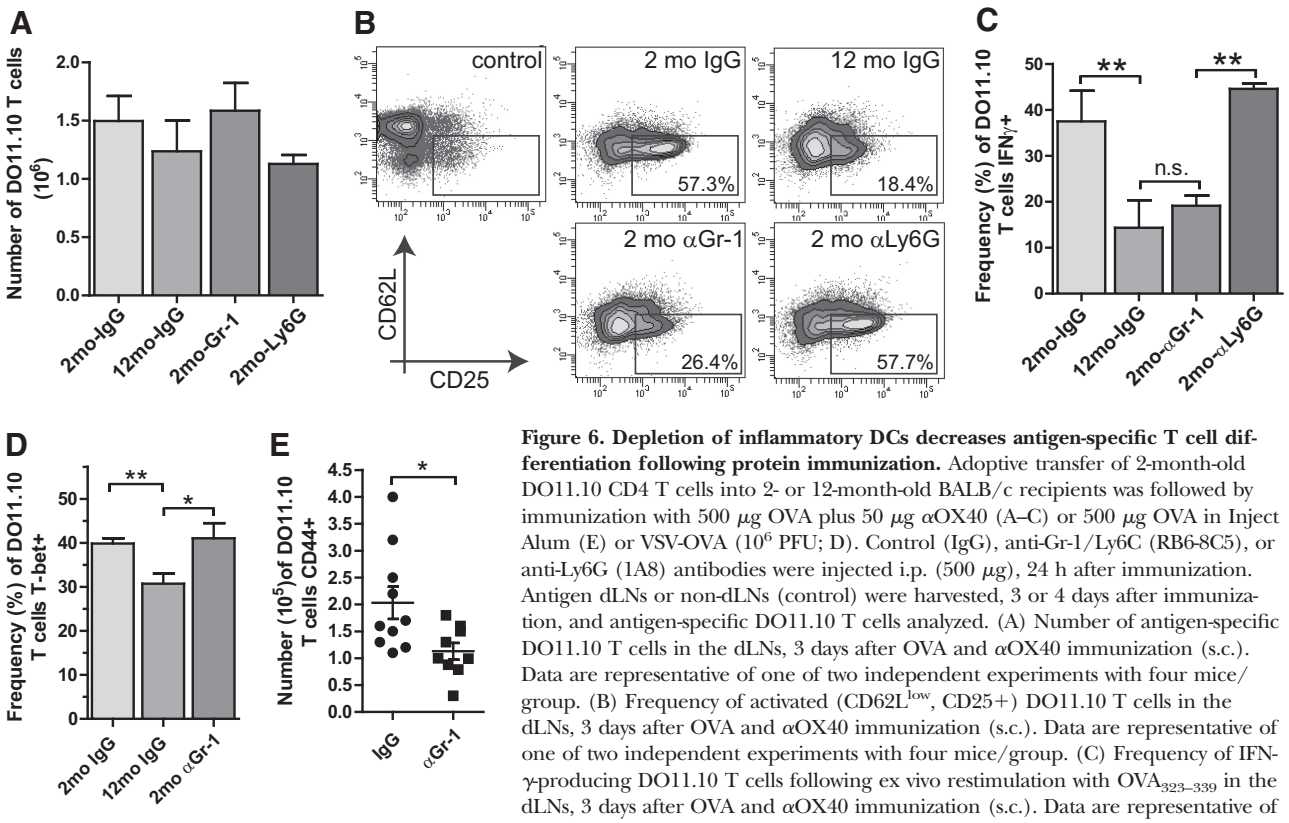

Figure 6. Depletion of inflammatory DCs decreases antigen-specific T cell differentiation following protein immunization. Adoptive transfer of 2-month-old DO11.10 CD4 T cells into 2- or 12-month-old BALB/c recipients was followed by immunization with 500 μg OVA plus 50 μg αOX40 (A–C) or 500 μg OVA in Inject Alum (E) or VSV-OVA (10^6 PFU; D). Control (IgG), anti-Gr-1/Ly6C (RB6-8C5), or anti-Ly6G (1A8) antibodies were injected i.p. (500 μg), 24 h after immunization. Antigen dLNs or non-dLNs (control) were harvested, 3 or 4 days after immunization, and antigen-specific DO11.10 T cells analyzed. (A) Number of antigen-specific DO11.10 T cells in the dLNs, 3 days after OVA and αOX40 immunization (s.c.). Data are representative of one of two independent experiments with four mice/group. (B) Frequency of activated ($CD62L^{low}$, CD25+) DO11.10 T cells in the dLNs, 3 days after OVA and αOX40 immunization (s.c.). Data are representative of one of two independent experiments with four mice/group. (C) Frequency of IFN-γ-producing DO11.10 T cells following ex vivo restimulation with $OVA_{323–339}$ in the dLNs, 3 days after OVA and αOX40 immunization (s.c.). Data are representative of one of two independent experiments (n=4). (D) Frequency of DO11.10 T cells expressing T-bet in the dLNs, 3 days after VSV-OVA infection. Data are representative of one of two independent experiments (n=4). (E) Number of activated (CD44+) DO11.10 T cells in the dLNs, 4 days after immunization (s.c.) with OVA/Alum. Data are the combination of two independent experiments.

of an inflammatory DC subset (CD11c+ CD11b+ Gr-1/Ly6C+) after s.c. OVA protein immunization. In contrast, VSV-OVA-infected 12-month-old mice did not exhibit a significant change in inflammatory DC numbers. Instead, the LNs in these mice displayed an increase in pDCs and concomitant decrease in mDCs after infection. Depletion of inflammatory DCs following OVA protein immunization verified their ability to affect Th1 differentiation in our model and taken in a broader sense, highlight the role that inflammatory DCs can play in immunity, depending on the immunological challenge. Thus, age-impaired T cell responses may not necessarily be the result of the same age-related defect in the host environment and more likely reflect a more complex disruption of the network of priming DCs that depends on the type of immune challenge.

We have revealed several early, middle-aged changes in the composition of DC subsets in the CD4 T cell priming environment. With DCs acting as an important bridge between innate and adaptive immune responses, early DC subset changes could be a driver of age-mediated remodeling of the adaptive arm of the immune system. Indeed, the DC alterations that we observed at 12-months of age coincide with the initiation of age-related changes to T cell responses. The findings—that middle-aged mice experience an age-related decrease in Th1 T cell responses in middle-aged mice after immunization with whole protein/peptide—are in line with previously described studies in elderly mice [8, 9]. One of these related studies assessed the contribution of the aged host environment (20–24 months old) on T cell responses after immunization with OVA with Alum. In this study, the priming and proliferation of antigen-specific T cells in the spleen were delayed in the older hosts [8]. Although we did not see a change in the dilution of CSFE—a measure of proliferation—we observed a significant decrease in the number of antigen-specific T cells at later time-points in the response, suggesting an impairment in proliferation and not a delay [1]. These contrasting findings could be the result of the differing routes of immunization, i.p. versus s.c., as well as potential differences in responses occurring in the spleen compared with LN. Although DCs were analyzed in these related studies, our results differed from the previous findings [8, 9]. We did not observe a significant change in younger, 12-month-old mouse expression of DC maturation markers (e.g., CD40), DC-mediated T cell proliferation, or in the number of CD11c+ DCs (Supplemental Fig. 2). However, we did observe age-related changes in the number of inflammatory DCs in the dLNs. Our results, to our knowledge, are the first to describe an age-related deficiency in inflammatory DCs.

Inflammatory DCs are considered a crucial reservoir of APCs, recruited during various immune responses [28]. This subset can be identified by its expression of the surface molecules CD11c, CD11b, and Gr-1 ($Ly6C^{hi}$) and can also express CD206, CD115, and CD64 [29]. A defining feature of inflam-

matory DCs is their recruitment from the blood through the action of CCR2 and CCR7 into the LNs or sites of inflammation [25, 26, 29]. Inflammatory DCs, like most DCs, are potent activators of T cells in the dLNs, but have the additional ability to prime T cells at the site of inflammation in response to pathogens and vaccination [25, 30, 31]. More recently, this DC subset was found to be indispensible in the induction of antitumor immune responses by the chemotherapeutic mitroxantrone, as blocking the recruitment of this subset to the tumor bed and not other DC subsets (e.g., CD8+ or pDCs) abolished tumor immunity [32]. The ability of this DC subset to migrate to sites of inflammation or lymphoid tissue and activate T cells suggests that these DCs are flexible contributors to the network of DCs, and their ultimate role depends on the location and type of immunological insult. In fact, our results support a highly varied role of this DC subset. Inflammatory DCs were found to be critical for T cell differentiation in a s.c. immunization, whereas in a virus model (VSV), these DCs were dispensable (Fig. 6D).

Infection of 12-month-old hosts with VSV resulted in an impaired CD4 Th1 phenotype, which to our knowledge, has not been reported and was similar to the T cell changes seen following protein immunization. In contrast to the age-related change to an inflammatory DC subset in the protein immunization model, we observed an unexpected increase in pDCs and concomitant decrease in mDCs (CD11c+ CD11b+ $CD8^{neg}$). pDCs are critical for the clearance of VSV [33], and an increase in these cells might be expected to enhance antiviral responses (e.g., IFN-γ), but instead, the virus antigen-specific T cell responses were impaired. A potential explanation is that pDCs in some conditions can inhibit CD4 T cell responses. In the context of aging, pDCs have been shown to impair the function of cDCs following *Encephalitozoon cuniculi* microsporidia parasite and *Toxoplasma gondii* infection, significantly impacting T cell responses [34]. Based on these findings, we depleted the potentially offending pDCs using an anti-PDCA-1 antibody, 24–36 h after VSV-OVA infection, with the goal of restoring responses in the 12 month-old hosts. Unfortunately, this approach did not alter the virus antigen-specific CD4 T cell response (data not shown), suggesting that pDCs may not inhibit the T cell response. An alternative explanation for the age-related immune suppression that we observed could be a result of a decrease in the mDC subset at this time. This subset is considered a LN-resident subset of cDCs that express CD11b but not CD8. This DC subset was shown previously, during protein immunization, to be unaltered by age (Fig. 3B), and the changes following virus infection could be a result of cell death or impaired retention or recruitment to the LN. Additional experiments are needed to determine the role of this DC subset.

Future studies will also need to determine the underlying mechanism that contributes to the age-related alteration in these specific DC subsets. One potential mechanism to be explored is the mobilization and migration of DCs. Inflammatory DCs have been shown to migrate in response to CCR7 and CCR2 ligands [25, 35]. Age-related changes in DC migration are highly likely, as previous studies have shown that aged hosts experience a decrease in the migration of DCs [36] and a decrease in the expression of CCL21, a CCR7 ligand [8]. Furthermore, our early, preliminary results show diminished expression of the CCL21 message (mRNA) in the LNs of 12-month-old mice (data not shown). Additional studies will be required to determine fully if this chemokine and possibly others (e.g., CCR2) are dysfunctional and impair the movement of the specific DC subsets found to be deficient in these studies.

Finally, the findings from these studies describe an early age onset defect in T cell priming and differentiation that could have a profound effect on systemic immunity and promote the accumulation of other age-related defects. The deficient or incomplete programming of T cells in middle-aged hosts has the potential to affect immunity in several ways. It could introduce improper epigenetic changes that would be passed on to memory or daughter cells. It is well understood that lineage differentiation involves the epigenetic silencing and amplification of important genes, such as IFN-γ and IL-4 [37]. Furthermore, the dysfunction in T cell differentiation at an earlier age has the potential to decrease the overall immune response and increase the duration of an infection. Chronic infection can contribute to immune senescence, memory inflation, and "inflamm-aging"—immunologic features documented in elderly subjects [38, 39]. Thus, the specific, middle-aged-related changes in the innate DC compartment could initiate significant remodeling of the adaptive immune response.

AUTHORSHIP

C.E.R., M.F., and Z.C. designed the research, analyzed the results, prepared the figures and the statistical analyses, and wrote the paper. J.N., C.E.R., M.F., and Z.C. performed the experiments. A.D.W. contributed critical reagents and support.

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DISCLOSURES

The authors declare no conflict of interest.

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