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CD8+ T Cell Responses following Replication-Defective Adenovirus Serotype 5 Immunization Are Dependent on CD11c+ Dendritic Cells but Show Redundancy in Their Requirement of TLR and Nucleotide-Binding Oligomerization Domain-Like Receptor Signaling

Ross W. B. Lindsay,* Patricia A. Darrah,† Kylie M. Quinn,* Ulrike Wille-Reece,* Lisa M. Mattei,‡ Akiko Iwasaki,‡ Sudhir P. Kasturi,‡ Bali Pulendran,‡ Jason G. D. Gall,§ A. Gregory Spies,§ and Robert A. Seder*

Replication-defective adenovirus serotype 5 (rAd5) is the most potent recombinant vector for eliciting CD8 T cell responses in humans. In this study, the innate mechanisms that influence T cell responses following rAd5 immunization were assessed in mice. Using rAd5 expressing enhanced GFP (eGFP-rAd5), we show that rAd5 transfects CD11c+ dendritic cells (DCs) in draining lymph nodes in vivo following s.c. or i.m. immunization. Among distinct DC subsets, eGFP expression was highest in CD11c+CD8+B220+ cells but showed redundancy in their pathways. Treatment with a lower frequency detected in CD11c+CD8+B220+ and CD11c+B220+ plasmacytoid DCs. CD11c+ DCs but not CD11c- cells from mice immunized with rAd5 encoding the SIINFEKL peptide induced proliferation of naive OT-I CD8 T cells. Furthermore, CD11c+CD8+B220+ was the most potent DC subset for eliciting naive OT-I CD8 T cell proliferation. Of note, mice with pre-existing immunity to rAd5 had a substantial decrease in eGFP expression in DCs, which was associated with -2-fold decrease in Th1 and complete inhibition of CD8 responses. Thus, pre-existing rAd5 immunity has a greater influence on CD8 compared with CD4 T cell responses.

In terms of how innate cytokines and signaling pathways influenced T cell immunity following rAd5 immunization, we show that the magnitude and quality of CD8 T cell responses are partially dependent on MyD88 but independent of IL-12, type I IFN, apoptosis-associated speck-like protein, nucleotide-binding oligomerization domain-like receptor protein 3, and IL-1. Taken together, these data demonstrate a critical role for CD11c+ DCs for CD8 responses but striking redundancy for innate cytokines and signaling by TLR and nucleotide-binding oligomerization domain-like receptor pathways. The Journal of Immunology, 2010, 185: 000–000.

Development of an effective vaccine against HIV, malaria, and tuberculosis will require the generation of durable humoral and/or cellular immune responses. Although multiple vaccine formulations are capable of generating strong Ab responses, there are far fewer vectors that elicit strong Ag-specific T cell immunity. Among these, replication-defective adenovirus serotype 5 (rAd5) vectors are especially notable for their ability to induce potent CD8 T cell responses. Indeed, in both mouse and nonhuman primate studies, rAd5 induces substantially higher CD8 responses than other adenovirus serotypes (1–3). Thus, a more thorough understanding of the innate mechanisms by which rAd5 induces T cell immunity in vivo may be useful for optimizing current and future vaccines.

A number of in vitro and in vivo studies in mice and humans showed that rAd5 activates various TLR and nucleotide-binding oligomerization domain-like receptor (NLR) signaling pathways to produce cytokines such as IL-1, IL-6, TNF, IL-12, IFN-α, and certain chemokines. The source of the cytokines and chemokines include mouse splenic macrophages and dendritic cells (DCs) isolated ex vivo following i.v. immunization with rAd5 (4, 5). In addition, similar cytokines have been detected following in vitro stimulation of cultured or freshly isolated mouse or human DCs with rAd5 (6, 7). In assessing which of these innate pathways and cytokines influence T cell immunity, prior studies showed a partial role for TLR9, MyD88, or IFN-α on CD8 T cell responses following i.v. immunization (5, 8, 9). In terms of the cellular tropism, in vitro studies using mouse splenic and bone marrow-derived DCs (BMDCs) showed efficient uptake of rAd5 (6, 10). In addition, freshly isolated human conventional DCs (cDCs) show enhanced transfection by rAd5 compared with plasmacytoid (pDCs) (7). Uptake of rAd5 in vivo following i.v. injection shows increased uptake by Kupffer cells in the liver (11) or macrophages in the marginal zone of the spleen (4, 5). Finally, although there is indirect evidence that DCs are...
activated in vivo by rAd5 following i.v. administration (5), their role in priming T cell responses in vivo is undefined. Collectively, although prior studies provide insight into how rAd5 activates various APCs, cytokines, and innate signaling pathways in vitro or in vivo following i.v. immunization, it remains unclear how these variables are integrated in vivo at sites of immune priming, such as draining lymph nodes (LNs) to influence both CD4 and CD8 T cell immune responses following s.c. or i.m. immunization. Moreover, there is little direct evidence to show a direct role of DCs in priming naïve T cells.

In this study, the innate mechanisms that influence the magnitude and quality of T cell responses were assessed following immunization with rAd5. On the basis of the functional heterogeneity of DC subsets and their critical role in initiating T cell immunity (12), an extensive analysis of the tropism of rAd5 for DCs in draining LNs and spleen was performed following immunization. Moreover, the ability of distinct CD11c+ DCs subsets isolated ex vivo following rAd5 immunization to present Ag to naïve CD8 T cells was determined. Finally, the influence of innate cytokines and signaling pathways on T cell immunity was assessed. Overall, the data presented highlight an important role for CD11c+ DCs in CD8 immunity following rAd5 immunization but reveal substantial redundancy in the requirement of TLR, NLR signaling, and the major T cell-polarizing cytokines IL-12 and type 1 IFN in vivo.

Materials and Methods

Animals

C57BL/6, IL-12p35−/−, IL-12p40−/−, and OT-I TCR transgenic mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained in the Vaccine Research Center Animal Care Unit (Bethesda, MD) under specific pathogen-free conditions. The generation of mice deficient in TLR9 (13), IFN-α/βR (14), MyD88 (15), apoptosis-associated speck-like protein (ASC), and NLR protein 3 (NLRF3) (16) has been reported previously. All experimental protocols were approved by the Vaccine Research Center Animal Care and Use Committee.

Immunization

Animals were immunized with 1 × 10^{10} adenovirus particle units (PUs) replication defective empty rAd5 (E1-, E3-, and E4-deleted) as a control or with replication defective rAd5 expressing GFP (eGFP-rAd5, E1-, E3-, and E4-deleted) or replication defective rAd5 expressing SIV-Gag protein (SIV-Gag-rAd5, E1- and E3-deleted) either s.c. in the quadriceps muscle, i.m. in the tibialis anterior, or i.v. in the tail vein. For depletion of pDCs, IL-12 or NK cells, 0.5 mg anti-pDCs (clone 927; provided by G. Trinchieri, National Cancer Institute, Frederick, MD), 1 mg anti–IL-12 (C17.8; provided by F. Finkelman, University of Cincinnati, Cincinnati, OH), or 0.5 mg anti-NK1.1 (provided by B. Chambers, Karolinska Institutet, Stockholm, Sweden) were given i.p. 1 d before rAd5 immunization. To assess the role of pre-existing immunity to adeno-virus, animals were immunized i.m. with 1 × 10^{10} PU empty rAd5 6 wk before immunization with eGFP-rAd5. To assess innate cytokine production, 1 × 10^{10} PU eGFP-rAd5 and 50 μg CpG 1826 (Coley Pharmaceutical Group, Düsseldorf, Germany) were administered s.c. and i.v. before animals were sacrificed at indicated time points for serum collection.

DCs and staining

At various time points after immunization, individual spleens, lungs, or pooled LNs (n = 5–10 animals) were gently homogenized and incubated with 1 mg/ml collagenase D (Roche, Nutley, NJ) for 30 min at 37°C. Tissues were further homogenized, washed, and enriched for CD11c+ DCs by positive selection (clone N418) using a MACS cell separation, according to the manufacturer’s protocol (Miltenyi Biotec, Auburn, CA). Cells were then stained for viability with LIVE/DEAD Fixable Violet Dead Cell Stain (Molecular Probes, Carlsbad, CA), CD3 (145-2C11; BD Biosciences, San Jose, CA), CD19 (6D5; BioLegend, San Diego, CA), and NK1.1 (PK136; BioLegend). DCs were also stained with an alternative clone CD11c Ab (HL3; BD Biosciences), B220 (RA3-6B2; BD Biosciences), CD11b (M1/70; BD Biosciences), CD8 (53-6.7; BioLegend), F4/80 (CLA-3; AbD Serotec, Raleigh, NC), and DEC205 (NLCDC-145, Miltenyi Biotec). Cells were resuspended in 0.5% parafomaldehyde before being acquired on a modified BD LSR II flow cytometer and analyzed using FlowJo software version 8.8.3 (Tree Star, Ashland, OR). A gating strategy was adopted to exclude dead cells, B cells, T cells, and NK cells.

Imaging
eGFP transcript in situ

Naive mice were immunized with 1 × 10^{10} PU empty rAd5 or eGFP-rAd5 i.m. or s.c. After 24 h, 7 d, and 40 d, legs were removed, and skin was peeled back before being imaged on a fully automated M205 FA stereomicroscope equipped with a ×0.63 Plan Apo objective and controlled by Leica LAS-AF software. High-resolution (1600 × 1200 pixel) fluorescent or bright-field images were acquired using a Leica DFC 340 FX monochrome camera. A GFP2 filter set (480/40, 510lp) was used for fluorescence imaging. A motorized stage was used to collect a tile scan of higher magnification images, and the final montage was assembled using Adobe Photoshop. Immunization of the animals was staggered so that all legs were imaged on the same day with the same fluorescence source intensity and camera exposure settings.

DC presentation to OT-I CD8 T cells

Mice were immunized s.c. or i.v. with 1 × 10^{10} PU SIIN-fAd5 (provided by S. E. Hensley, National Institute of Allergy and Infectious Disease, National Institutes of Health, Bethesda MD) or empty rAd5. After 24 h, the draining popliteal LNs and spleens were removed and enriched for CD11c+ DCs, CD11c+ DCs or CD11c- cells were incubated with 2 × 10^{6} CFSE-labeled CD8+ OT-I T cells at 37°C for 3 d. In addition, CD11c+ enriched cells were sorted on a FACS Aria II by expression of CD8 and B220 before coculture with CD8+ OT-I T cells. As a positive control, 0.1 μg/ml SIIN- FEKLP peptide and proximal LN DCs from animals immunized with empty rAd5 were incubated with OT-I T cells. CD8+ OT-I T cells were positively enriched by MACS cell separation, according to the manufacturer’s protocol (Miltenyi Biotec), from the spleen of transgenic mice expressing a TCR that recognizes OVA residues 257–266 (SIINFEKL) in the context of H-2Kb and labeled with 0.5 μM CFSE, according to the manufacturer’s protocol (Invitrogen).

ELISA and cytometric bead array

Serum samples were assayed for levels of IFN-α by ELISA (PBL Biomedical Laboratories, Piscataway, NJ) and IFN-γ, IL-10, IL-12p70, IL-6, MCP-1, and TNF by Cytometric Bead Array infection kit (BD Biosciences). Serum samples were collected 1, 2, 6, and 24 h following s.c. or i.v. immunization with 1 × 10^{10} PU eGFP-rAd5 or 50 μg CpG. IL-12p40 was measured by ELISA (R&D Systems, Minneapolis, MN) in supernatants from 1 × 10^{6} BMDCs or splenocytes incubated ex vivo with 1 × 10^{10} PU eGFP-rAd5 or 100 μg CpG for 24 h. BMDCs were prepared as described previously (17).

Multiparameter flow cytometry

At various times following immunization with rAd5, 1.5 × 10^{6} leukocytes from the spleen were incubated with 2 μg/ml anti-CD25 (BD Biosciences), 10 μg/ml brefeldin A (Sigma-Aldrich), and a 15-mer overlapping GFP peptide pool or 2 μg/ml each of the AL11 (CD8 epitope) and DD13 (CD4) SIV gag peptides for 5 h at 37°C before intracellular cytokine staining. Cells were stained with LIVE/DEAD Fixable Violet Dead Cell Stain (Molecular Probes), CD3, CD4, IFN-γ, IL-2, TNF (BD Biosciences), and CD8 (BioLegend). Cells were resuspended in 0.5% parafomaldehyde before being acquired on a modified BD LSR II flow cytometer and analyzed using FlowJo software version 8.8.3 (Tree Star) and SPICE version 4.2.2 (M. Roederer, Vaccine Research Center, National Institute of Allergy and Infectious Disease, National Institutes of Health).

Statistical analysis

T cell data are expressed as means of individual plots, and statistical significance was calculated using a two-tailed Student’s t test assuming unequal variance. Analysis was performed using SPICE version 4.2.2 (M. Roederer).

Results
eGFP-rAd5 transfects multiple DC subsets in vivo

To assess the innate mechanisms that influence the generation of T cell responses induced by rAd5 vaccines, the tropism for APCs in vivo following s.c. or i.m. immunization with an rAd5 vector
expressing eGFP (eGFP-rAd5) was assessed. On the basis of the potential role of DCs, a protocol was used to identify three distinct DC subsets: CD11c<sup>+</sup>CD8<sup>+</sup>B220<sup>−</sup>DCs, CD11c<sup>+</sup>CD8<sup>−</sup>B220<sup>−</sup> (cDCs), and CD11c<sup>+</sup>B220<sup>+</sup> (pDCs); the gating analysis excluded B, T and NK cells. Fig. 1 shows the relative frequencies of eGFP<sup>+</sup> cells among total CD11c<sup>+</sup> cells (histogram, left) and the expression of eGFP within distinct DC subsets (right) in the various immunization groups. As negative controls, there was no expression of eGFP in naive or mice immunized with PBS or empty rAd5 (Fig. 1A). Following s.c. immunization, eGFP was detected and significantly higher in total CD11c<sup>+</sup> DCs in the proximal draining LN (popliteal LN) (Fig. 1B) compared with the distal draining LN (inguinal LN) (Fig. 1C). In addition, uptake of eGFP-rAd5 was higher at 24 h (27.8 and 1.07%) compared with 72 h (9.92 and 0.61%) in both the proximal and distal LNs, respectively. Similarly, expression of eGFP was also higher in the proximal (8.71%) compared with distal LNs (2.66%) at 24 h as compared with 72 h (Fig. 1D, 1E) following i.m. immunization. Of note, the total percentage of CD11c<sup>+</sup> cells expressing eGFP in draining LNs is higher following s.c. compared with i.m. immunization. By 7 d after s.c. or i.m. immunization, eGFP expression was no longer detectable in either the proximal or distal LNs by flow cytometry (data not shown) but was visualized by in situ imaging (Supplemental Fig. 1). However, by 42 d after immunization, eGFP expression was no longer detected by in situ imaging (Supplemental Fig. 1B). Finally, in terms of other APCs, <1% eGFP was detected in CD11c<sup>+</sup> cells following s.c. or i.m. immunization (data not shown). These data show that CD11c<sup>+</sup> DCs are the major APCs taking up rAd5 following these physiologic routes of immunization.

With regard to uptake of specific DC subsets, although CD11c<sup>+</sup>CD8<sup>−</sup>B220<sup>−</sup> DCs had the largest relative percentage of eGFP expression following s.c. immunization, there was also uptake in CD11c<sup>+</sup>CD8<sup>+</sup>B220<sup>−</sup> DCs and CD11c<sup>+</sup>B220<sup>+</sup> pDCs (Fig. 1B-E). At 72 h postimmunization, the relative percentage of CD11c<sup>+</sup>CD8<sup>−</sup>B220<sup>−</sup> DCs expressing eGFP is diminished, and this is associated with an increase in the proportion of CD11c<sup>+</sup>CD8<sup>+</sup>B220<sup>−</sup> DCs. Similar data were seen in the DC subsets not expressing eGFP (data not shown). These data are consistent with differential migration of CD11c<sup>+</sup>CD8<sup>−</sup>B220<sup>−</sup> DCs compared with CD11c<sup>+</sup>CD8<sup>+</sup>B220<sup>−</sup> DCs into the LN resulting in alterations of the relative percentage of each subset. In terms of detecting rAd5 beyond the draining LNs, there were few eGFP expressing CD11c<sup>+</sup> DCs in the spleen or lungs 24 h after s.c. or i.m. immunization (Supplemental Fig. 2). Collectively, these data show that rAd5 is capable of transfecting all DC subsets in vivo, with the highest frequency detected in CD11c<sup>+</sup>CD8<sup>−</sup>B220<sup>−</sup> cells in all organs tested. Moreover, the results from both the imaging and uptake studies suggest that priming of T cells is local and limited to LN following s.c. and the LN and muscle following i.m. immunization.

CD11c<sup>+</sup>CD11b<sup>+</sup>F4/80<sup>+</sup>CD8<sup>−</sup> DCs are rapidly recruited to the draining lymph following eGFP-rAd5 immunization

The results above show that CD11c<sup>+</sup>CD8<sup>−</sup>B220<sup>−</sup> cells comprised the largest frequency of transfected DCs following eGFP-rAd5 immunization (Fig. 1). As CD11c<sup>+</sup>CD8<sup>−</sup>B220<sup>−</sup> DCs contain several additional subsets, the staining panel was expanded to include analysis of cells expressing CD11b, F4/80, and DEC205. These additional markers have been used to delineate whether CD11c<sup>+</sup> cells are blood-derived, monocyte-derived, or dermal DCs (Table I). As shown in Fig. 2A, following s.c. immunization with PBS or eGFP-rAd5, there was enhanced expression of CD11b and F4/80 in quadrant 4 (Q4; orange) with a decrease in the number of cells expressing DEC205 compared with PBS-immunized mice. In addition, there was an increase in the expression of DEC205 in CD11c<sup>+</sup>CD8<sup>−</sup>B220<sup>−</sup> (Q1; red) cells in rAd5 compared with PBS-immunized mice. The change in the relative percentages of the various DC populations was accompanied by a ~2-fold increase in the total number of CD11c<sup>+</sup> cells following immunization with empty rAd5 or eGFP-rAd5 compared with PBS (data not shown). In addition, the changes observed in the level of cell surface expression for CD11b and F4/80 are reflected in the median fluorescence intensity (Fig. 2B). Taken together, these data suggest that the influx of CD11c<sup>+</sup>CD11b<sup>+</sup>F4/80<sup>+</sup> cells into Q4 upon rAd5 immunization is in large part due to blood-derived DCs (Fig. 2). Indeed, uptake of eGFP at 24 h after s.c. immunization was highest in CD11c<sup>+</sup>CD8<sup>−</sup>B220<sup>−</sup> DCs, which were predominantly CD11b<sup>+</sup>F4/80<sup>+</sup>DEC205<sup>+</sup> (Fig. 2) and thereby blood-derived CD11b<sup>+</sup> DCs (Table I). At 72 h following immunization, the relative eGFP expression was still highest in CD11c<sup>+</sup>CD8<sup>−</sup>B220<sup>−</sup> DCs with a notable increase in the number of eGFP cells with a CD11c<sup>+</sup>CD8<sup>−</sup>
CD11b<sup>+</sup>DECD205<sup>+</sup> phenotype. The phenotype of such cells is consistent with migrating Langerhans cells, dermal DCs, and/or monocyte-derived DCs. Although these cell populations containing eGFP were also observed following i.m. immunization, they were of a far lower frequency compared with s.c. immunization (Supplemental Fig. 3). Overall, these data show that the large increase in the number and relative percentage of CD11c<sup>+</sup>CD8<sup>+</sup> B220<sup>−</sup> cells in the draining LN following s.c. immunization with rAd5 is heterogeneous.

**CD11c<sup>+</sup>CD8<sup>+</sup> DCs induce proliferation of naive CD8 T cells following rAd5 immunization**

Because rAd5 vectors are notable for their potent induction of CD8 responses, the next series of studies assessed how the DC subsets differ functionally for activating such cells. To demonstrate whether CD11c<sup>+</sup> DCs could present Ag directly to naive CD8 T cells, animals were immunized s.c. with an rAd5 vector encoding the SIINFEKL peptide (SIIN-rAd5). Twenty-four hours after immunization, total CD11c<sup>+</sup> DCs enriched from draining LNs or spleens were incubated with CFSE-labeled naive OT-I CD8<sup>+</sup> T cells. As shown in Fig. 3A, CD11c<sup>+</sup> DCs isolated from draining LNs stimulated division of naive OT-I CD8<sup>+</sup> T cells in vitro. By contrast, even very high numbers of CD11c<sup>+</sup> DCs were unable to stimulate division of naive OT-I CD8<sup>+</sup> T cells (Supplemental Fig. 4A). CD11c<sup>+</sup> DCs from the spleen also had the ability to prime OT-I T cells after s.c. immunization but only in stimulatory conditions containing the highest number of DCs (Fig. 3B). Following i.v. immunization, enriched CD11c<sup>+</sup> DCs from the spleen efficiently stimulated CD8 T cell proliferation (Fig. 3B). These data are consistent with the increased expression of eGFP in spleen cells following i.v. immunization (Supplemental Fig. 2A). Thus, both LN and spleen CD11c<sup>+</sup> DCs isolated ex vivo following immunization are capable of presenting Ag to naive OT-I CD8<sup>+</sup> T cells.

To assess whether distinct CD11c<sup>+</sup> DC subsets differed in their capacity to activate naive CD8 T cells, splenic CD11c<sup>+</sup> DCs were sorted by their expression of CD8 and B220 (Supplemental Fig. 4B) following i.v. immunization of SIIN-rAd5. As shown in Fig. 3C, CD11c<sup>+</sup>CD8<sup>+</sup>B220<sup>−</sup> DCs are the major DC subset presenting Ag to naive CD8 T cells. These data substantiate the well-established role of this distinct DC subset for induction of CD8 T cell immunity directly or through cross-presentation with protein Ags and certain viral infections (18–21).

**Pre-existing rAd5 immunity limits uptake of rAd5 in CD11c<sup>+</sup> DCs and generation of CD8 T cell responses**

A major limitation of using rAd5 vectors for inducing T cell responses is prevalence of pre-existing immunity from prior natural adenovirus exposure or by vaccination (1, 22–28). Accordingly, several studies have shown that pre-existing humoral and cellular

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<th>DC Subtype</th>
<th>Alternative Name</th>
<th>CD11c</th>
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**FIGURE 2.** Phenotypic analysis of DCs following s.c. eGFP-rAd5 immunization. Expression of various DC phenotypic markers that define previously identified DC subsets (Table I) was assessed in proximal draining LNs from C57BL/6 mice (n = 10) following s.c. immunization with 1 × 10<sup>10</sup> PU eGFP-rAd5. Histograms show the expression of these markers in Q1 (red) CD11c<sup>+</sup>CD8<sup>+</sup>B220<sup>−</sup> DCs, Q2/Q3 (blue/green) CD11c<sup>+</sup>CD8<sup>+</sup>B220<sup>−</sup> pDCs, and Q4 (orange) CD11c<sup>+</sup>CD8<sup>+</sup>B220<sup>−</sup> DCs after immunization. The expression of various DC markers on CD11c<sup>+</sup>-enriched cells from proximal LN of PBS or eGFP-rAd5–immunized animals was assessed (A), with dot plots representing total CD11c<sup>+</sup> cells (PBS) or CD11c<sup>+</sup>eGFP<sup>+</sup> cells (eGFP-rAd5). Arrows highlight changes in expression following immunization with GFP-rAd5. In addition, the expression of CD11b, F4/80, and DEC205 was expressed as median fluorescence intensity of protein expression (B). LNs were pooled from 10 animals before CD11c<sup>+</sup> enrichment; data are representative of three independent experiments.
Immunity can influence Ad5 immunogenicity through various mechanisms (1, 22–28). However, the effect that prior Ad immunity exerts vis-a-vis uptake of rAd by CD11c+ DCs in vivo has not been studied. To investigate this, pre-existing anti-rAd5 immunity was induced in mice following immunization with $1 \times 10^{10}$ PU empty rAd5 several weeks prior to vaccination with eGFP-rAd5. As shown in Fig. 4A, there was a striking decrease in the frequency ($\sim 10$-fold) of eGFP-expressing CD11c+ DCs in the proximal draining LNs of mice with pre-existing immunity compared with naive animals following s.c. or i.m. immunization with eGFP-rAd5. Moreover, there was a significant reduction in the number of CD11c+CD8+B220+ DCs transfected with eGFP-rAd5 in such mice. To assess the mechanism by which pre-existing immunity could influence uptake of eGFP-rAd5, serum of naive mice or mice previously immunized with rAd5 was transferred into naive animals before immunization with eGFP-rAd5. Consistent with the data in Fig. 4A, there was a 10-fold decrease in the frequency of eGFP-expressing CD11c+ DCs in mice that had received the rAd5 immune serum (Supplemental Fig. 5). These data clearly show that pre-existing humoral immunity is sufficient to limit uptake of eGFP-rAd5 in CD11c+ DCs.

We next determined how pre-existing anti-rAd5 immunity influenced the magnitude and quality of T cell responses following eGFP immunization. In terms of magnitude of the cytokine response, there is a 2-fold decrease in the frequency of total IFN-γ- or TNF-specific CD4+ T cells (Fig. 4B) with low to undetectable CD8 cytokine responses in the mice with pre-existing Ad5 immunity (Fig. 4C). In assessing the quality of the CD4 T cell response that comprises any combination of IFN-γ, TNF, and IL-2 at the single-cell level, mice with pre-existing immunity had a relative increase in the frequency of IFN-γ/TNF and IL-2 triple-positive subset, whereas IFN-γ/TNF double-positive and IFN-γ single-positive subsets were reduced (Fig. 4D). These data suggest that pre-existing Ad5 neutralizing responses lower the effective dose of rAd5 vaccination (29), resulting in a more multifunctional Th1 cytokine response (30).
CD8 T cells (Fig. 5B) measured by intracellular cytokine staining 12 d after eGFP-rAd5 immunization. The quality of the Th1 response was assessed in the spleen (cytometry for eGFP expression (assessment of DC transfection, LNs were pooled (are representative of two experiments; 10

C57BL/6 mice were first immunized i.m. with 1 × 10^10 PU empty rAd5. Six weeks later, naïve or mice with pre-existing immunity were immunized with 1 × 10^10 PU eGFP-rAd5 s.c. or i.m. Twenty-four hours later, draining proximal LNs were harvested, enriched for CD11c+ DCs, and analyzed by flow cytometry for eGFP expression (A). The magnitude of eGFP-specific CD4 (B) and CD8 (C) IFN-γ, IL-2–, or TNF-specific responses in the spleen was measured by intracellular cytokine staining 12 d after eGFP-rAd5 immunization. The quality of the Th1 response was assessed in the spleen (D). For assessment of DC transfection, LNs were pooled (n = 10) before CD11c+ enrichment. For T cell responses, individual spleens (n = 4) were processed; data are representative of two experiments; *p < 0.05; p < 0.01; or p < 0.005 as indicated, significantly different from animals without pre-existing immunity.

FIGURE 4. Effect of pre-existing rAd5 immunity on expression of eGFP by CD11c+ DCs and T cell immunity. To establish pre-existing immunity, C57BL/6 mice were first immunized i.m. with 1 × 10^10 PU empty rAd5. Six weeks later, naïve or mice with pre-existing immunity were immunized with 1 × 10^10 PU eGFP-rAd5 s.c. or i.m. Twenty-four hours later, draining proximal LNs were harvested, enriched for CD11c+ DCs, and analyzed by flow cytometry for eGFP expression (A). The magnitude of eGFP-specific CD4 (B) and CD8 (C) IFN-γ, IL-2–, or TNF-specific responses in the spleen was measured by intracellular cytokine staining 12 d after eGFP-rAd5 immunization. The quality of the Th1 response was assessed in the spleen (D). For assessment of DC transfection, LNs were pooled (n = 10) before CD11c+ enrichment. For T cell responses, individual spleens (n = 4) were processed; data are representative of two experiments; *p < 0.05; p < 0.01; or p < 0.005 as indicated, significantly different from animals without pre-existing immunity.

existing immunity substantially limits eGFP expression in CD11c+ DCs and has a far greater role in limiting CD8 than CD4 responses.

**Th1 and CD8 T cell responses have a partial dependence on TLR signaling following rAd5 immunization**

To gain further insight into how other innate immune mechanisms influence T cell responses following rAd5 immunization, the effect of inhibiting specific cytokines, innate signaling pathways, and cell populations was assessed. In terms of specific cytokines, IFN-α was the only cytokine we detected following rAd5 immunization in vivo with modest amounts of IL-12 detected from DCs in vitro (Supplemental Fig. 6). Remarkably, inhibiting IL-12 or type I IFN signaling alone or together did not influence the magnitude or quality of T cell responses (Supplemental Fig. 7). Moreover, depletion of pDCs or NK cells, which could alter T cell responses through a variety of mechanisms, also had no effect (Supplemental Fig. 7). These data show that rAd5 immunity is independent of two prominent T cell-polarizing cytokines, IL-12 and type I IFN, as well as pDCs in vivo.

In terms of innate signaling pathways, MyD88 and TLR9 have been reported to have some effect on limiting the frequency of CD8 T cell responses following immunization with rAd5 (6, 8). Thus, we extended these findings by assessing the influence of these TLR signaling pathways on the magnitude and quality of CD8 as well as CD4 T cell responses following rAd5 s.c. immunization. In addition to assessing eGFP-specific T cell responses after immunization with eGFP-rAd5, a rAd5 vector expressing SIV-Gag protein was used to provide data with a physiologically relevant Ag. As shown in Fig. 5, there was a 2- to 3-fold reduction in the frequency of CD4 (Fig. 5A, 5C) and CD8 T cells (Fig. 5B, 5D) secreting IFN-γ, IL-2, or TNF in MyD88−/− compared with WT mice for rAd5 vectors expressing eGFP or SIV-Gag. Finally, because rAd5 is a DNA virus, we used TLR9−/− mice to determine whether the partial dependence on MyD88 for T cell immunity was due to this specific TLR, which responds to bacterial and viral DNA. Although there was a modest decrease in the frequency of Ag-specific CD4 T cells in TLR9−/− mice, there was no effect on the CD8 cytokine response as compared with WT mice (Fig. 5E, 5F). As pDC recognition of rAd5 and IFN-α production has been shown to be mediated by TLR9 signaling (6, 10), these additional data showing that CD8 responses are not decreased in TLR9−/− mice substantiate our findings that pDCs and type I IFN have little role in rAd5-induced CD8 immunity.

**Th1 and CD8 T cell responses induced by rAd5 are independent of inflammasome and IL-1 signaling**

Because T cell immunity was still robust in the absence of TLR signaling, it was possible that other innate signaling pathways were important for mediating the potency of rAd5 on T cell immunity. Indeed, adenoviral DNA has been shown to induce innate cytokine or chemokine production in vitro or in vivo through the inflammasome pathway (31). A recent report, however, demonstrated that induction of proinflammatory cytokine and chemokine responses from splenic macrophages in vivo in response to rAd5 given i.v. was independent of NLRP3 but dependent on IL-1 (4). Nevertheless, despite these discordant results, the influence of the inflammasome or IL-1 on T cell immunity in vivo following rAd5 immunization has not been determined. As shown in Fig. 6, there was no significant difference in Th1 (Fig. 6A, 6C) or CD8 (Fig. 6B, 6D) T cell responses in ASC−/− or NLRP3−/− animals compared with WT mice to either Ag. Furthermore, SIV-Gag–specific Th1 and CD8 responses were also independent of IL-1 signaling.
show a clear preference of eGFP-rAd5 to transfect CD11c+ DCs, however, the evidence for the role for DCs during induction of immunity in vivo remains unclear (40). In this study, we show that these specific DCs are the most potent for inducing CD8 T cell immunity with protein Ags and certain viral infections (18–21). The data presented in this paper raise the question of whether rAd5 is inducing CD8 responses by direct or cross-presentation. Although sorting DCs into GFP-positive and -negative populations following rAd5 immunization could provide insight into these two mechanisms, the rAd5 vector expressing SIINFEKL and GFP was not suitable for separating DC populations based on uptake. Future studies using SIINFEKL expressing eGFP should enable us to address this question.

pDCs were the final subset of CD11c+ DCs to be extensively examined for their role in T cell immunity following rAd5 immunization. rAd5 has been shown to induce IFN-α in a TLR-dependent and -independent manner from pDCs and non-pDCs, respectively, ex vivo (9, 10). In terms of Ag presentation, whereas pDCs can present or cross-present Ag to CD4 or CD8 T cells in vitro (36–39), their ability to induce or modulate primary T cell immunity in vivo remains unclear (40). In this study, we show that a relatively small frequency of CD11c+ B220− pDCs were devoid of Ag presentation, prior studies have shown that macrophages or Kupffer cells are transfected by rAd5 in spleen or liver, respectively, following i.v. administration (4, 5). In these studies, uptake of rAd5 by cDCs compared with pDCs has been observed from in vitro studies with mouse or human DC subsets (6, 7). The preferential transfection of cDCs is not likely a reflection of a difference in the expression levels of the coxsackievirus-adenovirus receptor on the DCs, because adenovirus has been shown to transfect human and mouse DC subsets independently of coxsackievirus-adenovirus receptor (34, 35).

Among CD11c+ DC subsets, cDCs (CD11c+CD8−B220−) had markedly enhanced uptake of rAd5 compared with CD11c+CD8−B220− and pDCs (CD11c+B220+) in vivo. In additional analysis of the CD11c+CD8−B220− DCs subset, the highest proportion of cells were CD11b+F4/80−DEC205+, which have been classified as blood-derived DCs. In addition, a population of CD11b+F4/80−DEC205+ was most notable at 72 h postimmunization. The phenotype of such cells is consistent with migrating Langerhans cells, dermal DCs, or monocyte-derived DCs (32, 33). The increased uptake of rAd5 by cDCs compared with pDCs has been observed from in vitro studies with mouse or human DC subsets (6, 7). The preferential transfection of cDCs is not likely a reflection of a difference in the expression levels of the coxsackievirus-adenovirus receptor on the DCs, because adenovirus has been shown to transfect human and mouse DC subsets independently of coxsackievirus-adenovirus receptor (34, 35).

Analysis of additional CD11c+ DC subsets showed a relatively small frequency of CD11c+CD8−B220−DCs transfected following eGFP-rAd5 s.c. or i.m. immunization in the draining LN compared with CD11c+CD8−B220−DCs. However, on a per cell basis, we show that these specific DCs are the most potent for inducing proliferation of naive CD8 T cells. This unique subset of DCs has been shown to be critical for cross-presentation and induction of CD8 T cell immunity with protein Ags and certain viral infections (18–21). The preferential transfection of cDCs is not likely a reflection of a difference in the expression levels of the coxsackievirus-adenovirus receptor on the DCs, because adenovirus has been shown to transfect human and mouse DC subsets independently of coxsackievirus-adenovirus receptor (34, 35).

This study focused on the role of DC subsets, T cell-polarizing cytokines (IL-12 and IFN-α), and TLR (MyD88 and TLR9) and NLR (NLRP3 and ASC) signaling pathways on the magnitude and quality of T cell responses following rAd5 immunization. In terms of Ag presentation, prior studies have shown that macrophages or Kupffer cells are transfected by rAd5 in spleen or liver, respectively, following i.v. administration (4, 5). In these studies, however, the evidence for the role for DCs during induction of rAd5 immunity was indirect. The data presented in this paper show a clear preference of eGFP-rAd5 to transfect CD11c+ DCs in draining LNs following s.c. or i.m. immunization, which are the routes used in clinical studies using rAd5 as a preventive vaccine.

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Discussion

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FIGURE 5. Ag-specific Th1 and CD8 T cell responses in MyD88−/− and TLR9−/− mice following rAd5 immunization. C57BL/6, MyD88−/−, and TLR9−/− mice (n = 4 or 5) were immunized s.c. with 1 × 1010 PU eGFP-rAd5. Additional MyD88−/− mice were immunized with 1 × 1010 PU SIV-Gag-rAd5. Ag-specific T cell responses from individual spleens were measured by intracellular cytokine staining in response to a GFP peptide pool (A, B, E, F) or SIV-Gag DD13 and AL11 peptides (C, D) 14 d (TLR9−/−) or 21 d (MyD88−/−) after immunization. Data are representative of three independent experiments; *p < 0.05 significantly different from WT mice.

FIGURE 6. Ag-specific Th1 and CD8 T cell responses to rAd5 immunization in ASC−/−, NALP3−/−, and IL-1R1−/− mice. C57BL/6, ASC−/−, NALP3−/−, and IL-1R1−/− mice (n = 3 or 4) were immunized s.c. with 1 × 1010 PU eGFP-rAd5 or SIV-Gag-rAd5. Ag-specific responses in the spleen were measured by intracellular cytokine staining in response to a GFP peptide pool (A, B) or SIV-Gag DD13 and AL11 peptides 13 d (C, D) and 28 d (E, F) after immunization. Data are representative of two independent experiments; *p < 0.05 significantly different from WT mice.
transfected by eGFP-rAd5. pDCs were unable to present Ag to naive CD8 T cells in vitro and depleting CD8+ pDCs in WT or IFN-αβR−/− mice did not alter T cell immunity compared with WT mice. Our finding that IFN-α has little significant influence on the magnitude of Th1 and CD8 responses differs from a prior study showing a modest effect of IFN-α on CD8 responses as assessed by CTL activity or proliferation in vitro (10). This discrepancy may reflect differences in the route of immunization, Ag used in the rAd5 vector, the background of the IFN-αβR−/− mice, or the assays used to detect CD8 T cell responses.

In terms of TLR signaling, prior studies reported a ∼2-fold decrease in CD8+ peptide-specific IFN-γ ELISPOTs in MyD88−/− mice using a high dose of rAd5 (1.5 × 1011 PFU) given i.v. (8). Moreover, we also observed a ∼2-fold decrease in both Th1 and CD8 T cells responses in MyD88−/− mice in response to both eGFP and SIV-Gag Ags. Taken together, the data presented in this paper show that after a high dose of rAd5 given s.c., MyD88 plays a contributing role in Th1 and CD8 immunity. As Ag dose may also contribute to the magnitude and quality of the T cell response at high dose (30, 41), it is possible that TLR signaling pathways will have a more demonstrative role in T cell immunity at lower doses of rAd5, because Ag would be limiting.

The inflammasome is the other major innate signaling pathway that could be involved in T cell immunity following rAd5 immunization. In this regard, cytoplasmic rAd5 DNA has been shown to induce IL-1 and IL-6, but these cytokines were reduced in splenocytes from NLRP3−/− mice following i.p. administration of rAd5 (31). A recent study, however, reported that production of IL-1α from murine macrophages in the marginal zone of the spleen was independent of the inflammasome components ASC, caspase-1, and NLRP3 (4). Neither of these studies assessed the effect of the inflammasome signaling proteins on the adaptive immune response in vivo. The findings reported in this paper show that T cell responses are not influenced by the absence of NLRP3, ASC, or IL-1α signaling, at least with the dose and route of rAd5 used. Because T cell responses are NLR independent and only partially dependent on TLR signaling, it highlights a substantial redundancy in the innate immune requirements for generating T cell immunity by rAd5 immunization.

A final yet critical issue for using rAd5 vectors in humans is the high seroprevalence of anti-rAd5 immunity, especially within the populations throughout the world requiring T cell-based vaccines. Indeed pre-existing immunity from natural adenoviral infection or experimentally induced immunity from vaccination would engender less optimal primary responses and limit repetitive use of rAd5 to maintain or boost T cell immunity (1, 2, 24). The mechanisms by which pre-existing immunity would limit adaptative immunity include preventing transfection of cells in vivo by Ab or killing of transfected cells. In this regard, transfer of serum from animals previously exposed to rAd5 into naïve animals limited expression of transfected cells (42), whereas transfer of CD8 T cells limited immunity (24). In this study, we show that serum from mice immunized with rAd5 is sufficient to substantially limit expression of eGFP in the DCs of mice (Fig. 4, Supplemental Fig. 5). The striking decrease in eGFP expression observed in CD11c+ DCs within 24 h following s.c. immunization in draining LNs provides the first evidence for how pre-existing neutralizing Ab limits uptake of rAd5 in CD11c+ DCs in vivo. Pre-existing immunity caused a ∼50% decrease in the frequency of Th1 responses with no detectable CD8 T cell responses in response to rAd5 immunization. These data suggest there may be a different threshold or requirement for the number and type of CD11c+ DCs required to induce Th1 and CD8 T cell responses by rAd5 in the setting of pre-existing immunity. Alternatively, it is possible that Th1 responses are induced by non-DCs depending on the route of immunization and site of priming. Hence, following i.m. immunization, muscle cells transfected by rAd5 could express the Ag encoded by the vector, and Ag could be taken up by other APCs that have migrated to the muscle because of the local inflammation caused by the injection. A final possibility is that prior Ad5 immunity induces a population of CD4+CD25+ regulatory T cells that suppresses CD8 T cell responses (43). The overall clinical implications of these findings are that pre-existing immunity would have a greater impact on the generation of CD8 T cell responses than on CD4 T cell responses to a specific gene insert incorporated into the rAd5 vector. As Ad vectors may be especially useful for infections in which CD8 T cells have a role in protection such as HIV, malaria, and tuberculosis, it will be critical to evaluate whether prior Ad immunity would exclude such vectors entirely. There are two major variables that may impact the degree that pre-existing immunity would have on generating CD8 T cell responses with rAd5 and possibly other adenoviral vectors. First, a low level of Ab would allow a sufficient number of DCs to be transfected to induce a response. Second, it is possible that priming with another vaccine platform (e.g., DNA or heterologous viral vector) that induces a CD8 T cell response could allow boosting with rAd in the setting of pre-existing immunity based on a lower threshold of Ag presentation required to expand a memory CD8 T cell compared with priming naïve T cells.

In conclusion, the data presented in this paper show that CD11c+ DCs have a defined role in priming CD8 T cell responses following s.c. or i.m. rAd5 immunization with striking redundancy in the requirement of TLR and NLR signaling. The finding that pre-existing immunity to rAd5 seems to have a preferential effect on limiting CD8 but not CD4 responses suggests differing mechanisms by which these responses are induced. Because of the potency of rAd5 for eliciting CD8 immunity, these data should provide a useful benchmark to compare the mechanisms by which other serotypes are eliciting responses.

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Disclosures
J.G.D.G is an employee of GenVec, Inc. All other authors have no financial conflicts of interest.

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