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B Lymphocytes Participate in Cross-Presentation of Antigen following Gene Gun Vaccination

Huiming Hon,* Alp Oran,* Thomas Brocker,† and Joshy Jacob2*

Although endocytosed proteins are commonly presented via the class II MHC pathway to stimulate CD4\(^+\) T cells, professional APCs can also cross-present Ags, whereby these exogenous peptides can be complexed with class I MHC for cross-priming of CD8\(^+\) T cells. Whereas the ability of dendritic cells (DCs) to cross-present Ags is well documented, it is not known whether other APCs may also play a role, or what is the relative contribution of cross-priming to the induction of acquired immunity after DNA immunization. In this study, we compared immune responses generated after gene gun vaccination of mice with DNA vaccine plasmids driven by the conventional CMV promoter, the DC-specific CD11c promoter, or the keratinocyte-specific K14 promoter. The CD11c promoter achieved equivalent expression in CD11c immunization. In this study, we compared immune responses generated after gene gun vaccination of mice with DNA vaccines.

responses are driven by direct or by cross-presentation. The conventional CMV promoter is ubiquitously active, and results in dermal and epidermal expression of the plasmid-encoded transgene (25). By contrast, tissue-specific promoters can target Ag production to a distinct cell type resident at the site of immunization. To limit Ag expression to epidermal Langerhans cells and dermal DCs resident in the skin, we used DNA vaccines driven by the promoter of the β2 integrin CD11c, whose activity has been previously described to be limited to DCs in spleen and thymus, including these two skin DC populations (26, 27). The 5.3-kb 5′ untranslated region (UTR) portion of the CD11c gene (including the promoter and enhancer region) was cloned upstream of the cDNA of an Ag of interest. To target non-APCs, we cloned the same Ag cDNA downstream of the K14 promoter, whose activity in the skin is restricted to keratinocytes in the basal epidermis (21, 25). Ag-specific immune responses after gene gun vaccination with K14 promoter-driven plasmids would be solely due to APCs presenting peptides derived from endocytosis of the directly transfected keratinocytes.

In this study, we gene gun immunized cohorts of mice with DNA vaccine constructs driven by either the CMV, CMV, or K14 promoter, and evaluated the Ag-presentation capacity of the professional APCs in the dLN, and the endogenous Ag-specific T and B cell responses. We evaluated humoral responses with the mouse influenza hemagglutinin (HA) model, and measured T cell responses to full-length OVA. Despite demonstrating equivalent strength and duration of protein expression in DCs as a conventional CMV CMV promoter-driven vector, CD11c promoter-driven plasmids elicited poor T and B cell immunity in vivo. Furthermore, cell-sorting experiments and parallel studies in B cell-deficient (μMT) mice revealed the critical role of B cells in direct and cross-presentation for the generation of both CD4+ and CD8+ T cell responses after gene gun vaccination.

Materials and Methods

Mice

C57BL/6 and BALB/c mice were obtained from Charles River Laboratories, and OT-1, OT-2, and the B cell-deficient μMT mice from The Jackson Laboratory. Age- and sex-matched mice used for experiments were between 4 and 10 wk of age, and all animals were maintained under standard germfree housing conditions at the Emory Vaccine Center vivarium with the approval of the Institutional Animal Care and Use Committee.

Immunizations

Cohorts of mice were gene gun immunized, as described previously (18). Immunizations were performed bilaterally on nonoverlapping sites on shaved abdominal skin. To boost endogenous T cell responses, a second round of immunizations was performed after 7 days, as noted in text. A hand-held Accell gene delivery system (Auragen) or a Helios gene gun (Bio-Rad) was used to immunize mice with doses of 0.5 μg of plasmid DNA coated on 1 mg of gold beads (DeGussa-Huls) at a helium pressure of 400 psi.

DNA vaccine plasmids

DNA vaccine plasmids were constructed using routine molecular biology techniques. We used the pG1 expression vector, which contains the CMV immediate early promoter and intron A, as well as the bovine growth hormone polyadenylation sequence, a kind gift from H. Robinson (Emory University, Atlanta, GA) (28). Model Ags β-galactosidase (β-gal) (pCMV-β-gal), full-length OVA cDNA (pCMV-OVA), and influenza transmembrane HA cDNA (pCMV-HA), and influenza transmembrane HA cDNA (pCMV-HA) were blunt-end ligated downstream of the CMV promoter and upstream of the bovine growth hormone polyadenylation. The analogous CD11c promoter-driven plasmids and K14 promoter-driven plasmids were similarly constructed. The CD11c promoter consists of the 5.3-kb 5′ UTR of the murine CD11c gene with rabbit β-globin cDNA expression cassette on a pBluescript background (29, 30). The K14 promoter was kindly provided by I. Williams (Emory University), and consists of the 2-kb K14 5′ promoter and enhancer with a hGX polyadeny-

DC and B cell enrichment

Bilateral superficial inguinal lymph nodes (LN) were removed and digested with 1 mg/ml collagenase D (Roche) for 45 min at 37°C. Cells were then passed through a nylon mesh filter and incubated for 30 min with 24.52 Fc block (kind gift from R. Mittler, Emory University). Magnetic enrichment of CD11c+ cells was performed by the use of anti-CD11c (N418)-coupled magnetic beads (Miltenyi Biotec). The negative fraction was incubated with FITC-conjugated anti-CD19 (eBioscience), and then enriched for CD19+ cells using anti-FITC microbeads (Miltenyi Biotec).

Flow cytometry

For flow cytometric analysis of β-gal expression, LN cells were hypotonically loaded with 1 mM fluorescein di-β-n-galactopyranoside (FDG) ( Molecular Probes) in dH2O for 2 min at 37°C, as previously described (17, 32, 33). Aliquots of FDG-loaded cells were stained with fluorochrome-conju-
gated Abs (anti-CD11c allophycocyanin, anti-CD40 PE, anti-CD86 biotin, anti-IA-AF6–PE; BD Biosciences). If needed, a secondary streptavidin PerCP (BD Biosciences) Ab staining was performed. Cells were then directly run through a FACSCalibur (BD Biosciences), and data were analyzed with Flowjo software (Tree Star). We sorted cells with a FACSVantage (BD Biosciences), from C57BL/6 mice immunized 5 days earlier with pCMV-OVA, pCD11c-OVA, pK14-OVA, or an empty vector control. The CD11c+ , F4/80+, and CD19+ populations were sorted to >94% purity.

T cell proliferation

T cells were magnetically purified from spleens of OVA-expressing OT-1 or OT-2 TCR transgenic mice by autoMACS (Miltenyi Biotec). Stimulator cell populations isolated from dLNs of gene gun-immunized mice were irradiated with 5000 rad gamma radiation and plated with responder T cells in a 96-well microtiter plate. Irradiated accessory feeder cells at a 10-fold higher concentration were also added to each well. As controls, responder cells were cultured with OT-1 (OVA257–264) and OT-II (OVA233–241) peptides. After a 48-h culture, cells were pulsed with 1 μCi of [3H]thymidine. Subsequent to an overnight incubation, cells were harvested and assayed for incorporation of [3H] thymidine in a Microbeta plate reader (PerkinElmer Wallac).

ELISPOT assay

Seven days after primary or secondary vaccination with full-length OVA-expressing plasmids, the draining superficial LN were removed, made into single cell suspensions, and tested for Ag-induced IFN-γ secretion in a standard cytokine ELISPOT assay. Briefly, cells were stimulated in vitro in media with OT-I (OVA257–264; H-2 Kd), OT-II (OVA233–241; H-2 Kd), or an irrelevant (lymphocytic choriomeningitis virus gp33–41; H-2 Kd) peptide, along with 2 × 105 irradiated splenocytes and 2 μg/ml anti-CD28 and anti-CD49d (RB6-8C5 and R1–2, respectively; BD Biosciences), in polyvi-

nylidyne difluoride multiscreen 96-well plates (C.T.L.) coated with 1 μg/ml anti-mouse IFN-γ Ab (R4-6A2; BD Biosciences). We detected cyto-
kine by overnight incubation with 0.5 μg/ml biotinylated rat anti-mouse IFN-γ Ab (BD Biosciences), followed by binding to an avidin D-HRP conjugate (eBioscience). Spots were developed with a stable diaminoben-
zydine solution (Stable DAB; Research Genetics), and spot-forming units (SFU) were counted using a C.T.L. automated immunoscope (C.T.L.) and normalized for 106 dLN cells.

Serum ELISA

At days 15 and 30 after gene gun immunization, ~50 μl of blood was obtained from mice via retro-orbital bleeds, and serum was subsequently assayed for production of Ag-specific Abs on ELISA plates coated with sacrose gradient-purified A/PR/8/34 (H1N1) influenza virus, as described previously (34). Quantitation of Ab titers was performed by comparison with a standard curve using goat anti-mouse IgG (BD Biosciences), and data were analyzed with Microplate Reader software (Bio-Rad).

Influenza challenge

Forty days following DNA vaccination with constructs expressing influenza Enza HA, animals were challenged with the mouse-adapted influenza virus A/PR/8/34 (H1N1) (kind gift from H. Robinson, Emory University). All mice were housed in isolator units. Intranasal fluid containing influenza virus was serially diluted in sterile PBS with 0.2% BSA (Sigma-Aldrich), and a 50-μl volume containing 3× LD50 (0.03 hemagglutinating units) viral dose was given intranasally to anaesthetized mice. Intranasal inoculation of 3× LD50 influenza virus leads to...
Results

DNA vaccines can be engineered with different promoters for cell type-restricted Ag expression

DNA vaccines have conventionally used the human CMV immediate early promoter and enhancer, due to its ability to generate strong gene expression in a wide variety of cell types (35, 36). Independent of the route of delivery, CMV promoter-driven plasmids are expressed in all cells at the site of immunization. In order for Ag-specific T and B cell activity to be elicited, the expressed protein needs to be processed and presented on MHC Ag by a professional APC that can then stimulate naïve Ag-specific T cells resident in lymphoid tissues. This professional APC could have been directly transfected with the CMV promoter-driven plasmid at the site of immunization, leading to translation of the DNA vaccine-encoded protein and subsequent processing of Ag. Alternatively, the APC could have endocytosed another directly transfected cell or cellular material, shutting its Ag to both the class I (via cross-presentation) and class II MHC pathways. Thus, to determine the role of cross-presentation in the generation of Ag-specific immunity after gene gun vaccination, we engineered a series of DNA vaccine plasmids with the same Ag, but with different expression profiles: vaccines that would induce Ag expression in all cell types (CMV promoter), in DCs alone (CD11c promoter), or in keratinocytes alone (K14 promoter) (Fig. 1). We cloned antigenic cDNA (e.g., full-length OVA or influenza HA) between each promoter and a polyadenylation sequence, and used these plasmids as DNA vaccines to immunize mice.

Unlike the ubiquitously active CMV promoter, the 5.3-kb 5′ UTR of the murine CD11c gene, which encompasses the putative promoter and enhancer regions, would restrict Ag expression exclusively to CD11c+ DCs resident in the skin at the site of gene gun immunization, including Langerhans cells and dermal DCs. We and others have previously shown that the K14 promoter is specific to basal epidermal keratinocytes in the skin, and is not active in DCs (17, 25, 37). Furthermore, the frequency of other cells, such as T and B lymphocytes, in the skin is negligible, making it unlikely that effects shown are due to direct transfection of these cell types (data not shown). As keratinocytes do not express appreciable levels of class II MHC or T cell costimulatory molecules, and do not migrate to draining lymphoid tissues, immune responses generated from vaccination with K14 promoter-driven constructs would thus represent cross-priming of T cells by DCs or other APC.

The CD11c promoter induced equivalent protein expression over time in DCs as compared with a conventional CMV promoter-driven DNA vaccine plasmid

To measure the strength of the CD11c promoter vs the CMV promoter in vivo, we immunized C57BL/6 mice with pCMV-lacZ, pCD11c-lacZ, or an empty vector control, and quantified the frequency and absolute number of β-gal+ DCs in the dLN by flow cytometric analysis at various time points after gene gun vaccination. At 2.5 and 6.5 days after immunization, there was an equivalent frequency of CD11c+ β-gal+ cells in the dLN of mice immunized with pCMV-lacZ (9.1 ± 0.5% and 5.5 ± 0.3%, respectively) or with pCD11c-lacZ (8.8 ± 0.1% and 5.9 ± 0.2%) (Fig. 2a). Both groups of mice also demonstrated no statistical difference in the absolute number (6093 ± 519 vs 7077 ± 1217 at day 2.5 (p = 0.45), and 2135 ± 321 vs 1996 ± 314 at day 6.5 (p = 0.92), for pCMV-lacZ and pCD11c-lacZ, respectively) or geometric mean fluorescence intensities (GMFI) of β-gal expression at each time point (86.23 ± 2.1 vs 86.9 ± 6 at day 2.5 (p = 0.91), and 83.3 ± 0.1 vs 84.9 ± 2.5 at day 6.5 (p = 0.58) for pCMV-lacZ and pCD11c-lacZ, correspondingly) (Fig. 2, b and c).

There was no β-gal detected in the CD11c− population in any of the cohorts (data not shown). These studies indicate that within the time period analyzed, the strength of the CD11c promoter was equivalent to that of the conventional CMV promoter in DCs that emigrated from skin to the dLN.

Keratinocyte-restricted, but not DC-restricted Ag expression induced protective humoral immune responses

To determine whether humoral responses would be dependent upon cross-presentation of Ag, we used the mouse influenza virus infection model. Immunization of mice with DNA vaccines encoding influenza HA induces high titers of long-lasting, virus-specific Abs (38). Briefly, we gene gun immunized cohorts of BALB/c mice with DNA vaccines expressing transmembrane HA derived from an H1 influenza virus (pCMV-HA, pCD11c-HA, or pK14-HA), or an empty vector control. Anti-influenza Ab titers were determined by a standard ELISA on serum samples collected at days 15 and 30 after immunization. Vaccination of mice with pCD11c-HA did not produce anti-influenza IgG Abs, similar to that seen after immunization with an empty vector control DNA (Fig. 3a). In contrast, the groups of mice immunized with pCMV-HA or with pK14-HA both produced large amounts of Ab by day 30 after vaccination (101.3 ± 17.4 and 144.1 ± 25.7 μg/ml, respectively).

To test whether the Ab titers generated would be protective, we challenged each group of mice with 3× LD50 of a mouse-adapted strain of influenza virus (A/PR/8/34) at 40 days after immunization, and monitored for weight loss and survival over the subsequent 10 days. As would be predicted from the serum anti-influenza Ab levels, both the pCD11c-HA- and the control DNA-immunized groups quickly succumbed to infection, losing weight precipitously after day 4 postchallenge until all were dead by day 9 (Fig. 3, b and c). By contrast, the pCMV-HA and pK14-HA groups of mice both exhibited an initial minimal weight loss (<5% decrease from baseline weight), and all the mice in these two groups recovered and survived the virus challenge. These studies suggested that despite the presence of Ag-bearing DCs in dLNs (Fig. 2), B cell responses were not generated after immunization with DC-restricted DNA vaccines. Interestingly, immunization

FIGURE 1. Schematic of DNA vaccines used in these studies. a, The CMV promoter-driven DNA vaccines in this study use the ubiquitously active 1.8-kb CMV immediate early promoter and intron A to drive expression of the downstream Ag. The polyadenylation (pA) sequence was derived from the bovine growth hormone gene. b, In the DC-restricted DNA vaccines, the Ags were cloned between the 5.3-kb 5′ UTR of the murine CD11c gene and a rabbit β-globin pA. c, The keratinocyte-restricted DNA vaccines contain the 2-kb K14 5′ promoter and enhancer.
with keratinocyte-restricted vaccines did elicit protective humoral immunity to a similar level seen with the conventional CMV promoter-driven vaccine.

A DC-restricted DNA vaccine failed to elicit endogenous T cell responses

To determine whether T cell responses would be similarly deficient as B cell responses after DC-restricted vaccine immunizations, we gene gun vaccinated cohorts of C57BL/6 mice with constructs expressing full-length OVA, and analyzed the endogenous Ag-specific CD4⁺ and CD8⁺ T cell responses by quantifying the OVA-specific IFN-γ production by the T cells isolated from the dLN of immunized mice.

At 5 days after immunization, cells were stimulated with OVA or irrelevant peptides for 36 h, and we detected cytokine secretion with an ELISPOT assay. The bulk dLN cells from pCD11c-OVA-immunized mice did not produce measurable IFN-γ in response to either CD8 (OT-I, OVA257–264; H-2 Kb) or CD4 (OT-II, OVA323–339; I-Ab) immunodominant peptides (Fig. 4). In contrast, cells isolated from pCMV-OVA-immunized mice demonstrated a significant level of Ag-specific cytokine secretion, ~1.5–2 times more than was produced by cells from pK14-OVA-immunized mice, for both the CD8 (80 ± 16 vs 55.5 ± 4 SFU/10⁶ cells, respectively) and the CD4 (48 ± 16 vs 23 ± 6 SFU/10⁶ cells, respectively) T cell epitopes. Thus, not only did mice that received DC-restricted vaccines fail to induce humoral responses, they only mounted weak endogenous T cell responses against the immunogen. By contrast, effector T cells from pK14-OVA-immunized mice exhibited Ag-specific IFN-γ secretion for both CD4 and CD8 T cell epitopes.

FIGURE 2. Vaccination of C57BL/6 mice with plasmids encoding lacZ driven either by the CMV promoter or the CD11c promoter led to equivalent expression of β-gal in CD11c⁺ cells isolated from dLN. We immunized groups of C57BL/6 mice with pCMV-lacZ, pCD11c-lacZ, or an empty vector control. Single cell suspensions from superficial inguinal LNs were prepared 2.5 or 6.5 days after vaccination, hypotonically loaded with FDG, and stained with anti-CD11c allophycocyanin. Cells were then analyzed by flow cytometry for FDG and CD11c expression (a). Plots shown were gated on the CD11c⁺ live cell population, and are representative of three separate experiments, with five mice per group. There was no statistical difference in the absolute number of CD11c⁺β-gal⁺ cells per LN at 2.5 days (p = 0.45) and 6.5 days postvaccination (p = 0.92) (b), nor in the expression of β-gal per cell, as measured by the geometric mean of FDG fluorescence intensity at 2.5 days (p = 0.91) and 6.5 days (p = 0.58) (c) in mice vaccinated with pCMV-lacZ or pCD11c-lacZ.
Diminished immune responses after immunization with DC-restricted plasmids were not due to a defect in the maturation status of the transfected DCs

Although DC-restricted DNA vaccines resulted in an equivalent marking of directly transfected DCs, they were less able than ubiquitously active or keratinocyte-specific DNA vaccines to generate T and B cell immunity in vivo after gene gun immunization. To rule out a defect in the functional capacity of the DCs transfected by the CD11c promoter-driven vaccines, we analyzed the maturation status of the transfected β-gal⁺ DCs in dLN of mice immunized 2.5 days earlier with pCMV-lacZ, pCD11c-lacZ, or an empty vector control. In the process of migrating from the periphery to lymphoid tissues, DCs undergo a maturation process that transforms them from immature cells specialized in the uptake of Ag, to mature professional APCs that can stimulate T cells. This development includes the up-regulation of class II MHC (I-Ab) and T cell costimulatory molecules upon the DC cell surface. β-gal⁺ DCs marked by the pCMV-lacZ or by the pCD11c-lacZ plasmid both exhibited a similar maturation status, as seen in the increased expression of I-Aⁿ, CD40, and CD86 (B7.2) (Table I). Both experimental groups mounted a ∼10-fold increase in I-Aⁿ GMFI, 2-fold higher CD40 GMFI, and 6-fold higher CD86 GMFI at 2.5 days after DNA vaccination. Thus, despite the lack of B and T cell responses generated after DC-restricted vaccination, the use of the CD11c promoter in DNA vaccines achieved an equivalent level and kinetics of Ag expression (Fig. 2) in migrant DCs as the conventional CMV promoter, and gene gun immunization induced a comparable maturation of DCs.

Direct ex vivo capacity of DCs to stimulate Ag-specific T cell proliferation was not altered by use of the CD11c promoter in DNA vaccines

As no Ag-specific immune responses could be detected after DC-restricted DNA vaccination despite the presence of mature, Ag-bearing DCs in the dLN, we assessed the capacity of CD11c⁺ cells from dLN of immunized mice to induce T cell proliferation directly ex vivo, without addition of exogenous peptide. Briefly, we immunized groups of C57BL/6 mice with pCMV-OVA, pCD11c-OVA, pK14-OVA, or an empty vector control. At 5 days after vaccination, CD11c⁺ cells were magnetically enriched from the draining superficial LN, pooled, and used as stimulator cells to induce proliferation of responder naive T cells in a direct ex vivo [³H]thymidine incorporation assay. Stimulator cells were plated at a 1:1 ratio with responder T cells.

Direct ex vivo proliferation assays revealed no difference (p > 0.1) in the ability of CD11c⁺ cells, isolated after immunization with pCMV-OVA, pCD11c-OVA, or pK14-OVA, to specifically stimulate proliferation of naive OT-II OVA-specific CD4⁺ T cells isolated from the spleen of a TCR transgenic mouse (Fig. 5). Responder cells pulsed with cognate (OT-II) or irrelevant (OT-I) peptides served as positive and negative controls, respectively, for the assay (data not shown). In addition, CD11c⁺ cells isolated from mice immunized with a control empty DNA vector were not able to stimulate T cell proliferation.

As the K14 promoter is only active in keratinocytes at the site of vaccination, the ability of CD11c⁺ cells from pK14-OVA-immunized animals to induce naive T cell proliferation indicated that in addition to presenting Ag after direct transfection with the DNA vaccine, DCs also endocytosed and presented keratinocyte-derived peptide.
Ag to prime T cell responses after gene gun immunization. Moreover, the fact that the cells derived from pCD11c-OVA-immunized mice induced the same level of OT-II T cell proliferation directly ex vivo demonstrates that there were functional Ag-bearing DCs in the dLN after immunization with DC-restricted vaccines, although they were unable to elicit protective immune responses. Therefore, we postulated that another cell type may be serving as potent APCs to drive T cell responses after gene gun vaccination.

**Titration of bulk dLN proliferation assay showed presence of a CD11c<sup>+</sup> Ag-bearing APC**

To identify the APC involved in cross-priming T cells in the dLN, we repeated the [3H]thymidine incorporation assay using titrated amounts of irradiated bulk dLN cells as stimulators for naive OT-II TCR transgenic cell proliferation. We titrated the stimulator cells to evaluate the total number of Ag-bearing cells present in the dLN. As before, at 5 days after immunization with pCMV-OVA, pCD11c-OVA, pK14-OVA, or an empty vector control, we plated isolated irradiated bulk dLN cells at various ratios to stimulate proliferation, without addition of exogenous peptide, to stimulate proliferation of naive OT-II T cells at various responder:stimulator ratios in a [3H]thymidine incorporation assay.

As shown previously in Figs. 5 and 7a, the CD11c<sup>+</sup> DCs isolated from the dLN of mice immunized with OVA-expressing vaccines exhibited comparable levels of T cell stimulation, irrespective of cell type protein expression (Fig. 7a). No T cell proliferation was induced by coculture with F4/80<sup>+</sup>-activated macrophages from any of the four cohorts. Interestingly, the CD19<sup>+</sup> B cell populations from both the pCMV-OVA- and the pK14-OVA-immunized cohorts were able to stimulate Ag-specific T cells, unlike B cells from the pCD11c-OVA-immunized cohort.

Next, we titrated the numbers of DCs and B cells, and evaluated their ability to stimulate Ag-specific T cells. Briefly, we immunized mice with pCMV-OVA, pCD11c-OVA, pK14-OVA, or an empty vector control, and isolated and pooled dLNs 5 days later. The dLN suspensions were then magnetically purified into DCs (CD11c<sup>+</sup>) and B cells (CD19<sup>+</sup>). Equal cell numbers from each of these two cell populations were used as APCs directly ex vivo, without addition of exogenous peptide, to stimulate proliferation of naive OT-I T cells at various responder:stimulator ratios in a [3H]thymidine incorporation assay.

As shown previously in Figs. 5 and 7a, the CD11c<sup>+</sup> DCs isolated from the dLN of mice immunized with pCMV-OVA, pCD11c-OVA, or pK14-OVA stimulated comparable amounts of T cell proliferation at a 1:1 responder:stimulator ratio. However, at a 2-fold lower concentration of CD11c<sup>+</sup> DCs, there was a significantly higher proliferation ($p = 0.017$) induced by the APCs isolated after pCMV-OVA, indicating the presence of more Ag-bearing DCs (Fig. 7b). There was no difference ($p = 0.95$) between the

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**FIGURE 5.** Intact exogenous T cell priming by isolated dLN CD11c<sup>+</sup> cells after pCMV-OVA, pCD11c-OVA, or pK14-OVA immunization. Co-horts of C57BL/6 mice ($n = 5$) were gene immunized with pCMV-OVA, pCD11c-OVA, pK14-OVA, or an empty vector control, dLN were obtained for each of the cohorts immunized with an OVA-expressing vaccine. CD11c<sup>+</sup> DCs from dLN by magnetic sorting, and used directly, without addition of exogenous peptide, to stimulate proliferation of naive CD4<sup>+</sup> T cells from an OT-II OVA TCR transgenic mouse in a [3H]thymidine incorporation assay. Stimulator cells pulsed with cognate OT-II peptide or irrelevant OT-I peptide served as positive and negative controls, respectively. Values represent mean radioactivity incorporation ± SD of triplicate wells. Comparable data were achieved from three separate experiments.

**FIGURE 6.** CD11c<sup>+</sup> population of cells can induce Ag-specific T cell proliferation after gene gun vaccination. At 5 days after immunization of C57BL/6 mice ($n = 5$) with pCMV-OVA, pCD11c-OVA, pK14-OVA, or an empty vector control, we plated isolated irradiated bulk dLN cells at various ratios to stimulate proliferation, without addition of exogenous peptide, of naive OT-II T cells in a standard [3H]thymidine incorporation assay. The amount of radioactivity incorporation by responder T cells was measured after 72-h culture. In this assay, responder T cells were pulsed with irrelevant (OT-I) or cognate (OT-II) peptide as negative and positive controls, with radioactivity uptake levels of 24.1 ± 1.7 and 0.21 ± 0.04 × 10<sup>3</sup> cpm, respectively. Values represent mean [3H]thymidine incorporation ± SD of triplicate wells; *, indicates $p < 0.05$; **, indicates $p < 0.001$. Comparable data were achieved from two separate experiments.
CD11c+ DCs from the cohorts immunized with pCD11c-OVA or pK14-OVA, suggesting that the level of Ag is equivalent in these two groups of DCs. As shown previously, the CD11c+ B cell populations from both the pCMV-OVA- and the pK14-OVA-immunized cohorts induced Ag-specific T cell proliferation, unlike B cells from the pCD11c-OVA-immunized cohort. There was no statistical difference at all responder:stimulator cell ratios between the amount of T cell proliferation induced by the CD19+ B cell populations isolated after immunization with an ubiquitously active or a keratinocyte-restricted vaccine (Fig. 7c), suggesting a model whereby CD19+ B cells acquire Ag from the keratinocytes that have been transfected with DNA at the site of gene gun vaccination to stimulate T cell responses in the dLNs. Furthermore, the levels of [3H]thymidine incorporation by the OT-I T cells induced by these B cells were only 3-fold lower than the CD11c+ populations isolated from the same dLNs. The fact that the CD19+ B cells were able to induce CD8+ T cell proliferation indicated that these cells were capable of cross-presentation of the endocytosed Ag derived from keratinocytes, albeit at a lower efficiency, on a per cell basis, than DCs.

The endogenous T cell responses are diminished in μMT mice following gene gun vaccination with pK14-OVA

To verify that B cells participate in the generation of Ag-specific immunity after gene gun vaccination, we examined endogenous T cell responses generated in B cell-deficient μMT mice. The μMT mouse strain is deficient for IgM transmembrane tail exons, thus blocking B cell development at the pro-B stage (39, 40). We immunized cohorts of μMT mice with pCMV-OVA, pCD11c-OVA, pK14-OVA, or an empty vector control. At 5 days following the second immunization, we isolated dLNs and evaluated for IFN-γ secretion in response to CD8 (OT-I, OVA257–264) (a) and CD4 (OT-II, OVA323–339) (b) epitopes in a cytokine ELISPOT assay. Data are represented as mean SFU/10⁶ dLN cells ± SEM of triplicate wells.

FIGURE 7. CD19+ cells participate in naive T cell priming after gene gun vaccination. We gene gun vaccinated groups of C57BL/6 mice (n = 5 per group per sorting strategy) with pCMV-OVA, pCD11c-OVA, pK14-OVA, or an empty vector control. At 5 days after immunization with these OVA-expressing vectors, dLN from each group of mice were isolated and sorted into CD11c+, F4/80+, and CD19+ populations (a), or magnetically purified into CD11c+ (b) and CD19+ (c) populations. We used titrated numbers of irradiated cells from each sorted population as APCs to stimulate proliferation directly ex vivo (without the addition of exogenous peptide) of an equal number of naive CD3+ splenocytes from an OT-I OVA TCR transgenic mouse. After 72 h of culture, proliferation was quantified as the mean [³H]thymidine incorporation ± SD of triplicate wells. Responder T cells pulsed with irrelevant (OT-II) or cognate (OT-I) peptide served as negative and positive controls, respectively (data not shown). * Indicates p < 0.05.
evaluated the endogenous T cell responses generated in the dLN to OVA by an IFN-γ ELISPOT assay.

T cells from the cohort of μMT mice immunized with pCMV-OVA showed high levels of IFN-γ secretion in response to both the CD8 (OT-I) (Fig. 8a) and CD4 (OT-II) (Fig. 8b) immunodominant peptides. This level of cytokine secretion was only slightly less than that shown by control C57BL/6 mice. Interestingly, very low levels of IFN-γ-secreting cells were detected in μMT mice immunized with pK14-OVA, suggesting that B cells are responsible for the endogenous T cell responses shown in C57BL/6 mice. Neither the μMT mice nor the C57BL/6 mice that were immunized with pCD11c-OVA were able to induce much cytokine production over background. Collectively, these data indicate that B cells participate in the cross-priming of T cell responses after gene gun vaccination.

**Discussion**

In recent years, DCs have been recognized as professional APC capable of priming naïve T cells. Due to their pivotal role in the generation of Ag-specific immune responses, the targeting of vaccines to DCs has been proposed as an approach to improving vaccine efficacy (41). However, our data suggest that despite the potential ability of DCs to drive T cell function, limiting Ag expression to a small number of DCs results in the failure to generate protective immunity after gene gun vaccination.

Although DNA vaccines driven by a DC-specific promoter, CD11c, showed comparable level and duration of expression in DCs as analogous vaccines using a conventional CMV promoter, subsequent studies found that DC-restricted Ag expression failed to induce endogenous T and B cell responses. T cells isolated from dLNs after immunization with pCD11c-OVA produced minimal IFN-γ upon peptide stimulation. Similarly, mice immunized with pCD11c-HA demonstrated scarce anti-influenza Ab responses, and all perished following a lethal virus challenge. However, the Ag-bearing DCs that appeared in the dLN after immunization with a DC-restricted vaccine were phenotypically mature, and functioned as APCs in an in vitro T cell proliferation assay, discouraging the notion that T cells were being tolerized in this model. Furthermore, the observation that K14 promoter-driven plasmids were more immunogenic than their CD11c promoter-driven counterparts suggests that a broad expression of DNA vaccine-encoded Ag is critical, and that cross-priming plays a vital role in the generation of immunity after gene gun immunization.

The inability of DC-restricted vaccines to induce immune responses is most likely a consequence of the limited production of Ag by the small numbers of directly transfected DCs at the site of immunization. Although we demonstrated that expression of a model Ag (β-gal) was equivalent in DCs that had migrated to dLN, the level of Ag expression after CMV promoter-driven vaccination is undoubtedly higher than after CD11c promoter-driven vaccination, due to Ag production by the directly transfected keratinocytes. After immunization of mice with DNA vaccines driven by the CMV or the K14 promoter, a high level of Ag expression occurs in the many keratinocytes of the basal epidermis directly transfected by the DNA-coated gold particles. As Ag-specific endogenous T and B cell responses were detected in the cohorts of animals immunized by K14 promoter-driven vaccines, the keratinocyte-derived Ag must have been taken up by professional APC that then stimulate naïve T cells in the secondary lymphoid tissues. The fact that CD11c DCs isolated from the dLNs of pK14-OVA-immunized mice induced a similar level of OVA-specific T cell proliferation in vitro as CD11c DCs from pCMV-OVA- or pCD11c-OVA-immunized mice clearly demonstrates that DCs do play a role in cross-priming T cell responses, presenting keratinocyte-derived Ag on both class I and class II MHC. However, the level of Ag expression after CD11c promoter-driven vaccination is insufficient to induce T cell responses. In addition, it is clear that B cells are also implicated in the cross-presentation of Ag. The CD19 B cell population from dLN of both pCMV-OVA- and pK14-OVA-immunized C57BL/6 mice induced a significant level of OVA-specific CD8+ T cell proliferation. Furthermore, only minimal endogenous T cell responses were detected in pK14-OVA-immunized B cell-deficient μMT mice, similar to that shown by pCD11c-OVA-immunized C57BL/6 or μMT mice. Collectively, our data indicate that both B cells and DCs mediate cross-presentation of Ag after gene gun vaccination.

These results are in line with a study conducted by Cho et al. (24), who compared T cell responses in mice after gene gun immunization with a pan-APC-targeted vaccine (plasmids driven by the CD11b promoter, which is active in macrophages and a subset of DCs) vs a non-APC-targeted vaccine (plasmids driven by the K14 promoter). They found that non-APC-specific gene expression was markedly better at inducing both T and B cell responses than the pan-APC-targeted expression. They concluded that cross-priming is indeed the predominant mechanism for generation of immunity after DNA vaccination.

Although the ability of DCs to cross-present Ag is well documented (1, 3, 10, 42), the role of B cells in cross-priming T cells remains unclear. B cells can internalize Ag via their surface Ig receptors, and drive priming of CD4+ T cells in vivo (12, 13). Many groups have shown that covalent linkage of OVA protein to the immunostimulatory CpG-oligodeoxynucleotide resulted in DNA receptor-mediated endocytosis and stimulation of CD4+ and CD8+ T cells by both DCs and B cells (43–46). The presentation of the CD8 T cell epitope by B cells was, however, less efficient than that seen by DCs (6). Nevertheless, adoptive transfer of CpG-OVA-loaded B cells into mice was able to induce Ag-specific CTL responses. This model may be analogous to that of gene gun vaccination, in which immunostimulation is thought to be due, in part, to CpG DNA from the bacteria-derived plasmids used to transfect cells.

The Ag-bearing cells produced after immunization of μMT mice with pCMV-OVA are both directly transfected DCs and cross-presenting DCs. The data suggest that the involvement by both directly transfected and cross-presenting DCs provides enough stimulation to endogenous T cells such that the absence of the B cell contribution in μMT mice is not substantial. This is supported by the fact that when the numbers of stimulator DCs are titrated against a constant number of responder OT-I CD8+ T cells, there is a significant difference in the amount of T cell proliferation induced at a 2:1 responder-stimulator ratio (Fig. 7b). However, in situations of low Ag load or low numbers of Ag-bearing DCs, B cells become important APCs in the generation of immune responses. This results in the loss of endogenous T cell responses after immunization of μMT mice with pK14-OVA.

Our results demonstrate that B cells, in addition to DCs, play a strong role in cross-primer T cell responses from endocytosed, keratinocyte-derived Ag, and that broad Ag expression is vital to the generation of T cell immunity after DNA vaccination. Previous studies have also attempted to limit Ag expression to DCs with cell type-specific promoters. Gene gun delivery of a plasmid driven by the dectin-2 promoter was able to target Ag expression to DCs (47). Use of a Langerhans-limited promoter, fascin, in DNA vaccines resulted in a marked absence of Ag-specific Ab production, as was seen in our studies (48). However, these studies showed Ag-specific IFN-γ secretion in an ELISPOT assay after immunization with DC-restricted plasmids, albeit at lower levels than was
achieved by a CMV promoter-driven vector, while we demonstrated an inability of CD11c promoter-driven vectors to mediate endogenous T cell priming. This may be due to differences in the strength of the dendin-2 and fascin promoters vs the CD11c promoter. Both the fascin and dendin-2 promoters have been shown to be highly active in the maturing Langerhans cell (48, 49). In contrast, the β2 integrin CD11c is most likely constitutively expressed at lower levels. Moreover, the schedule of immunizations used by the other studies may play a role in augmenting the T cell responses seen by increasing the amount of Ag available to DCs at the site of immunization.

Cross-priming of T cells is therefore a major mechanism by which immune responses are generated after DNA vaccination. This work adds to previous data showing that B cells are capable of cross-presentation of Ag by shutting endocytosed proteins into the class I MHC pathway (6). Furthermore, these Ag-bearing B cells have the ability to stimulate both naive CD4+ and CD8+ T cells in vivo. These results have direct implications for the rational design of vaccines, which should not be restricted to immunization of specific cell types or APC, but should instead be as broad as possible for the optimal generation of the initial clonal T cell burst that leads to protective immunity. It is thus of vital importance to develop means to enhance the level and duration of Ag expression in vaccines, such as by engineering DNA vaccines with strong promoters and regulatory elements (34).

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Disclosures
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