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Conventional Dendritic Cells Are Required for the Activation of Helper-Dependent CD8 T Cell Responses to a Model Antigen After Cutaneous Vaccination with Lentiviral Vectors

Hugh D. Goold^{*}, David Escors[†], Thomas J. Conlan^{*}, Ronjon Chakraverty^{*}, and Clare L. Bennett^{*}

^{*}Dendritic Cell Immunotherapy and Transplant Immunology Groups, Division of Cancer Studies, Department of Haematology, University College London, Royal Free Campus, London NW3 2PF, United Kingdom

[†]Division of Infection and Immunity, Department of Immunology, University College London, London W1T 4JF, United Kingdom

Abstract

Cutaneous vaccination with lentiviral vectors generates systemic CD8 T cell responses that have the potential to eradicate tumors for cancer immunotherapy. However, although s.c. immunization with <1 million lentiviral particles clearly primes cytotoxic T cells, vaccination with much higher doses has routinely been used to define the mechanisms of T cell activation by lentiviral vectors. In particular, experiments to test presentation of lentiviral Ags by dendritic cells (DC) require injection of high viral titers, which may result in aberrant transduction of different DC populations. We exploited inducible murine models of DC depletion to investigate which DC prime the lentiviral response after s.c. immunization with low doses of lentiviral particles. In this article, we demonstrate that conventional DC are required to present Ag to CD8 T cells in draining lymph nodes. Langerhans cells are not required to activate the effector response, and neither Langerhans cells nor plasmacytoid DC are sufficient to prime Ag-specific T cells. Immunization drives the generation of endogenous long-lived memory T cells that can be reactivated to kill Ag-specific targets in the absence of inflammatory challenge. Furthermore, lentiviral vaccination activates expansion of endogenous CD4 Th cells, which are required for the generation of effector CD8 T cells that produce IFN- γ and kill Ag-specific targets. Collectively, we demonstrate that after cutaneous immunization with lentiviral particles, CD4-licensed lymph node conventional DC present Ag to CD8 T cells, resulting in the generation of protective endogenous antitumor immunity that may be effective for cancer immunotherapy.

Third-generation lentiviral vectors have been developed as efficient gene-delivery tools for novel vaccination strategies. Improved safety measures and the lack of immunity to virus-derived proteins mean that lentiviral vectors are the most attractive viral vector system for clinical use (1). In particular, much research is focused on the potential of lentiviral-based immunization strategies in cancer immunotherapy. However, a thorough understanding of the mechanisms that result in T cell immunity after immunization with lentivirus is essential

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Address correspondence and reprint requests to Dr. Clare Bennett, University College London, Royal Free Campus, Rowland Hill Street, London NW3 2PF, United Kingdom. c.bennett@medsch.ucl.ac.uk.

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to devise novel strategies to overcome the dominant immunosuppressive environment in cancer patients.

Dendritic cells (DC) are essential to prime and orchestrate T cell immunity. One hypothesis to explain the potency of immunization with lentiviral vectors is that the efficient transduction, and therefore Ag loading, of DC in vivo leads to optimal priming of naive T cells (2). In addition, there is accumulating data on the activation of DC by lentivectors (3, 4). Subcutaneous vaccination with high numbers of lentiviral particles results in integration of the vector into the genome of DC purified from lymph nodes (LN) draining the injection site (2, 5), and GFP- and luciferase-transduced DC can be visualized in LN of immunized mice when titers of lentivirus significantly higher than those needed to activate T cells were used (4-7). In addition, DC isolated from mice vaccinated via cutaneous routes presented lentiviral Ag to T cells in vitro (2, 8). These experiments led to the assumption that DC are required to prime the immune response after lentiviral vaccination. However, although increasing the Ag load by enhancing lentiviral uptake by DC, or activating transduced DC, enhances the CD8 T cell response (7, 9, 10), restricting Ag expression to DC results in reduced immune responses compared with immunization with a lentivector encoding a ubiquitously expressed Ag (6). In addition, CD11c⁺ DC were not sufficient to prime expansion of activated T cells after immunization with a DNA vaccine (11). Therefore, it is not known whether DC are required to prime T cell responses to the encoded Ag in vivo after immunization.

The successful use of lentiviral vectors in cancer immunotherapy will depend on their ability to activate long-term memory responses, which are rapidly reactivated upon re-emergence of previously eradicated tumors. The priming of functional effector CD8⁺ T cells, as well as the expansion of a durable memory response, depends on the provision of CD4 T cell help, via the licensing of DC presenting MHC class I (MHC I) and MHC class II (MHC II) Ags from the infectious agent (12-14). Dullaers et al. (15) showed that the importance of T cell help for priming of the lentiviral response may depend on the method of lentiviral Ag delivery, whereas Esslinger et al. (5) demonstrated that CD4 T cells were required for maximal expansion of tetramer⁺ CD8 T cells after cutaneous immunization with a lentivector expressing a melanoma mini-gene. However, the function of the “helpless” CD8⁺ T cells was not investigated in that study, and the Ag specificity of the CD4 response was not discussed, given that a minimal CD8 T cell epitope sequence alone was encoded in the lentiviral vector (5). In addition, visualization of the CD4 response has depended on adoptive transfer of high numbers of TCR transgenic T cells (15, 16), and the activation of an Ag-specific endogenous CD4 T cell response in vivo has not been shown after immunization with lentiviral vectors.

Although s.c. immunization with <1 million lentiviral particles clearly primes cytotoxic T cell responses (2), vaccination with much higher doses has routinely been used to test presentation of lentiviral Ags by DC. For example, He et al. (2) used vaccination doses of 0.5–1 million transduction units to prime Ag-specific T cells, but they used 10 million transduction units to track presentation of lentiviral Ag by DC. Increased lentiviral doses will be more immunogenic, in particular as the result of DC activation with higher viral numbers (17), and these doses may not reflect the mechanisms of activation of the T cell response elicited by fewer lentiviral particles. In this study, we exploited murine models of DC depletion to determine the role of DC in priming lentiviral immunity. To our knowledge, we show for the first time that conventional DC (cDC) are required to present low concentrations of lentiviral Ag to CD8 T cells in draining LN and, therefore, prime a protective antitumor response. In addition, we found that immunization with lentivirus activates expansion of endogenous CD4⁺ T cells, which are critically required for the development of IFN- γ -producing cytotoxic CD8 T cells.

Materials and Methods

Mice

Animals were used under protocols approved by local institutional research committees and in accordance with U.K. Home Office guidelines. C57BL/6 (B6) and OT-I/Rag2^{-/-} mice were bred in-house. CD11c-DTR mice (on the B6 background) were bought from Jackson Laboratories. Langerin-DTR mice (on a B6 background) were kindly provided by A. Kissenpfennig and B. Malissen (18). Both lines were bred in our facility. cDC were depleted upon i.p. injection of 100 ng diphtheria toxin (DT; Sigma) in PBS, and Langerin⁺ DC were depleted using an increased dose of 500 ng DT [according to published protocols (19, 20)]. Depletion of cDC or Langerhans cells (LC) was confirmed in all experiments by staining of splenic and epidermal single-cell suspensions, respectively. MHC II-deficient mice were a kind gift of George Kassiotis (National Institute of Medical Research, London, U.K.) (21).

Generation of syngeneic CD11c-DTR chimeras

C57BL/6 mice were myeloablated by irradiation with two split doses of 5.5 Gy separated by 48 h. Following the second irradiation, the mice were injected with 5×10^6 bone marrow cells from CD11c-DTR mice via the tail vein. All mice were bled after 4 wk to verify reconstitution of the hematopoietic system and were used at 8 wk posttransplant.

Preparation of lentiviral particles and immunization of mice

The lentivectors pSIN-DUAL-empty-Ii:OVA (LV-Ii:OVA) or a GFP control were used for these experiments based on previously described transfer vectors (9). VSV-G pseudotyped lentivectors were produced as described (16, 22). Lentivectors were concentrated 100-fold by ultracentrifugation through a 20% sucrose cushion in PBS, resuspended in PBS containing 10% glycerol, and stored as aliquots at -80°C . The titers of all lentivectors were determined using a colorimetric reverse-transcriptase (RT) ELISA kit (Roche), as described previously (23). Lentivector stocks for vaccination were diluted in PBS and injected s.c. at the base of the tail at a dose of 14 ng RT activity per injection. Direct comparisons of the RT activity of lentivectors containing traceable markers, with flow cytometric analysis of transduced 293T cells, indicated that 14 ng RT activity was the equivalent to injection of $<10^6$ lentiviral particles. In some experiments, CD8⁺ OVA-specific T cells were purified from the spleens of OT-I mice by immunomagnetic separation with a Miltenyi CD8 T cell kit (Miltenyi Biotec). Titrated numbers of T cells were injected i.v. 1 d before immunization.

Tumor experiments

Mice were immunized with PBS or with 14 ng RT activity of LV-Ii:OVA s.c. Nine or forty-two days later, mice were challenged with 2×10^6 OVA-transfected EL4 (EG7) thymoma cells injected s.c. into the shaved flank. Tumor scores were calculated by measuring the width and height of the tumor at successive time points with a caliper. Mice were killed when tumor areas exceeded 150 mm².

In vivo cytotoxicity

B cells were isolated from splenocytes by immunomagnetic separation. Some cells were then incubated with 5 mM OVA_{257–264} peptide (SIINFEKL) for 30 min at 37°C and washed. Target cells were labeled with low-dose (0.5 μM) CFSE and mixed at a 1:1 ratio with non-Ag-pulsed control cells labeled with high-dose (5 μM) CFSE. A total of 10^7 cells was injected i.v. into recipients. Fifteen hours later, cells recovered from the LN and spleen were analyzed by flow cytometry for the presence of the two populations of CFSE-labeled cells, and specific lysis was calculated compared with the ratio of the injection mix or the ratio of injected cells in PBS-immunized mice, as previously described (24).

Flow cytometry

The following mAbs were used: anti-CD8-PE or anti-CD8-allophycocyanin (clone 53-6.7), anti-B220-PE-Cy5 (clone RA3-6B2), anti-V α 2-PE (clone B20.1), and anti-IFN- γ -allophycocyanin (XMG1.2) (from eBiosciences or BD-Pharmingen). Intracellular staining of cytokines was performed after overnight ex vivo stimulation of LN cells or splenocytes with 0.5–5 μ M MHC I OVA_{257–264} peptide. Four hours before harvesting, brefeldin A (Sigma) was added to the cells at a final concentration of 10 μ g/ml. Cells were fixed and permeabilized using the BD fix and perm kit (BD-Biosciences). Nonspecific IFN- γ production by cells restimulated in the absence of Ag was subtracted from all samples. Samples were acquired using FACSCalibur or FACScan flow cytometers (BD Bioscience) and analyzed using FlowJo software (Treestar).

In vivo Ag-presentation assay

At defined time points after immunization, mice were injected i.v. with $4\text{--}5 \times 10^6$ CFSE-labeled CD8⁺ OT-I cells. Sixty-five hours later, LN draining the site of injection were harvested, and LN cells were stained for flow cytometry. OT-I cells were identified by expression of CD8 and V α 2, and proliferation was analyzed by dilution of CFSE among these cells.

Staining with OVA-specific pentamer

Cells were washed and incubated with PE-conjugated H-2K^b-SIINFEKL pentamers in HBSS/2% FBS (ProImmune, U.K.) for 10 min at 37°C, washed, and incubated with anti-CD8-allophycocyanin for 15 min at 4°C.

ELISPOT assays

IFN- γ production by spleen and LN cells was measured 7 d after immunization. ELISPOT plates (Millipore) were coated with anti-IFN- γ Ab (clone R4-6A2; BD Biosciences) overnight at 4°C. Serial dilutions of total spleen or LN cells were plated in duplicate in RPMI 1640 supplemented with 5% heat-inactivated FBS, L-glutamine, penicillin/streptomycin, and 2-ME. Cells were plated without Ag or with 10 μ M MHC II-restricted OVA peptides (OVA_{265–280} [TEWTSSNVMEERKIKVY] and OVA_{323–339} [ISQAVHAAHAEINEAGR]) for 20 h. The assay was developed according to the manufacturer's instructions, and spots were counted using an AID ELiSpot reader and counter (Autoimmun Diagnostika, Germany).

Depletion of CD4 T cells

Mice were injected with 300 μ g anti-CD4 clone GK1.5 and PBS or a rat IgG2b isotype control on days –3, –1, and +7 of the experiment (Bio X cell), according to published protocols (25). The mice were immunized on day 0 and sacrificed 8 d later.

Statistical analysis

The two-tailed Mann–Whitney *U* test was used for statistical analyses. A *p* value ≤ 0.05 was regarded as significant.

Results

Cutaneous immunization with lentiviral vectors elicits potent systemic CD8⁺ T cell immunity

To test the potency of lentiviral vectors as vaccination vehicles, we immunized C57BL/6 mice with a single s.c. injection of 14 ng RT activity (23) of lentiviral particles; this was the

lowest dose of lentiviral vector that routinely activated T cell expansion in our hands. For these experiments, we used the transfer vector pSIN-DUAL expressing the model Ag OVA fused to the invariant chain, to enhance targeting of Ags to the MHC II pathway (LV-Ii:OVA) (9, 16) (Fig. 1A). As shown in Fig. 1B–D, a single s.c. injection of LV-Ii:OVA primed an endogenous IFN- γ -secreting OVA-specific CD8⁺ T cell response in draining LN 7 d after immunization. To investigate the development of effector cytotoxic function by CD8⁺ T cells, CFSE-labeled peptide-coated B cells were transferred into immunized recipients as T cell targets. Fig. 1E demonstrates the development of Ag-specific killing in the spleen and LN of immunized mice. This response peaked in the spleen at days 10–11 postimmunization and persisted for 3 wk postimmunization, as previously described (2) (data not shown). Therefore, our data demonstrated that a single s.c. injection with 14 ng RT activity of LV-Ii:OVA is sufficient to prime a systemic, endogenous CD8⁺ effector response in vivo.

Endogenous antilentiviral immunity is sufficient to protect against tumor challenge

To test whether the immune responses activated by vaccination with 14 ng RT activity of lentiviral particles were sufficient to protect mice against challenge with tumor cells, we initially injected 2×10^6 OVA-transfected EL4 thymoma cells (EG7) (26) into vaccinated mice 9 d after immunization with LV-Ii:OVA. Fig. 2 shows that EG7 cells grafted and expanded in PBS-immunized mice but not in those vaccinated previously with lentivirus. When the mice were challenged at a later time point, without boost, tumor growth was significantly delayed. Fig. 2C and 2D show tumor growth in PBS- or lentivector (LV)-immunized mice challenged with EG7 cells 6 wk later (median day of tumor appearance in PBS-versus LV-injected mice: 12.5 and 23.0; $p = 0.010$). These data demonstrated that a single vaccination with low doses of lentiviral particles is sufficient to induce durable T cell immunity against cutaneous tumor challenge.

Conventional DC are required to present Ag to OVA-specific CD8⁺ T cells after immunization with low doses of lentiviral vectors

Putative skin-derived DC from mice immunized with high doses of lentivectors were shown to present Ag to OVA-specific T cells in vitro, after cutaneous footpad injection (2). However, whether DC are required to activate the immunization response in vivo has not been demonstrated, particularly when mice are injected with reduced numbers of lentiviral particles. To address this question, we exploited the CD11c-DTR model, in which conventional CD11c^{high} DC express a high-affinity DT receptor and can be specifically ablated upon injection of DT (19). To deplete cDC for the duration of the experiment, syngeneic CD11c-DTR $\rightarrow$ C57BL/6 chimeras were generated, in which the CD11c-DTR transgene is restricted to the hematopoietic system, and that tolerate repeated injections of DT (27, 28). To measure presentation of lentiviral Ag to T cells, CFSE-labeled OVA-specific CD8⁺ OT-I cells were transferred into immunized mice, and proliferation in lymphoid organs was compared in mice that had or had not been depleted of cDC. At least 80% of cDC were routinely depleted from CD11c-DTR chimeras (Fig. 3A), which was sufficient to ablate Ag presentation to OVA-specific CD8 T cells in immunized mice (Fig. 3B). We observed that dividing OT-I cells accumulated in LN but not the spleen, suggesting that the dominant site of Ag presentation by cDC is in draining LN (Fig. 3C; as well as at later time points postimmunization [data not shown]). Therefore, these data demonstrated that depletion of cDC ablated presentation of Ag to, and proliferation of, OVA-specific T cells after injection of 14 ng RT activity of LV-Ii:OVA.

LC are not required for activation of the effector T cell response

In addition to dermal cDC, the skin contains two populations of Langerin⁺ DC: LC in the epidermis and Langerin⁺ CD103⁺ DC in the dermis (29). LC are not depleted from CD11c-

DTR mice due to low-level expression of CD11c in the epidermis (30) (C.L. Bennett, unpublished observations). Therefore, our cDC-depletion data suggested that LC are not sufficient to present lentiviral Ag to T cells after s.c. vaccination (Fig. 3). However, to determine whether Langerin⁺ CD11c⁺ dermal DC and/or epidermal LC play an undefined role in the activation of optimal cytotoxic T cell immunity in this model, we repeated our in vivo cytotoxicity experiments in mice depleted of Langerin⁺ cells. Thus, CFSE-labeled T cell targets were transferred into immunized Langerin-DTR mice that had been previously injected with DT to ensure depletion of LC and dermal Langerin⁺ DC (Fig. 4A) (31, 32). Our data showed that depletion of Langerin⁺ DC did not reduce activation OVA-specific cytotoxic T cells, indicating that Langerin⁺ DC are not required for activation of the CD8⁺ effector response in vivo (Fig. 4B).

Induction of endogenous CD8⁺ T cell memory responses by lentiviral vaccination

To test whether a single cutaneous vaccination with 14 ng RT activity of LV-Ii:OVA induced CD8⁺ T cell memory, we measured OVA-specific cytotoxicity in mice immunized 9 wk previously. Persistence of lentiviral Ag resulted in prolonged presentation of Ag after cutaneous vaccination, as well as activation of effector T cells for 3 wk postimmunization (2, 15). Therefore, to visualize activation of memory T cells in our model, we chose a time point that was 6 wk after the detectable presentation of Ag in our system (Fig. 5A). At this time, low levels of Ag-specific CD8 T cells could be detected in the blood of immunized mice (Fig. 5B). Transfer of peptide-coated target B cells 9 wk after immunization, without an additional challenge, was sufficient to activate robust Ag-specific killing by CD8⁺ memory T cells (Fig. 5C). Therefore, these data demonstrated that a single s.c. immunization with lentivirus is sufficient to induce an endogenous CD8⁺ T cell memory response that can be activated without the need for a prime-boost vaccination strategy.

Activation of an endogenous CD4⁺ T cell response in immunized mice

The generation of good CD8 T cell memory, particularly in antiviral immunity, is critically dependent on CD4⁺ T cell help. However, activation of an endogenous CD4⁺ T cell response after lentiviral vaccination has not been demonstrated. Therefore, we investigated whether a single vaccination with LV-Ii:OVA could expand detectable endogenous Ag-specific CD4⁺ T cells. The C terminus of OVA contains two defined I-Ab-restricted CD4 T cell epitopes: OVA_{323–339}, which is recognized by TCR transgenic T cells from the OT-II mouse (33), and OVA_{265–280}. LV-Ii:OVA was shown to prime transferred OT-II cells (16), but activation of the endogenous CD4 response has not been documented. We immunized mice and restimulated LN and spleen cells 7 d later with either of the MHC II-specific peptides. T cell activation was measured as IFN- γ production by ELISPOT. Fig. 6 demonstrates that a low, but consistent, endogenous CD4 response was elicited in the LN and spleen after vaccination with LV-Ii:OVA. Restimulation with the OVA_{265–280} peptide resulted in increased production of IFN- γ compared with the OVA_{323–339} epitope. Collectively, these data showed that immunization with LV-Ii:OVA activated expansion of endogenous CD4⁺ T cells.

Activation of cytokine-producing CD8 T cells depends on CD4 T cell help

Because endogenous CD4⁺ T cells were activated by immunization, we next determined whether these cells were required to provide help for generation of the CD8 effector response. In initial experiments, the requirement for CD4 T cell help was tested by depletion of CD4⁺ T cells after injection of the anti-CD4 Ab clone GK1.5. The efficiency of T cell depletion was confirmed using anti-CD4 clone RM4-4, which binds to a distinct epitope on the CD4 molecule from GK1.5 (Fig. 7A). Mice depleted of CD4 T cells generated significantly reduced cytotoxic T cell responses in the LN and spleen after immunization with LV-Ii:OVA (Fig. 7B). In addition, Ag-specific production of IFN- γ was abrogated in

the absence of CD4 T cell help (Fig. 7C). Therefore, these data indicated that the CD8 response to LV vaccination is helper dependent and that T cell help is required for cytokine production and cytotoxic activity by CD8 T cells.

Experiments in which CD4⁺ T cells are transiently depleted by injection of GK1.5 are complicated by the loss of other CD4-expressing cells. In particular, the depletion of CD4⁺ cDC, which may play a role in the priming of the T cell response, is often ignored. This is particularly important given our data showing that cDC are required to prime CD8⁺ T cells. Therefore, to exclude a requirement for CD4⁺ cDC, we repeated our immunization experiments in MHC II knockout (KO) mice that lack circulating CD4⁺ T cells (21) but retain normal development of DC populations. CD8⁺ T cells from immunized MHC II KO mice did not efficiently kill Ag-specific targets (Fig. 7D). To determine whether the defect in CD8 T cell function was due to reduced accumulation of T cells in MHC II KO mice, we compared the frequency of Ag-specific CD8 T cells in the spleens of immunized recipients. Fig. 7E shows that there was no significant difference in the accumulation of OVA-specific CTL in wild-type (WT) or KO mice. Taken together, these data showed that endogenous CD4⁺ T cells activated by cutaneous lentiviral immunization are strictly required for the activation of functional cytokine-producing and cytotoxic CD8⁺ effector T cells.

Discussion

Lentiviral vectors induce potent CD8 T cell immunity; as such, they are being actively developed as vaccination vehicles for cancer immunotherapy. However, a thorough understanding of the mechanisms by which these vectors activate the T cell response is vital to devise novel strategies to overcome the many hurdles toward achieving productive antitumor T cell responses in cancer patients. We demonstrated that, following s.c. immunization with a low dose (14 ng RT activity) of LV-Ii:OVA, cDC are required to prime helper-dependent CD8 T cells that protect mice from cutaneous tumor challenge.

The skin contains multiple populations of DC. In addition to the classical LC in the epidermis and cDC populations in the dermis and LN, Langerin⁺ (CD103⁺) dermal DC were recently characterized (34-36) as the result of the development of in vivo LC-labeling and -depletion models (18, 20). These Langerin⁺ dermal DC are now known to be the dominant cross-presenting DC population of the skin (37, 38). All of these DC may interact with lentiviral particles after cutaneous injection, in situ or in draining LN, and it is not known which cutaneous DC prime the immune response to lentiviral Ags. Tracking of lentivirally transduced DC requires injection of numbers of viral particles that are at least a log higher than those used to elicit a T cell response in vivo (5-7); however, injection of large amounts of virus may alter the repertoire of transduced cells. Therefore, we exploited a model of inducible DC depletion in vivo to determine which DC presented lentiviral Ag after s.c. immunization with low doses of lentiviral particles. To our knowledge, we demonstrated for the first time that conventional CD11c^{hi} DC are required to present s.c.-injected lentiviral Ag to CD8 T cells in vivo. Langerin⁺ DC are not required to prime the cytotoxic T cell response, in agreement with recently published data (8). Concentration of high titers of lentiviral Ag in the skin by intradermal injection results in transduction of migratory DC that present Ag to T cells in vitro (2). In addition, Furmanov et al. (8) recently showed that Langerin⁺ DC are not required to prime T cell responses to intradermally injected lentiviral Ag, therefore suggesting that migrating dermal Langerin⁻ cDC are the dominant T cell-priming DC when lentivirus is injected directly into the skin. However, s.c. injection of lentivirus in our experiments may result in the direct transfer of lentiviral particles to draining LN (39), and future experiments will be conducted to distinguish between the importance of Ag presentation by LN-resident and migratory dermal cDC in our model.

It was demonstrated that plasmacytoid DC (pDC) are activated by lentiviral vectors and may restrict lentivector-induced immunity by activation of innate antiviral mechanisms (40, 41). However, it is not known whether pDC also play a role in initiating the adaptive arm of the lentiviral response. Our data demonstrated that Ag presentation by activated pDC, which are not depleted from the CD11c-DTR chimeras (42), is not sufficient to prime the T cell response to lentiviral Ag. Therefore, our data support a model in which efficient processing and presentation of Ag by cDC, but not Langerin⁺ DC or pDC, are sufficient for T cell priming by lentiviral vectors. This finding will help the rational design of lentivectors specifically targeted toward cDC, which could maximize immune responses while reducing lentivector titers.

We further showed that immunization with LV-Ii:OVA activates expansion of endogenous OVA-specific CD4⁺ T cells, which are required to provide help for functional IFN- γ -producing and cytotoxic CD8 T cells in this system. These data are in accord with other published work showing that T cell help is required for T cell cytotoxicity in immunized mice (5, 15). The clear dependency on T cell help during the priming phase of the lentiviral response likely reflects the limited inflammatory response elicited on injection of lentiviral vectors (43). It is possible that the OVA-specific CD4 T cell response is only a fraction of the CD4 T cell repertoire that is activated after lentiviral vaccination. Indeed, data by Esslinger et al. (5), showing that expansion of CD8⁺ tetramer⁺ T cells after vaccination with lentivirus expressing the Melanin A mini-gene was dependent on the presence of CD4 T cells, although a CD4 epitope was not included in the Melanin A construct, strongly suggested that viral vector-derived sequences provide unidentified helper epitopes in these experiments. The activation of CD4 help in this model likely contributes to the development of systemic long-term memory CD8 T cell responses. This memory pool, which can be reactivated by transfer of peptide-coated cells in the absence of challenge, is particularly important for cancer immunotherapy, whereby memory T cells will be required to kill tumor cells that emerge in the absence of other inflammatory-activating signals. However, the observation that the memory responses elicited in this model were sufficient to delay, but not to prevent, tumor growth in immunized mice suggests that inclusion of CD4 epitopes, as well as a boost vaccination, may be required in a therapeutic lentiviral-vaccination strategy.

Defining the mechanisms by which lentiviral vectors prime T cell immunity is vital to devise novel approaches to improve the use of these vaccination vehicles for immunotherapy. Enhancing DC activation, in combination with the use of low viral titers, is an attractive approach to improving T cell responses to lentiviral vaccination (10). By defining a requirement for cDC to present LV Ag, strategies can be developed to specifically target Ag to, and activate, those DC to further augment the potency of lentivector-based immunization.

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Abbreviations used in this article

cDC	conventional dendritic cell
DC	dendritic cell

DT	diphtheria toxin
KO	knockout
LC	Langerhans cell
LN	lymph node
LV	lentivector
MHC I	MHC class I
MHC II	MHC class II
pDC	plasmacytoid dendritic cell
RT	reverse transcriptase
WT	wild-type

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FIGURE 1.

A single s.c. immunization with LV-Ii:OVA activates systemic CD8 T cell responses. *A*, Structure of the pSIN-DUAL lentivector. Δ U3 represents the HIV-1 LTR with a deletion covering the U3 region, resulting in an SIN vector. *B* and *C*, Mice were immunized s.c. at the base of the tail with PBS or 14 ng of RT activity of LV-Ii:OVA. Seven days later, mice were sacrificed, and production of intracellular IFN- γ was measured after overnight stimulation of LN cells with OVA_{257–264} peptide. Representative dot plots (*B*) and pooled data (*C*) from three separate experiments. Circles represent individual mice; horizontal lines represent the median IFN- γ production. $**p < 0.01$. *D*, ELISPOT data showing CD8 IFN- γ production by LN cells from mice immunized with a GFP control virus or LV-Ii:OVA 7 d previously. LN cells were restimulated with MHC I OVA_{257–264} peptide. Bars represent the mean number of spots $\pm$ SEM for three mice per group from one experiment. Data are representative of three independent experiments. *E*, The in vivo cytotoxicity assay was used to determine killing of T cell targets in the spleens of mice 9 d after immunization. Graphs in the first three panels show representative data from one experiment; the graph on the far right shows pooled summary data for specific lysis in the LN and spleen from two independent experiments. Circles represent individual mice; horizontal lines show median specific lysis. Ψ , HIV packaging signal; cPPT, HIV central polypurine tract; Ctl, control; LTR, HIV-1 long terminal repeat; RRE, HIV Rev response element; SFFV, spleen focus forming promoter; UBIQ, human ubiquitin promoter; WPRE, woodchuck hepatitis virus posttranscriptional regulatory element.

FIGURE 2.

Tumor protection in mice immunized with LV-Ii:OVA. Mice were immunized s.c. with PBS (A, C) or 14 ng of RT activity of LV-Ii:OVA (B, D). They were challenged 9 days (A, B; $n = 7$) or 42 days (C, D; $n = 8$) later by s.c. injection of 2×10^6 EG7 tumor cells. Graphs show the increase in tumor area over time; lines represent individual mice. Mice were killed when tumor areas exceeded 150 mm². Data are representative of two independent experiments.

FIGURE 3.

cDC are required to present lentiviral Ag to T cells in draining LN. *A*, CD11c-DTR syngeneic chimeras were injected with PBS or DT on days -4, -1, and +2 to deplete cDC. FACS plots show depletion of CD11c^{hi} GFP⁺ cDC from the spleen immediately following the final injection. *B*, On day 0, depleted or nondepleted chimeras were immunized with LV-Ii:OVA; 3 d later, CFSE-labeled OT-I cells were injected via the tail vein. The mice were sacrificed 65 h later, and OT-I cells were identified in draining LN by gating on CD8⁺ and V α 2⁺ cells. Proliferation was measured by dilution of CFSE. *Left panels* show representative CFSE profiles; *right panel* shows one representative experiment from three independent experiments. Circles represent individual mice, and horizontal lines represent median proliferation. * $p < 0.05$. *C*, Dot plots showing accumulation and proliferation of CFSE-labeled CD8⁺ V α 2⁺ OT-I T cells in the LN and spleen of nondepleted mice; these are representative of three independent experiments.

FIGURE 4.

Langerin⁺ cells are not required to activate the cytotoxic T cell response. *A*, Langerin-DTR mice were injected with PBS or DT on days -3 and +4. FACS plots show depletion of CD11b⁺ GFP⁺ LC from the epidermis. *B*, Langerin-DTR mice that had been injected with PBS or DT were immunized on day 0 with LV-Ii:OVA, and activation of CD8 T cells was measured in draining LN by in vivo cytotoxicity assay on day 8. *Left panels* show representative data; *right panel* shows pooled summary data from two independent experiments. Circles represent individual mice, and horizontal lines represent the median percentage of specific lysis. n.s., not statistically significant.

FIGURE 5.

Activation of memory T cells in the absence of inflammatory challenge. *A*, CFSE-labeled OT-I cells were injected via the tail vein at different time points after s.c. immunization with 14 ng RT activity of LV-Ii:OVA. Sixty-five hours later, the mice were sacrificed, and OT-I cells were identified in draining LN by gating on CD8⁺ and Vα2⁺ cells. Proliferation of OT-I cells is identified by dilution of CFSE. *B*, Nine weeks after immunization with LV-Ii:OVA or a control lentivector expressing GFP, mice were bled, and the presence of OVA-specific CD8⁺ T cells was measured by pentamer staining. Data are pooled from two independent experiments. Circles represent individual mice, and horizontal lines represent the median percentage of pentamer⁺ cells. ****p* < 0.001. *C*, The in vivo cytotoxicity assay was performed by transferring CFSE-labeled T cell targets into mice immunized 9 wk previously. *Left panels* show representative data from one experiment comparing specific lysis in the spleens of PBS- and LV-Ii:OVA-immunized mice; *right panel* shows pooled summary data from two independent experiments, with horizontal lines representing the median specific lysis per group. Ctl, control.

FIGURE 6.

Expansion of endogenous CD4 T cells after s.c. immunization with LV-Ii:OVA. Mice were immunized with LV-Ii:OVA or a GFP-expressing control virus. Seven days later, IFN- γ production was measured in LN (*left panel*) and the spleen (*right panel*) by ELISPOT after restimulation with the MHC II peptides OVA₂₆₅₋₂₈₀ or OVA₃₂₃₋₃₃₉. Data are pooled from two independent experiments, with a total of seven mice per group. Bars show the mean number of spots + SEM. * $p < 0.05$, *** $p < 0.001$. n.s., not statistically significant.

FIGURE 7.

OVA-specific CD8 T cell responses require CD4 help. *A*, Dot plots show depletion of CD4⁺ T cells from mice injected with three doses of 300 μg of the GK1.5 Ab (*Materials and Methods*). *B*, Mice were injected with PBS (open bars) or GK1.5 (filled bars) prior to immunization with LV-Ii:OVA; specific lysis of peptide-pulsed B cells was measured 9 d later. Injection of PBS or the isotype control showed no difference in control animals (data not shown). Data are pooled from two independent experiments, with a total of eight mice per group. Bars show the mean specific lysis + SEM. *C*, CD8⁺ IFN-γ production was measured after overnight stimulation with OVA₂₅₇₋₂₆₄ in cells from mice that had received peptide-coated B cells for the cytotoxicity assay. Control animals received PBS or the isotype control Ab. Data are pooled from two independent experiments, with a total of six or seven mice per group. Bars show the mean percentage of IFN-γ⁺CD8⁺ cells + SEM. *D*, WT or MHC II KO mice were injected with LV-Ii:OVA, and in vivo cytotoxicity was measured on day 9 after immunization. Data are pooled from two independent experiments, with a total of seven mice per group. Bars show the mean specific lysis + SEM. *E*, The presence of splenic OVA-specific CD8⁺ T cells was measured 9 d after immunization by pentamer staining in cells from WT or MHC II KO mice that had received peptide-coated B cells for the cytotoxicity assay. Data are pooled from two independent experiments. Circles represent individual mice, with horizontal lines representing the median percentage of pentamer⁺ cells. ***p* < 0.01, ****p* < 0.001. n.s., not statistically significant.