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Engineered lentivector targeting of dendritic cells for *in vivo* immunization

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We report a method of inducing antigen production in dendritic cells by *in vivo* targeting with lentiviral vectors that specifically bind to the dendritic cell–surface protein DC-SIGN. To target dendritic cells, we enveloped the lentivector with a viral glycoprotein from Sindbis virus engineered to be DC-SIGN–specific. *In vitro*, this lentivector specifically transduced dendritic cells and induced dendritic cell maturation. A high frequency (up to 12%) of ovalbumin (OVA)-specific CD8$^+$ T cells and a significant antibody response were observed 2 weeks after injection of a targeted lentiviral vector encoding an OVA transgene into naive mice. This approach also protected against the growth of OVA-expressing E.G7 tumors and induced regression of established tumors. Thus, lentiviral vectors targeting dendritic cells provide a simple method of producing effective immunity and may provide an alternative route for immunization with protein antigens.

Although immunization is one of the most productive tools in modern medicine, it still has limitations, and novel methods of immunization are likely to be needed[1]. One of the great advances in our understanding of the process of immunization was the recognition of the role of dendritic cells as specialized antigen-presenting cells[2]. This has led to attempts at dendritic cell–based immunization and/or vaccination in which dendritic cells are loaded with specific antigens[3,4]. Although substantial progress has been made on various aspects of this vaccination method, many challenges remain in rationally manipulating dendritic cells to achieve protective immunity[3–5].

There is growing interest in genetically modifying dendritic cells to make them either express antigens or produce immunostimulatory molecules[6]. Of these methods, viral vectors have been proven most effective for the delivery of genes into dendritic cells *in vitro*[7]. The most popular viral vectors capable of transducing and expressing transgenes in dendritic cells are adenoviral vectors[8–10], gammaretroviral vectors[11,12] and lentiviral vectors (lentivectors)[13–15].

Considerable effort has also gone toward direct immunization using recombinant viral vectors as vaccine carriers. In these protocols, viral vectors are injected directly into an animal with the hope that a fraction will target immune cells and stimulate the desired immunity. Direct injection of adenoviral vectors was shown to induce both innate and adaptive immune responses and is currently being evaluated as a subunit vaccine for several infectious diseases and cancer[16–18]. Lentivectors injected into mice do transduce dendritic cells, leading to antigen-specific CD8$^+$ T-cell responses and anti-tumor immunity[19–22]. However, these recombinant viral vectors usually have broad specificity and transduce multiple cell types. Development of viral vectors capable of transducing dendritic cells in a cell-specific manner *in vivo* could potentially improve the safety and efficacy of vaccination. Whereas there have been few attempts at targeting recombinant viral vectors to dendritic cells[23–25], success has been reported for targeting protein antigens directly to dendritic cells *in vivo* by conjugating antigens to the anti-DEC-205 antibody[26–28] and other dendritic cell–specific molecules[29,30].

Here we report a method of *in vivo* dendritic cell–targeting through the dendritic cell–specific surface molecule DC-SIGN (also known as CD209)[31,32] by a recombinant lentivector bearing an engineered glycoprotein derived from Sindbis virus. We show that the engineered lentivector can genetically modify dendritic cells *in vitro* with high specificity. Direct administration of the targeting lentivector induces a strong antigen-specific T-cell response and an antibody response. Finally, we have explored the applicability of this strategy to achieve cancer immunotherapy in mice and found that it is a promising approach for immunization against cancer.

RESULTS

Targeting of DC-SIGN–expressing cell lines *in vitro*

Certain subsets of dendritic cells bear on their surface the DC-SIGN protein[31,32], a C-type lectin-like receptor capable of rapid binding and endocytosis of materials[33] and an ideal targeting receptor on dendritic cells. Sindbis virus—a member of the *Alphavirus* genus and the *Togaviridae* family—is able to infect dendritic cells through DC-SIGN[34]. However, the canonical viral receptor for the laboratory strain of Sindbis virus is cell-surface heparan sulfate, which is expressed by many cell types[35,36].

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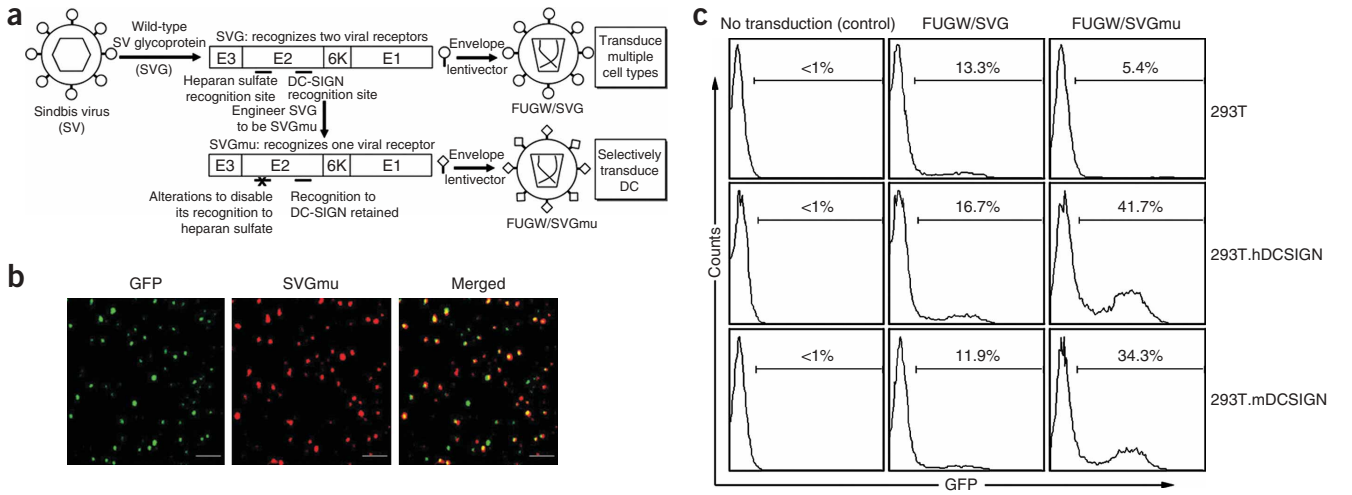

Figure 1 Lentivector bearing engineered Sindbis viral glycoprotein targets to DC-SIGN-expressing cells. (**a**) A schematic representation of the general strategy to engineer a lentivector system capable of targeting dendritic cells. (**b**) Viral supernatant harvested from virus-producing cells transiently transfected with GFP-vpr, SVGmu, and other necessary packaging constructs was coated to a poly-lysine containing coverslip by centrifugation. A hemagglutinin (HA) epitope tag (YPYDVPDYA) was engineered into SVGmu to facilitate its detection by antibody. The resulting coverslip was then rinsed and immunostained with an anti-HA tag antibody (red) to label SVGmu and imaged using a laser confocal microscope. The scale bar represents 2 μm. (**c**) One milliliter of fresh viral supernatants of FUGW/SVG and FUGW/SVGmu were used to transduce 293T cells ($2x10^5$) expressing human DC-SIGN (293T.hDCSIGN) or murine DC-SIGN (293T.mDCSIGN). The parental 293T cells lacking the expression of DC-SIGN were included as controls. Three days later, the transduction efficiency was measured by analyzing GFP expression using flow cytometry. The specific transduction titer of FUGW/SVGmu was estimated to be $\sim 1 \times 10^6$ TU/ml for 293T.hDC-SIGN and $\sim 0.8 \times 10^6$ TU/ml for 293T.mDC-SIGN.

Taking advantage of the physical separation of the two receptor-binding sites on the Sindbis virus envelope glycoprotein (designated SVG), we engineered the receptor to be blind to its canonical binding target, heparan sulfate, but left intact its ability to interact with DC-SIGN (**Fig. 1a**). Once it is incorporated onto a lentiviral surface, this mutant glycoprotein should be able to mediate infection of dendritic cells but not other cells. We and others have demonstrated that SVG can efficiently pseudotype lentiviruses and that alterations to SVG, including deletion of amino acids 61–64 of the SVG E3, mutations of 157KE158 into 157AA158 of the SVG E2, and an insertion of 10-amino acid tag sequence (MYPYDVPDYA) between amino acids 71 and 74 of the SVG E2, can disable its binding to heparan sulfate[37,38]; we designated this modified SVG as SVGmu. Using a green fluorescent protein (GFP)–viral protein R (vpr) labeling method, we found that over 70% of lentiviral particles that we produced displayed SVGmu (**Fig. 1b**).

To facilitate our study of targeted transduction, we constructed 293T cell lines that stably expressed either human DC-SIGN (293T.hDCSIGN) or murine DC-SIGN (293T.mDCSIGN) (**Supplementary Fig. 1** online). To assess transduction efficiency and specificity, we pseudotyped the lentiviral vector FUGW, which carries the GFP reporter gene under control of the human ubiquitin C promoter[39], with wild-type SVG or SVGmu to produce FUGW/SVG or FUGW/SVGmu. FUGW/SVG had similar transduction efficiency (11 ~ 16% transduction) toward the three target cell lines (293T, 293T.hDCSIGN and 293T.mDCSIGN) (**Fig. 1c**), indicating that SVG has broad specificity and the presence of DC-SIGN on the cell surface does not markedly alter the transduction ability of an SVG-pseudotyped lentiviral vector. In contrast, the FUGW/SVGmu vector could specifically transduce 293T.hDCSIGN and 293T.mDCSIGN cells, with 42% and 34% transduction efficiencies, respectively, but not the 293T cells (**Fig. 1c**). We confirmed the stable integration of the FUGW lentiviral vector in the transduced cells by PCR analysis of the genomic integration of the GFP reporter gene (data not shown). Adding soluble anti-human DC-SIGN antibody to the FUGW/SVGmu viral supernatant before its exposure to 293T.hDCSIGN cells reduced the transduction efficiency (data not shown). The specific titer of FUGW/SGVmu for 293T.mDCSIGN was estimated to be 1×10^6 TU (transduction units)/ml. FUGW/SVGmu could be concentrated by ultracentrifugation with a >90% recovery, indicating that SVGmu is a stable envelope for a lentivector.

Targeting of mouse and human dendritic cells *in vitro*

In a mixed mouse bone marrow culture, ~10% of the cells were $CD11c^+$ dendritic cells, of which ~80% were DC-SIGN high (data not shown). After FUGW/SVGmu transduction, 12% of the cells were GFP^+ (**Fig. 2a**, right). Within the GFP^+ cells, up to 95% of the transduced cells were $DC\text{-}SIGN^+CD11c^+$ dendritic cells, indicating that FUGW/SVGmu could specifically transduce dendritic cells expressing DC-SIGN. A blocking assay using anti-mouse DC-SIGN antibody confirmed that DC-SIGN plays an important role in the specific transduction (**Fig. 2b**). In contrast, although 68% of the cells were GFP^+ after exposure to lentivectors enveloped with an ecotropic murine leukemia virus glycoprotein (FUGW/Eco), only 9% of the transduced cells were dendritic cells, within which 6.5% were $DC\text{-}SIGN^+$ (**Fig. 2a**, lower). A similar nonspecific transduction was observed for lentivector enveloped with vesicular stomatitis viral glycoprotein (FUGW/VSVG, **Supplementary Fig. 2** online). FUGW/SVG could preferentially modify $CD11c^+$ cells, but it was less specific for $DC\text{-}SIGN^+$ cells (FUGW/SVG, **Supplementary Fig. 2**). Stable transduction by FUGW/SVGmu was verified by Alu PCR analysis[40] of the genomic integration of the LTR of the lentivector backbone (data not shown). In addition, attempts to use FUGW/SVGmu to transduce primary T and B cells harvested from mouse spleen completely failed (**Supplementary Fig. 3** online), indicating its transduction specificity.

We next tested the efficiency of the lentivector bearing SVGmu to transduce *in vitro*–cultured, mouse bone marrow–derived dendritic cells (mBMDCs). Flow cytometry analysis showed that FUGW/Eco transduced both $CD11c^+$ dendritic cells (33%) and $CD11c^-$ cells (7.6%) (**Fig. 2c**), which is consistent with the wide tropism of Eco. On the contrary, FUGW/SGVmu could only transduce $CD11c^+$ dendritic cells (32.7%) (**Fig. 2c**), indicating that FUGW/SGVmu can specifically modify mBMDCs. FUGW/SVGmu also had a greater specificity for transducing human monocyte–derived dendritic cells (hMoDCs) compared to FUGW/VSVG or FUGW/SVG; the transduction is closely correlated with DC-SIGN expression (**Fig. 2d**).

We further examined whether the targeted transduction could activate and mature dendritic cells[41]. Flow cytometry analysis of mBMDCs 3 d post-transduction showed that treatment with FUGW/SVGmu elevated the expression of dendritic cell activation markers, CD86 and $I\text{-}A^b$ (ref. 41), on GFP^+ dendritic cells, as compared to GFP^- dendritic cells (**Fig. 2e**, upper). Moreover, we found that the targeted transduction of mBMDCs could synergize with lipopolysaccharide (LPS) treatment to further mature dendritic cells (**Fig. 2e**, lower), suggesting that it can either work alone or in combination with other dendritic cell maturation stimuli, such as adjuvant, to induce dendritic cell activation.

Targeting of dendritic cells *in vivo* in mice

To test whether engineered lentivectors bearing SVGmu could target dendritic cells *in vivo* in mice, we injected 50×10^6 TU of FUGW/SVGmu subcutaneously on the right flank of a B6 mouse. On day 3, we observed a significant ($P < 0.01$) enlargement of the right inguinal

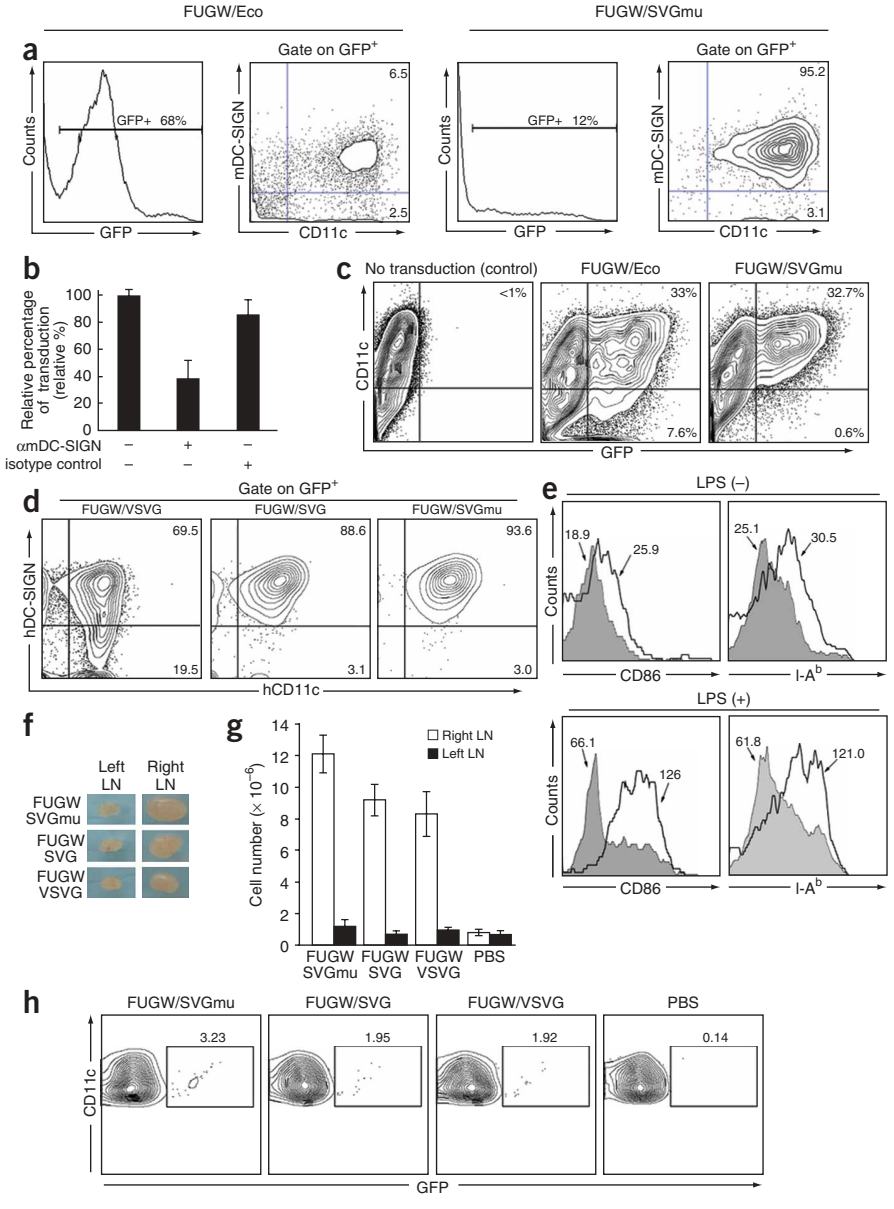

Figure 2 Lentivector encoding a reporter GFP gene and bearing SVGmu can selectively transduce dendritic cells *in vitro* and *in vivo*. (**a**) Whole bone marrow cells isolated from B6 mice were exposed to the fresh viral supernatant of FUGW/SVGmu. The FUGW lentivector pseudotyped with the ecotropic glycoprotein (FUGW/Eco) was included as a nontargeting control. Three days post-transduction, the cells were collected for flow cytometric analysis of GFP expression. Surface markers of the GFP-positive cells were assessed by staining with anti-CD11c and anti-DC-SIGN antibodies. (**b**) Anti-murine DC-SIGN antibody was added into viral supernatant during transduction of whole mouse bone marrow cells for 8 h. Then, the supernatant was replaced with fresh medium. The cells were analyzed for GFP expression after 2 d. Isotype-matched antibody was used as a control. (**c**) Murine bone marrow-derived dendritic cells (mBMDCs) were generated by culturing freshly isolated bone marrow cells in the presence of cytokine GM-CSF for 6 d. The resulting cells were transduced with the fresh viral supernatant of either the targeting FUGW/SVGmu or nontargeting FUGW/Eco vector. GFP and CD11c expression were measured by flow cytometry. (**d**) Human monocyte-derived dendritic cells (hMoDCs) were generated by culturing freshly purified $CD14^+$ peripheral blood moncytes in the presence of GM-CSF and IL-4. The cells from the day 2 culture were transduced with the fresh viral supernatant of either FUGW/SVGmu, or FUGW/VSVG, or FUGW/SVG vector. GFP expression was measured by flow cytometry at day 6. (**e**) Upon targeted transduction of mouse BMDCs with FUGW/SVGmu, dendritic cell activation was assessed by analyzing the surface expression of CD86 and $I\text{-}A^b$ using flow cytometry. The addition of LPS (1 μg/ml) overnight was used as a synergistic stimulator for the activation of transduced BMDCs. Shaded area, GFP negative cells (untransduced); solid line, GFP positive cells (transduced). The mean fluorescence is indicated. (**f**–**h**) *In vivo* dendritic cell–targeting using FUGW/SVGmu lentivector. B6 mice were injected with 50×10^6 TU of FUGW/SVGmu, FUGW/SVG, or FUGW/VSVG, and analyzed 3 d later. Mice injected with PBS were included as a control. Comparison of a representative inguinal lymph node close to the injection site (right) and the equivalent lymph node distant from the injection site (left) (**f**). Total cell number counts of the indicated lymph nodes (**g**). Representative flow cytometric analysis of $CD11c^+$ cells from the indicated lymph nodes that are close to the injection sites (**h**). The numbers indicate the fraction of GFP^+ dendritic cell populations.

lymph node close to the injection site (**Fig. 2f**), and found an approximately tenfold increase in cell number in this lymph node compared with the equivalent lymph node at the opposite side or lymph nodes from a mouse injected with PBS (**Fig. 2g**), indicating that vector administration can enhance trafficking of dendritic cells and proliferation of lymphocytes in a nearby lymph node. Flow cytometry showed that ~3.2% of the total $CD11c^+$ cells in the right inguinal lymph node cells were GFP^+ dendritic cells (**Fig. 2h**), which had presumably migrated from the injection site.

To compare the *in vivo* effects of vectors pseudotyped with SVGmu, SVG and the more commonly used VSVG, we injected into the flanks of mice FUGW enveloped with each of these proteins and found that FUGW/SVGmu produced a distinctly larger node, more cellularity and more transduced dendritic cells than the others (**Fig. 2f–h**). Thus, the increased infection efficiency mediated by the mutant SVG protein compared to wild type, which we found *in vitro* (**Fig. 1c**), is paralleled by an increased effectiveness *in vivo*.

To examine the *in vivo* specificity of the dendritic cell–targeted lentivector, we constructed a lentiviral vector encoding a firefly luciferase (designated as Fluc, **Supplementary Fig. 4a** online), which enabled us to visualize the *in vivo* transduction of the tissue cells using bioluminescence imaging. We found that Fluc/VSVG-treated mice had a strong and permanent signal at the injection site, whereas no marked signal was detected at the injection site of Fluc/SVGmu-treated mice (**Supplementary Fig. 4b**), indicating that the lentivector bearing SVGmu had a relatively stringent target specificity. At no time were we able to detect a luminescence signal in the targeted mice, probably owing to the rare and sparse distribution of the dendritic cells, which is beyond the sensitivity of the current bioluminescence imaging method. After a month, the mice injected with Fluc/SVGmu were subjected to biodistribution analysis by quantitative RT-PCR and no detectable copy of the lentivector was observed in all isolated organs (heart, liver, spleen, kidney, gonad, lung, skin, lymph node), verifying the lack of nonspecific infection in the animals and thus the specificity of the targeted vector for dendritic cells.

Induction of $CD8^+$ and $CD4^+$ T-cell responses *in vitro*

To determine whether the targeted transduction of dendritic cells by a recombinant lentivector could be used to effectively deliver antigen genes to dendritic cells for stimulation of antigen-specific $CD8^+$ and $CD4^+$ T cell responses, we constructed a lentivector expressing the model antigen, chicken OVA, which we designated FOVA (**Fig. 3a**). In C57BL/6J (B6) mice, OVA is a well-characterized target antigen for the $CD8^+$ TCR, OT1, which recognizes $OVA_{257\text{-}269}$ (designated as OVAp), and for the $CD4^+$ TCR, OT2, which recognizes $OVA_{323\text{-}339}$ (designated as OVAp*)[42]. mBMDCs were transduced on day 6 of culture with

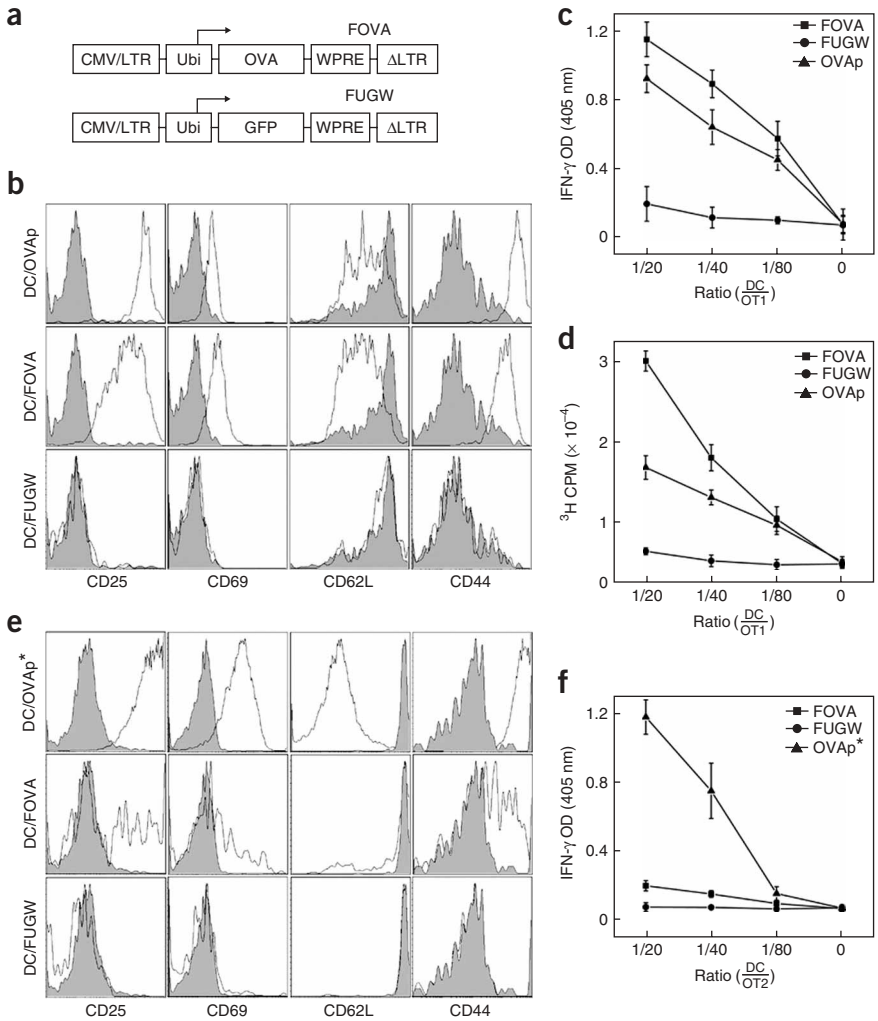

Figure 3 Mouse bone marrow-derived dendritic cells (mBMDCs) transduced by a SVGmu enveloped lentivector encoding an OVA gene can stimulate OVA-specific $CD8^+$ and $CD4^+$ T cells *in vitro*. (**a**) A schematic representation of the lentivector encoding the OVA antigen (FOVA) or the lentivector encoding GFP (FUGW) as a control. LTR: long terminal repeat; Ubi: human ubiquitin-C promoter; WPRE: woodchuck hepatitis virus posttranscriptional regulatory element. (**b–f**) OVA-specific, $CD8^+$ OT1 T cells and $CD4^+$ OT2 T cells were harvested from the spleens of OT1 TCR- or OT2 TCR-transgenic mice (The Jackson Laboratory) and were cocultured with FOVA/SVGmu-transduced mBMDCs (DC/FOVA) *in vitro* for 3 d. Non-transduced BMDC pulsed with either OVAp peptide (SIINFEKL) (DC/OVAp), recognized by OT1 T cells, or OVAp* peptide (ISQAVHAAHAEINEAGR) (DC/OVAp*), recognized by OT2 T cells, were included as positive controls. mBMDCs transduced with FUGW/SVGmu (DC/FUGW) were included as a negative control. Patterns of surface activation markers of OT1 T cells cocultured with various mBMDCs were assessed by antibody staining for CD25, CD69, CD62L, and CD44 (**b**). Shaded area: naive OT1 T cells harvested from transgenic animals; solid line: OT1 T cells cocultured with the indicated mBMDCs. OT1 T cells were mixed with various dilutions of mBMDCs transduced with FOVA/SVGmu (■), FUGW/SVGmu (●), or pulsed with OVAp peptide (▲) and cultured for 3 d. Secretion of IFN-γ was measured by ELISA (**c**). The proliferative responses of treated OT1 T cells from **c** were measured by a $[^3H]$ thymidine incorporation assay (**d**). Similar to **b** except for the analysis of activated $CD4^+$ OT2 T cells (**e**). Shaded area, naive OT2 T cells harvested from transgenic animals; solid line, OT2 T cells cocultured with BMDCs. Similar to (**c**) except for the responding cells being $CD4^+$ OT2 T cells (**f**).

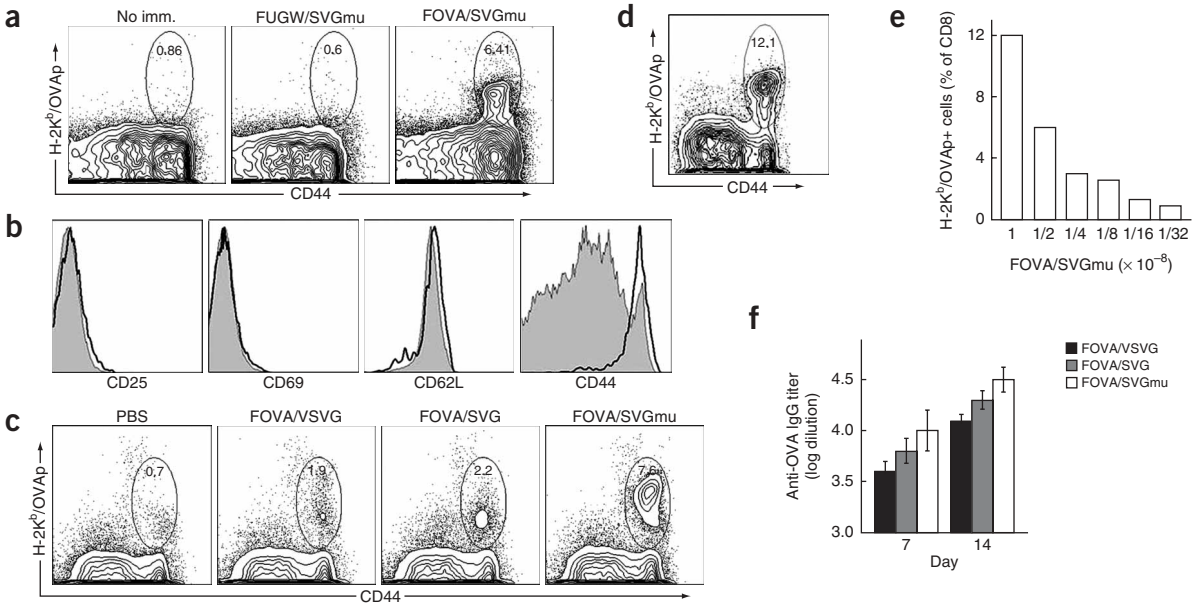

Figure 4 *In vivo* stimulation of antigen specific T cell and antibody responses in wild-type B6 mice following a subcutaneous injection of the dendritic cell–targeting lentivector FOVA/SVGmu. (**a**) B6 mice were immunized subcutaneously with 50 × 10^6 TU of either FOVA/SVGmu or FUGW/SVGmu (as a control). Mice without immunization (no imm.) were included as a negative control. Fourteen days post-immunization, spleen cells were harvested and analyzed for the presence of OVA-specific $CD8^+$ T cells measured by H-$2K^b$-SIINFEKL-PE tetramer and CD44 staining. Indicated percentages are a percent of total $CD8^+$ T cells. (**b**) Patterns of surface activation markers of OVA-specific $CD8^+$ T cells (identified as tetramer positive cells) isolated from immunized mice 2 weeks post-injection were assessed by antibody staining for CD25, CD69, CD62L and CD44. Solid line, $tetramer^+CD8^+$ T cells from FOVA/SVGmu-immunized mice; shaded area, $CD8^+$ T cells from nonimmunized mice. (**c**) Naive B6 mice were immunized by subcutaneous injection of 50 × 10^6 TU of the different lentivectors (FOVA/VSVG, FOVA/SVG or FOVA/SVGmu). The injection of PBS was included as a control. Two weeks later, spleen cells were harvested and analyzed for the presence of OVA-specific $CD8^+$ T cells measured by H-$2K^b$-SIINFEKL-PE tetramer and CD44 staining. Indicated percentages are a percent of total $CD8^+$ T cells. (**d**–**e**) OVA-specific T cell responses seen in mice receiving different subcutaneous doses of FOVA/SVGmu. OVA-specific T cells were identified by tetramer staining as described in **a**. Percentage of OVA-specific $CD8^+$ T cells following immunization with 100 × 10^6 TU of FOVA/SVGmu (**d**). Dose responses of OVA-specific $CD8^+$ T cells following injection of the indicated doses of FOVA/SVGmu (**e**). OVA-specific serum IgG titer of B6 mice following immunization with 50 × 10^6 TU of the different lentivectors (FOVA/VSVG, FOVA/SVG, or FOVA/SVGmu) (**f**). Sera were collected on day 7 and day 14 post-immunization and were analyzed for the titer of OVA-specific IgG using ELISA at serial 10× dilutions, starting at 1:100. The titer values were determined by the highest dilution at which the optical density was 2× standard derivations higher than that of the baseline serum at the equivalent dilution.

either FOVA/SVGmu or FUGW/SVGmu encoding a nonrelevant reporter gene GFP; the modified dendritic cells were designated as DC/FOVA and DC/FUGW, respectively. The capacity of vector-transduced dendritic cells to process and present the transgenic OVA antigen was examined by their ability to stimulate OVA-specific OT1 transgenic $CD8^+$ T cells and OT2 transgenic $CD4^+$ T cells. Unmodified mBMDCs pulsed with the OVAp (DC/OVAp) or OVAp* (DC/OVAp*) were included as positive controls. After a 3-d coculture with varying ratios of DC/FOVA to transgenic T cells, flow cytometry analysis showed that the activated OT1 T cells exhibited the typical effector cytotoxic T-cell phenotype ($CD25^+CD69^+CD62L^{low}CD44^{high}$) after stimulation by either DC/FOVA or DC/OVAp (**Fig. 3b**). As another indication of vigorous response, the treated OT1 T cells secreted IFN-γ (**Fig. 3c**) and proliferated (**Fig. 3d**). As expected, no detectable OVA response was seen using DC/FUGW (**Fig. 3c,d**). When we cocultured the dendritic cells with OT2 $CD4^+$ T cells, we also observed T-cell activation indicated by changes in the surface markers (**Fig. 3e**) and the production of IFN-γ (**Fig. 3f**). However, stimulation of $CD4^+$ cells was not as pronounced as that of $CD8^+$ cells, presumably because of the less efficient presentation of endogenous antigen peptides to the major histocompatibility complex (MHC) class II molecules. By modifying the cellular localization of OVA antigen to direct it to MHC class II presentation pathway, we have achieved an enhancement of CD4 stimulation, which was even better than that of peptide-pulsed dendritic cells (data not shown). Our results show that the method of dendritic cell targeting through lentivector infection can effectively deliver antigens to dendritic cells and stimulate both $CD8^+$ and $CD4^+$ T-cell responses.

Induction of CTL and antibody responses in mice

We focused our subsequent studies on the efficacy of *in vivo* dendritic cell targeting for inducing an antigen-specific $CD8^+$ cytotoxic T lymphocyte (CTL) and antibody responses after administration of the targeting lentivector to naive, wild-type mice. We injected a single dose of 50 × 10^6 TU of FOVA/SVGmu subcutaneously and monitored the presence of OVA-specific T cells by measuring cytokine secretion and tetramer staining (see **Supplementary Methods** online). At day 14 post-injection, T cells harvested from spleens were analyzed. The generated $CD8^+$ T cells could be primed to secrete IFN-γ upon OVAp restimulation (**Supplementary Fig. 5** online). Administration of the control vector FUGW/SVGmu failed to generate OVAp-specific responses (**Supplementary Fig. 5**). Using MHC class I tetramer staining, a high frequency of OVAp-specific T cells (>6%) was observed after a single-dose injection (**Fig. 4a**); no tetramer-positive cells were detected in the mice treated with FUGW/SVGmu (**Fig. 4a**), correlating well with the intracellular IFN-γ staining results (**Supplementary Fig. 5**). These cells displayed the surface characteristics of effector memory T cells ($CD25^{low}CD69^{low}CD62L^{high}CD44^{high}$)

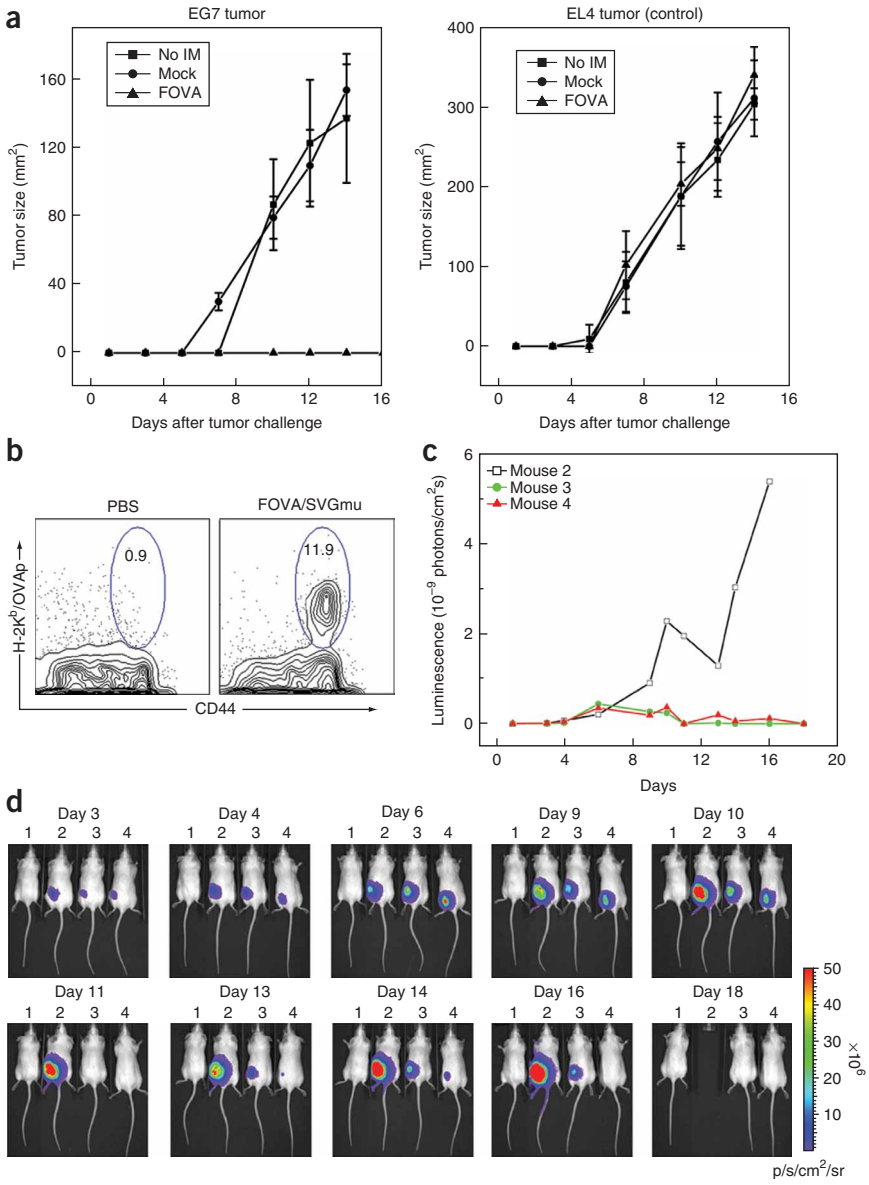

Figure 5 Preventive and therapeutic anti-tumor immune responses elicited through *in vivo* administration of the dendritic cell–targeted lentivector FOVA/SVGmu in a murine E.G7 tumor model. (**a**) B6 mice were immunized at day 0 by subcutaneous injection of 50x10^6 TU of either FOVA/SVGmu (▲) or mock vector FUW/SVGmu (●). No immunization (■) was included as a control. At day 14 post-immunization, the mice were challenged with 5 × 10^6 of either E.G7 tumor cells (expressing the OVA antigen) or EL4 tumor cells (lacking the OVA antigen, as a control) subcutaneously. Tumor growth was measured with a fine caliper and is shown as the product of the two largest perpendicular diameters (mm^2). Four mice were included in each group. The experiment was repeated for 3 times and the result for one representative experiment was shown. (**b**) Percentage of OVA-specific T cells present following immunization with 100 × 10^6 TU of FOVA/SVGmu, or PBS (control), in the albino strain of B6 mice. The analysis was as described in **Figure 4**. (**c**–**d**) B6 mice were implanted with E.G7 tumor cells stably expressing a firefly luciferase imaging gene (E.G7.luc) as described in **a**. A mouse (no. 1) without tumor implantation was included as a control. Mice bearing tumors were treated without immunization (no. 2), or with immunization (no. 3, no. 4) by the injection of 50 × 10^6 TU of FOVA/SVGmu at day 3 and day 10. The kinetic growth of the tumors was monitored by live animal imaging using bioluminescence imaging (**d**). The p/s/cm^2/sr represents photons/sec/cm^2/steridian. (**c**) Quantitation of luminescence signals generated by the E.G7 tumors in (**d**). (■) for mouse no. 2; (●) for mouse no. 3; (▲) for mouse no. 4.

(**Fig. 4b**). In addition, we also compared the level of CD8 T-cell responses generated by FOVA/SVGmu with that of other vectors (FOVA/VSVG and FOVA/SVG) and found that FOVA/SVGmu provided the strongest immune response (**Fig. 4c**).

To investigate the dose response of lentivector administration, we injected FOVA/SVGmu ranging from 100 × 10^6 TU to 3 × 10^6 TU subcutaneously and measured OVAp-specific T cells in the spleen at day 14 post-injection. Notably, a high frequency (12%) of OVAp-specific $CD8^+$ T cells was detected at the dose of 100 × 10^6 TU (**Fig. 4d**). The percentage of OVAp-specific cells correlated proportionately with the amount of recombinant vector administered (**Fig. 4e**). We may not have reached a plateau response with the doses we tested, suggesting that further enhancement may be possible by increasing the amount of vector injected and/or the frequency of injection.

We further examined the serum IgG levels specific for OVA in mice on the 7th and 14th days after immunization with FOVA/SVGmu (50 × 10^6 TU). The IgG serum titer was 1:10,000 on day 7 and 1:30,000 on day 14 (**Fig. 4f**). This is a substantial antibody response for a single-dose injection without additional adjuvant or other stimuli, suggesting that targeted lentivector immunization can also elicit significant B-cell secretion of antigen-specific antibodies. As a comparison, immunization with the same vector titer of either FOVA/VSVG or FOVA/SVG generated less serum IgG (**Fig. 4f**). These results show that *in vivo* administration of a dendritic cell–targeting lentivector can be an effective tool to induce both cellular and humoral immune responses against the delivered antigen.

Generation of anti-tumor immunity

As a measure of the effectiveness of this mode of immunization, we evaluated the anti-tumor immunity generated after an *in vivo* administration of dendritic cell–targeted lentivector. We used the E.G7 tumor model[42], in which OVA serves as the tumor antigen. Mice receiving a subcutaneous administration of 50 × 10^6 TU of FOVA/SVGmu as a single dose tumor vaccination were challenged two weeks later with 5 × 10^6 E.G7 tumor cells. Vaccination with FOVA/SVGmu completely protected the mice from the tumor challenge (**Fig. 5a**, left), whereas tumors grew rapidly in mice receiving a mock vaccination with a lentivector lacking the OVA transgene (**Fig. 5a**, left). This protection was OVA-specific because the vaccinated mice grew control EL4 tumors that lack expression of OVA (**Fig. 5a**, right).

We then reversed the times of tumor injection and lentivector administration to test whether an established tumor could be

eliminated, in a test of so-called therapeutic vaccination. To this end, we used E.G7 tumor cells expressing the firefly luciferase gene (E.G7.luc) to challenge mice, allowing us to closely monitor tumor growth kinetics in live animals using bioluminescence imaging. To facilitate imaging, an albino strain of B6 mice was used. Injection of these mice with FOVA/SVGmu showed a similar response to that observed in canonical B6 mice (**Fig. 5b**). E.G7.luc tumor cells (5×10^6) were implanted subcutaneously in the albino B6 mice. The mice were immunized by FOVA/SVGmu (50×10^6 TU per mice per time) twice on days 3 and 10 post-tumor challenge by subcutaneous injection. The experiment was repeated three times with a representative experiment shown in **Fig. 5c**,**d**. The mice receiving the dendritic cell–targeting lentivector immunization showed a decline of tumor growth starting at day 9, followed by tumor regression and a reduction of luminescence below the detection level on day 11 (**Fig. 5c**,**d**). Although minimal tumor recurrence was observed from day 12 to day 16, mice treated with FOVA/SVGmu were free of disease at the end of day 18 and thereafter; no tumor relapse was observed for as long as the experiment ran (>60 d). In contrast, tumors grew progressively in the mice receiving no treatment and the mice had to be removed from the experiment after day 16 owing to the large size of the tumors. It was an interesting observation that tumor regression started 7 d after the lentivector immunization. This timing correlates well with the kinetics of an antigen-specific immune response induced by vaccination.

DISCUSSION

This study describes a method to deliver genes of antigenic proteins to dendritic cells both *in vitro* and *in vivo*. By using a vector coated with a protein selectively targeted to a dendritic cell surface protein, we have achieved high specificity, as measured *in vitro,* and effective T-cell immunization through *in vivo* delivery of the vectored genes. One measure of this effectiveness is the complete protection achieved against a very rapidly growing tumor expressing a single protein targeted by the T cells that were stimulated by vector administration to a wild-type mouse.

DC-SIGN is one of many potential receptors predominantly expressed on the surface of dendritic cells for antigen uptake. Many viruses are able to interact with DC-SIGN, and some use it as a receptor to mediate viral entry[43]. This suggested to us that the glycoprotein derived from Sindbis virus could be engineered to target a lentiviral vector to dendritic cells. Many alterations were introduced to the wild-type SVG, resulting in SVGmu, a protein unable to interact with cell-surface heparin sulfate but retaining the ability to interact with DC-SIGN. Lentivectors pseudotyped with SVGmu proved to be highly specific and effective in modifying both murine and human dendritic cells expressing DC-SIGN.

Direct subcutaneous injection of the engineered lentivector into animals was sufficient, in the absence of any adjuvant, to produce a rapid and florid enlargement of the lymph node near the injection site with a few percent of transduced dendritic cells detected within 3 d. Because the injection was subcutaneous, ~10 mm from the lymph node itself, it would appear that dermal dendritic cells were transduced by the vector and then migrated to the nearby lymph node. It seems likely that it is the infection process by which the dendritic cells are transduced that stimulates dendritic cells to mature and migrate. The capacity of lentivectors to transduce nondividing cells may be especially advantageous for effective delivery of antigen genes into dermal dendritic cells, which are considered to be poorly proliferative[44].

In a mouse tumor model, we found that direct administration of a lentivector carrying the gene encoding a tumor antigen could not only protect the mice from tumor challenge but also lead to a complete regression of established solid tumors. These studies show that this *in situ* dendritic cell immunization method has the potential to improve the efficacy of current immunotherapy protocols.

Quantitation of T-cell responses after administration of a single dose of a targeted lentivector into naive mice showed that the method could elicit high levels of antigen-specific $CD8^+$ T cells (up to 12%). It is considerably more efficient than conventional methods using recombinant protein antigen or adoptive transfer of antigen-loaded or viral vector-transduced dendritic cells (**Supplementary Fig. 6** online)[45,46]. Although the exact mechanisms behind such an efficient immunization are still under investigation, it seems likely that the lentivector transduction through DC-SIGN can stimulate the maturation of immature dendritic cells that are resident in the dermis. Whatever the cause, these cells must take up the vector, express its genes, process the encoded proteins, present the peptides on MHC molecules, migrate to the local lymph nodes, present their MHC–peptides to lymphocytes and activate the lymphocytes to become effectors. The independence from an additional maturation signal represents a significant advantage of our targeting method. Unlike our approach, the protocol of anti-DEC-205-mediated delivery of protein antigens to dendritic cells requires the co-administration of anti-CD40 antibody as a maturation stimulus for the induction of immunity[27]. With our method, phenotypic analysis of the induced antigen-specific T cells showed them to be typical effector memory cells, which is important for therapeutic vaccination. Further studies are needed to quantify the efficiency of generating central memory T cells by the dendritic cell–targeting lentivector, which is essential for protective vaccination. Significant antibody responses were also detected in our experiments, and we found that co-delivery of CD40L could further enhance the antibody response (data not shown).

There is much left to learn about this system. For instance, we used the highly immunogenic E.G7, OVA-expressing tumor to test immunity. How well would the method work with less immunogenic tumors, viral antigens or antigens of other pathogens? Experiments are underway on these issues. A key question is the specificity of the mutated Sindbis glycoprotein. There is controversy regarding the functional difference between human and murine DC-SIGNs, and several studies have shown that murine DC-SIGN lacks the ability to interact with viruses, as opposed to human DC-SIGN[47]. We know that DC-SIGN can be the receptor for the SVGmu-enveloped lentivector to transduce cells, but more experiments are needed to examine the possibility that other C-type lectin receptors closely related to DC-SIGN, such as the non-DC-specific L-SIGN for humans and SIGNR1-8 for mice[48], could mediate transduction. In fairly insensitive experiments, we saw no off-target effects, but that could be a property of the subcutaneous route of inoculation, and there might be cells inaccessible by that route which could be transduced.

In conclusion, we demonstrate here a specific method of targeting lentivectors to dendritic cells *in vivo* by direct subcutaneous administration that elicits strong antigen-specific immune responses. This method expands the scope of genetic modification of dendritic cells for vaccines and may overcome the current limitations of the tedious and costly protocols used to induce immunity through adoptive transfer of *in vitro*–manipulated dendritic cells. Further studies using dendritic cell–specific lentivectors encoding clinically relevant antigens are underway to evaluate the therapeutic utility of this method against cancer and infectious diseases.

METHODS

Mice. C57BL/6J (designated as B6) female mice were purchased from Charles River Breeding Laboratories. The albino B6 female mice (B6(Cg)-*Tyr*$^{c\text{-}2J}$/J), OT1 transgenic mice (C57BL/6-Tg(TcraTcrb)1100Mjb/J) and OT2 transgenic mice (C57BL/6-Tg(TcraTcrb)425Cbn/J) were purchased from The Jackson Laboratory. All mice were housed in the animal facility in accordance with Institute regulations.

Plasmid construction. The cDNA for wild-type SVG was cloned into the pcDNA3 vector (Invitrogen) by PCR to generate plasmid SVG. SVGmu was generated by PCR mutagenesis of the E2 glycorprotein of SVG to introduce a 10-residue tag sequence (MYPYDVPDYA) between amino acids 71 and 74, and mutations of 157KE158 into 157AA158. Additional deletion was introduced to the E3 glycoprotein of SVG to remove amino acids 61-64. The cDNA of firefly luciferase was amplified from pGL4.2LucP (Promega) and cloned into FUGW[39] to replace GFP, yielding the construct Fluc. FOVA was constructed from FUGW by replacing the GFP with the cDNA of chicken ovalbumin.

Lentivector production. The lentiviral transfer vectors (FUGW and its derivatives) used in this study are the third generation HIV-based lentiviral vectors, in which most of the U3 region of the 3′ LTR was deleted, resulting in a self-inactivating 3′-LTR or SIN. Lentivectors were prepared by transient transfection of 293T cells using a standard calcium phosphate precipitation protocol. 293T cells cultured in 6-cm tissue culture dishes (Corning or BD Biosciences) were transfected with the appropriate lentiviral transfer vector plasmid (5 μg), along with 2.5 μg of the envelope plasmid (SVG, SVGmu, Eco, or VSVG) and the packaging plasmids (pMDLg/pRRE and pRSV-Rev). The viral supernatants were harvested 48 and 72 h post-transfection and filtered through a 0.45-μm filter (Corning). To prepare concentrated viral vectors for *in vivo* study, the viral supernatants were ultracentrifugated (Optima L-80 K preparative ultracentrifuge, Beckman Coulter) at 50,000*g* for 90 min. The pellets were then resuspended in an appropriate volume of cold PBS.

Confocal imaging of GFP-vpr labeled lentiviral vectors. GFP-vpr-labeled lentivectors were produced as described above with an additional plasmid encoding GFP-vpr (2.5 μg). Fresh viral supernatant was overlaid on polylysine-coated coverslips in a 6-well culture dish and centrifuged at 3,700*g* at 4 °C for 2 h using a Sorvall Legend RT centrifuge. The coverslips were rinsed with cold PBS twice and immunostained by anti-HA-biotin antibody (Miltenyi Biotec) and Cy5-streptavidin (Invitrogen). Fluorescent images were acquired by a Zeiss LSM 510 laser scanning confocal microscope equipped with filter sets for fluorescein and Cy5. A plan-apochromat oil immersion objective (63×/1.4) was used for imaging.

Cell line construction. The 293T.hDCSIGN and 293T.mDCSIGN cell lines were generated by stable transduction of parental 293T cells with a VSVG-pseudotyped lentivector. The cDNAs for human DC-SIGN and murine DC-SIGN were amplified from plasmidspUNO-hDCSIGN1Aa andpUNO-mDCSIGN (InvivoGen) and cloned downstream of the human ubiquitin-C promoter in the lentiviral plasmid FUW to generate FUW-hDCSIGN and FUW-mDCSIGN, respectively. The lentivectors were then pseudotyped with VSVG and was used to transduce 293T cells. The resulting cells were subjected to antibody staining (anti-human DC-SIGN antibody from BD Biosciences and anti-murine DC-SIGN from eBioscience) and cell sorting to yield a uniform population of DC-SIGN$^+$ 293T.hDCSIGN and 293T.mDCSIGN cell lines.

Targeted lentivector transduction of cell lines *in vitro*. Target cells (293T.hDCSIGN, 293T.mDCSIGN, or 293T cells; 0.2 × 10^6 per well) were seeded in a 24-well culture dish (Corning or BD Biosciences) and spin-infected with viral supernatants (1 ml per well) at 2,500 r.p.m. and 30 °C for 90 min using a Sorvall Legend centrifuge. Subsequently, the supernatants were replaced with fresh culture medium and incubated for 3 d at 37 °C with 5% of CO_2. The percentage of GFP$^+$ cells was measured by flow cytometry. The transduction titer was determined by the dilution ranges that exhibited a linear response.

Mixed bone marrow and BMDC culture and transduction. Total bone marrow cells were harvested from B6 mouse and BMDCs were generated as previously described[42]. Either bone marrow cells or BMDCs were plated in a 24-well culture dish (2 × 10^6 cells per well), and spin-infected with viral supernatant (1 ml per well) at 2,500 r.p.m. and 30 °C for 90 min using a Sorvall RT7 centrifuge. After the spin, the supernatant was removed and replaced with fresh RPMI medium containing 10% FBS and GM-CSF (1:20 J558L conditioned medium). The cells were cultured for 3 d and were analyzed for GFP expression using flow cytometry.

Murine T-cell and B-cell transduction *in vitro*. Spleen cells were harvested from B6 mice, and cultured in a 24-well culture dish (2 × 10^6 cells per well) in RPMI containing 10% FBS for 3 d in the presence of T-cell stimuli (0.5 μg/ml anti-mouse CD3 + 0.5 μg/ml anti-mouse CD28, BD Biosciences), or B-cell stimuli (50 μg/ml LPS, Sigma). On day 1 and 2, the cells were spin infected with viral supernatant (1 ml per well) at 2,500 r.p.m. and 30 °C for 90 min. After each spin, the supernatant was removed and replaced with the T cell–specific or B cell–specific medium. On day 3 post-infection, the cells were analyzed for GFP expression using flow cytometry.

***In vivo* assay of targeting of dendritic cells by lentivector.** The recombinant and concentrated lentivector FUGW/SVGmu (50 × 10^6 TU resuspended in 200 μl PBS) was injected subcutaneously into the right flank of the C57BL/6 mice close to an inguinal lymph node (within 1 cm range). The right inguinal lymph node and the equivalent lymph node at the opposite site were isolated for size examination on day 3 post-injection. The cells were harvested from these nodes and their total numbers were counted. The percentage of GFP$^+$ dendritic cells was analyzed by flow cytometry on cells stained with anti-CD11c antibody (BD Biosciences).

***In vitro* dendritic cell stimulation of OT1 and OT2 T cells and functional assays.** The day 6 BMDCs were spin infected with viral supernatant, and cultured for an additional 3 d. On day 9, the nonadherent cells were collected and recultured in RPMI medium containing 10% FBS, GM-CSF (1:20 J558L conditional medium), and 1μg/ml LPS). On day 10, the cells were collected and used for T-cell stimulation. In parallel, nonadherent cells were collected from nontransduced day 9 BMDC culture, and were recultured in the same medium (RPMI containing 10% FBS, GM-CSF and LPS). On day 10, the cells were collected and loaded with either OVAp ($OVA_{257\text{-}269}$, recognized by OT1 TCR) or OVAp* ($OVA_{323\text{-}339}$, recognized by OT2 TCR), and used for T-cell stimulation. Spleen cells were collected from the OT1and OT2 transgenic mice and cultured with the lentivector-transduced BMDCs, or BMDCs loaded with either OVAp or OVAp*, at the indicated ratio. Three days later, the supernatant was collected and assayed for IFN-γ production using enzyme-linked immunosorbent assay (ELISA) and the cells were collected and analyzed for their surface activation markers using flow cytometry. T-cell proliferation was assayed using [^{3}H] thymidine incorporation.

***In vivo* immunization of naïve mice.** Wild-type B6 mice or albino B6 mice were given a single injection of targeting lentivector subcutaneously on the right flank at the indicated dose. On day 7 and day 14 post-immunization, blood was collected from the immunized mice through tail bleeding, and the serum anti-OVA IgG was measured using ELISA. On day 14, spleen and lymph node cells were harvested and analyzed for the presence of OVA-specific T cells and their surface activation markers using flow cytometry.

Tumor challenge study. The tumor cell lines EL4 (C57BL/6J, H-2^b, thymoma) and E.G7 (EL4 cells stably expressing one copy of chicken OVA cDNA)[42] were used for the tumor challenge of mice. For the tumor protection experiment, B6 mice received a single injection of 50 × 10^6 TU of the targeting lentivector on the right flank. Two weeks later, 5 × 10^6 EL4 or E.G7 cells were injected subcutaneously into the left flank of the mice. Tumor size was measured every other day using fine calipers and was shown as the product of the two largest perpendicular diameters $a \times b$ (mm^2). For the tumor eradication experiment, albino B6 mice were injected subcutaneously with 5 × 10^6 E.G7.luc cells (E.G7 cells stably expressing a firefly luciferase) on the left flank. On day 3 and day 10 post-tumor-challenge, the mice received 50 × 10^6 TU of targeting

lentivector through subcutaneous injection on the right flank. The tumor growth was monitored using bioluminescence imaging. In both experiments, the mice were killed when the tumors reached 400 mm^2.

Note: Supplementary information is available on the Nature Biotechnology website.

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AUTHOR CONTRIBUTIONS

L.Y. and H.Y. designed research, performed experiments, discussed the results, wrote the paper. K.R., T.C., K.J., L.Z., A.E., A.W., D.Y. performed experiments. D.B. designed research, discussed the results, wrote the paper and provided financial support. P.W. designed research, performed experiments, discussed results, wrote the paper, provided financial support and coordinated the whole project.

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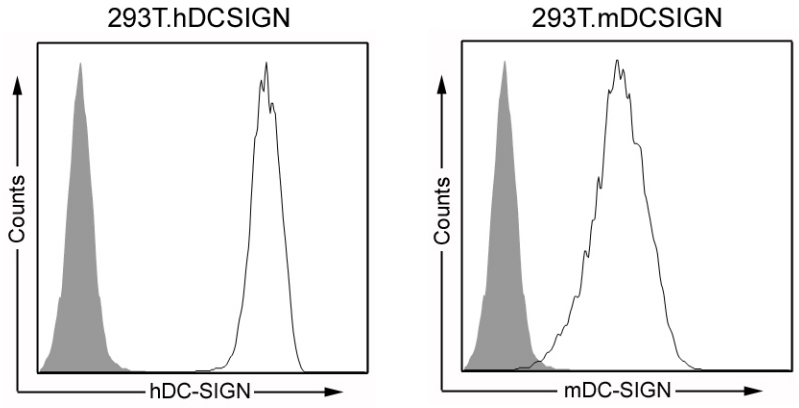
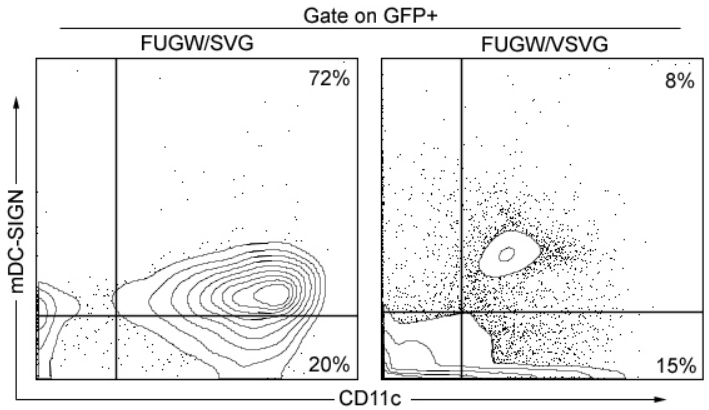
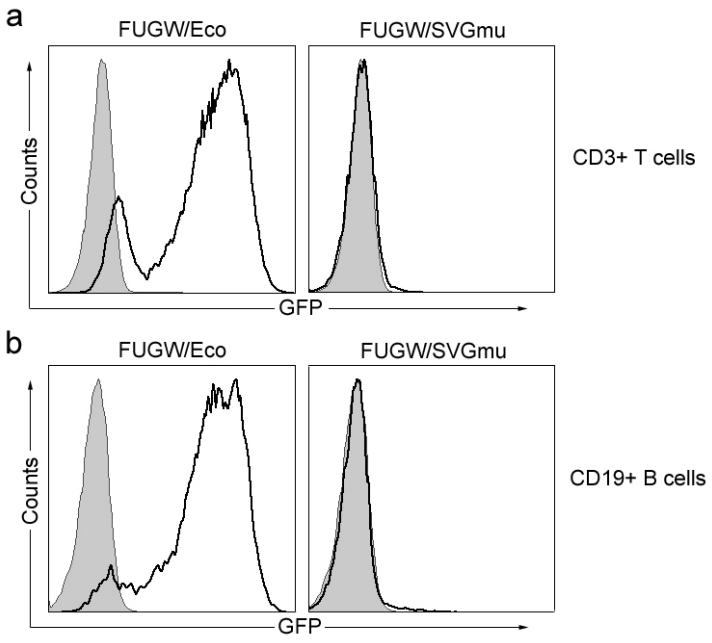
Supplementary Figures



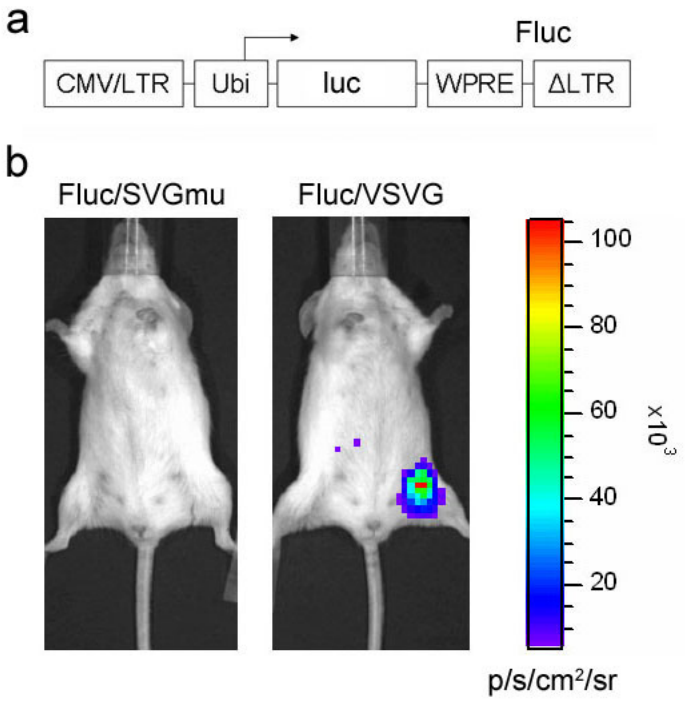
Supplementary Figure 1: Flow cytometry analysis of constructed target cell lines 293T.hDCSIGN expressing human DC-SIGN, and 293T.mDCSIGN expressing murine DC-SIGN. DC-SIGN expression was detected with either anti-human DC-SIGN or anti-murine DC-SIGN antibodies. Solid line, expression of DC-SIGN in target cell lines; shaded area, background staining in parental 293T cells.



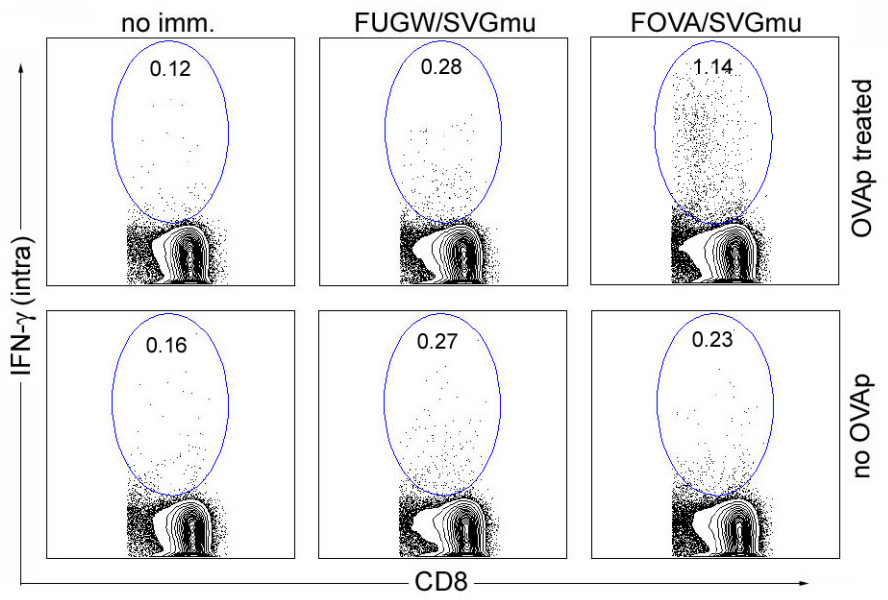
Supplementary Figure 2: Whole bone marrow cells isolated from B6 mice were exposed to the fresh viral supernatant of FUGW/SVG or FUGW/VSVG. Three days post-transduction, the cells were collected for flow cytometric analysis of GFP expression. Surface antigens of the GFP-positive cells were assessed by staining with anti-CD11c and anti-DC-SIGN antibodies.



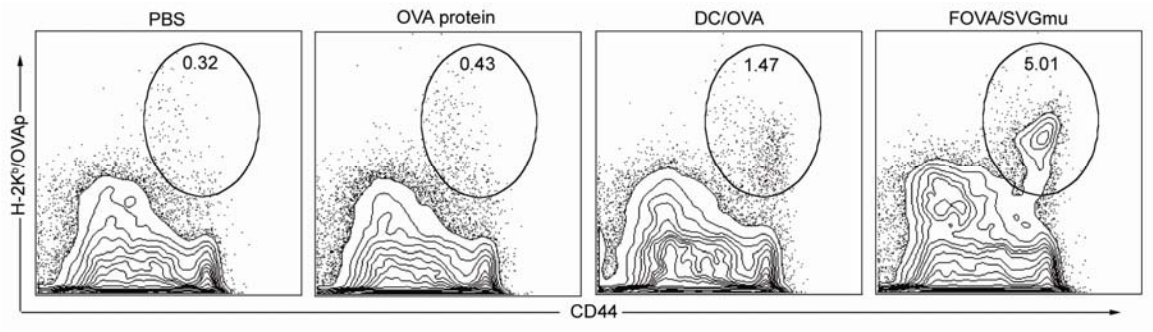
Supplementary Figure 3: Primary T cells ($CD3^+$, **a**) and B cells ($CD19^+$, **b**) isolated from the mouse spleen were transduced with the fresh viral supernatant of either the targeting FUGW/SVGmu or non-targeting FUGW/Eco vector. GFP expression was analyzed by flow cytometry. Solid line, cells exposed to indicated lentiviral vector; shaded area, cells without transduction (as a negative control).



Supplementary Figure 4: (**a**) A schematic representation of the lentivector (Fluc) encoding an imaging gene (firefly luciferase). (**b**) Bioluminescence imaging of mice injected subcutaneously with $50x10^6$ TU of either the DC-specific Fluc/SVGmu lentivector or the non-specific Fluc/VSVG lentivector. The representative image was obtained at day 30 post-injection using IVIS200 (Xenogen).



Supplementary Figure 5: Wild-type B6 mice were immunized by subcutaneous injection of $50x10^6$ TU of the DC-specific lentivector FOVA/SVGmu. Mice injected with the same dose of FUGW/SVGmu were included as a control. Two weeks later, the spleen cells were harvested and analyzed by intracellular IFN-γ staining with or without OVAp peptide restimulation. Indicated percentages are the percent of IFN-γ^+CD8^+ T cells.



Supplementary Figure 6: Naïve B6 mice were immunized by subcutaneous injection of OVA protein (100 μg), or $1x10^6$ DCs that were transduced by FOVA/Eco, or $50x10^6$ TU of FOVA/SVGmu. Mice injected with PBS were included as a control. Two weeks later, spleen cells were harvested and analyzed for the presence of OVA-specific T cells measured by H-2K^b-SIINFEKL-PE tetramer and CD44 staining. Indicated percentages are a percent of total CD8$^+$ T cells.

Supplementary Methods

Live animal imaging. We anesthetized mice and injected them intraperitoneally with D-luciferin (3 mg/mouse, Xenogen). The mice were placed in the chamber of the IVIS200 imaging instrument (Xenogen). The images were obtained using a cooled IVIS CCD camera and analyzed with Living Imaging 2.50 Software (Xenogen). We performed data acquisition after 10 min of D-luciferin injection.

Antibodies and FACS Analysis. Fluorochrome-conjugated antibodies specific for mouse CD11c, CD4, CD8, CD25, CD69, CD62L, CD44, TCRVα2, and TCRVβ5.1,5.2 were purchased from BD Biosciences. Human DC-SIGN antibody was purchased from BD Bioscience. Mouse DC-SIGN antibody was purchased from eBioscience. H-2K^b/OVAp tetramer was purchased from Beckman Coulter. Surface staining was performed by blocking with anti-CD16/CD32 (mouse Fc receptor, BD Biosciences) followed by staining with fluorochrome-conjugated antibodies. Intracellular staining of IFN-γ was performed using the Cytofix/Cytoperm Kit from BD Pharmingen following the protocol provided by the manufacturer. FACScan and FACSort (BD Bioscience) were used for all the flow cytometry analysis.

Biodistribution analysis of mice injected with the targeting lentivector. Mice received subcutaneous injection of the recombinant lentivector Fluc/SVGmu (50x10^6 TU). One month later mice were sacrificed by CO_2 inhalation and various organs were isolated for genomic DNA exaction using a DNeasy kit (Qiagen) following the manufacture's

protocol. Detection of vector integration was performed by using SYBRgreen real-time PCR kit (Qiagen) and a Bio-Rad MyiQ real-time PCR detection system. The firefly luciferase-specific primers for the analysis were Flucfw (5'-CCACGCTGGGCTACTTGATC-3') and Flucbw (5'-GCAAGAATAGCTCCTCCTCGAA-3').

Serum Anti-OVA IgG ELISA. Mouse sera at serial 10-fold dilutions, starting from 1:100, were added to ELISA plates (96-well C bottom, Nunc) pre-coated with 10μg/ml of chicken egg albumin (Sigma). The plates were then washed and the bound IgG were detected with biotinylated goat anti-mouse IgG (Vector), streptavidin conjugated HRP (R&D), and TMB peroxidase substrate (Kirkegaard and Perry). The TMB reaction was stopped by the addition of 2M H_2SO_4 at a uniform time point for each set of the assay, and the absorbance at 450nm was assayed using a VERSAmax plate reader (Molecular Devices). Titer values were assigned as the highest dilution at which the optical density was 2 SDs higher than the optical density of the control serum at equivalent dilutions.

IFN-γ ELISA. Cell culture supernatant was added to ELISA plates (96-well C bottom, Nunc) pre-coated with 1μg/ml of anti-mouse IFN-γ antibody (Clone R4-6A2, BD Biosciences). The plates were then washed and the captured mouse IFN-γ was detected with biotinylated anti-mouse IFN-γ antibody (Clone XMG1.2, BD Biosciences), streptavidin conjugated AKP and SIGMA FAST^{TM} p-Nitrophenyl Phosphate Tablets (Sigma). The absorbance at 405nm was assayed using a VERSAmax plate reader (Molecular Devices) with the maximal value not exceeding 1.500.

Intracellular IFNγ Staining. Mouse spleen or lymph node cells were cultured in 24-well plate ($2x10^6$ cells per well) in RPMI containing 10% FBS and 0.1μg/ml OVAp in the presence of 0.67μg/ml of GolgiStop™ (BD Biosciences) for 6 hours. The cells were then collected and stained for surface markers, followed by intracellular staining of IFN-γ using the Cytofix/Cytoperm Kit from BD Biosciences following the protocol provided by the manufacturer, and analyzed using flow cytometry.

T Cell Proliferation Assay. Mouse T cells were stimulated for 3 days. In the last 12 hours, [^{3}H]thymidine was added at 0.01mCi/ml (1Ci= 37 GBq). The proliferation was measured by [^{3}H]thymidine incorporation using a Wallac (Gaithersburg, MD) ^{3}H counter.