Supporting Information

Backer et al. 10.1073/pnas.0909541107

SI Materials and Methods

Splenectomy and Autotransplantation of the Spleen. Mice were anesthetized using 2% isofluorane (Forene; Abbott). The splenic vessels were ligated by 5.0 monophilic suture (Prolene; Ethicon) and the spleen was excised. Peritoneal cavity and skin were closed by 5.0 natural silk (catgut). For autotransplantation the spleen was removed, divided into four parts, and placed between pancreas, kidney, and renal blood vessels.

Adenovirus Infection, Tumor Injection, and In Vivo Imaging. Mice were infected with 5×10^9 VP recombinant adenovirus expressing OVA, GFP, and luciferase (AdLOG), recombinant adenovirus expressing GFP and the melanoma tumor Ag TRP2 (AdgfpTRP2), or 5×10^7 pfu recombinant adenovirus expressing ovalbumin (1). B16 melanoma tumor cells expressing luciferase, ovalbumin, and GFP were provided by Günther J. Hämmerling (Heidelberg, Germany). Mice were injected with 5×10^4 cells/50 μL into the portal vein. In vivo imaging was performed by using IVIS200 (Xenogen). Mice were injected with 200 μL of 50 mM luciferine potassium salt (Caliper Life Science) and then anesthetized with 2% isofluorane.

In Vivo Cytotoxicity Assay. $CFSE^{high}$, specific target cells were spleen cells pulsed with 1 μM $OVA_{257–264}$ peptide for 35 min and labeled with 1 μM CFSE (Invitrogen) for 20 min at 37°C. $CFSE^{low}$, nonspecific target cells were labeled with 0.1 μM CFSE. The two target populations were mixed in equal numbers and injected i.v. into effector mice. Mice were killed 4 h later and the ratio between peptide-loaded and empty target cells was quantified by flow cytometry.

Depletion of Macrophages and Dendritic Cells. Mice were injected i.v. with 200 μL Cl_2MBP (clodronate; Roche Diagnostics)-containing liposomes (2). After 7 days, AdLOG or mAb-OVA (2.5 μg/mouse plus 25 μg/mouse αCD40 mAb) was injected i.v. and, 16 h later, DCs were isolated and ex vivo Ag-presentation assays were performed. Data were summarized by the mean and standard error of the mean (SEM). DCs were depleted in CD11cDTR mice by injecting 800 ng diphtheria toxin. After 24 h, AdLOG was injected in these mice and the induction of in vivo cytotoxicity was determined.

PTx Treatment of Mice. Mice were injected i.p. with 500 ng/mouse pertussis toxin (from *Bordetella pertussis*) (Sigma). After 8 h, mice were immunized with mAb-OVA and, 16 h later, $CD11c^+$ DCs were isolated for an ex vivo Ag-presentation assay.

Immunizations and Detection of T-Cell Responses. Mice were injected with 1 μg mAb-OVA plus 25 μg activating αCD40 mAb (1C10) intravenously. After 7 days, splenocytes were restimulated in vitro for 5 h in the presence of Brefeldin A (Golgiplug; BD Biosciences) and MHC class I $OVA_{257–264}$ peptide (100 ng/mL) for $CD8^+$ T cells. $CD4^+$ T cells were incubated with MHC class II $OVA_{323–339}$ peptide (100 μg/mL) for 2 days at 37°C followed by 5 h incubation with Brefeldin A. Cells were stained with CD11a-FITC and CD8-PE or CD4-PE, and for intracellular cytokine expression using IFNγ-allophycocyanin (BD Pharmingen) and reagents provided in the Cytofix/Cytoperm kit according to the manufacturer's instructions (BD Pharmingen). The percentage of IFNγ-producing T cells was measured by flow cytometry (Becton Dickinson). Data were summarized by the mean and standard error of the mean.

Isolation of T Cells. $CD8^+$ T cells and $CD4^+$ T cells were purified from lymph nodes and spleens from OT-I and OT-II transgenic mice, respectively, by negative depletion using bead-based T-cell isolation kits (Dynal Biotech ASA) following the manufacturer's protocols. Purity of T-cell preparations was between 80% and 90%.

DC Isolation and Ex Vivo Ag-Presentation Assays. Mice were injected i.v. with 2.5 μg/mouse mAb-OVA together with 25 μg/mouse αCD40. After 16 h, DCs were purified by positive selection with anti-CD11c MACS microbeads (Miltenyi Biotec), and $CD8^+$ and $CD8^-$ DC subsets were purified by FACS sorting as previously described (3). Sorting for $CD8^-$ and $CD8^+$ DC populations resulted in 85–90% pure populations. To detect OVA presentation, 3×10^5 (plus 1:3 titrations) $CD11c^+$ DCs or FACS-sorted DCs were coincubated with 10^5 OT-I $CD8^+$ T cells or OT-II $CD4^+$ T cells in triplicate in flat-bottomed 96-well plates (Greiner). Cells were cultured in Iscove's modified Dulbecco's medium (Gibco) with supplements as described before (3). After 48 h incubation, plates were pulsed for 16 h with 1 μCi/well of [^{3}H]thymidine. [^{3}H]Thymidine incorporation was measured on an LKB Wallac Betaplate 1205 liquid scintillation counter. Data were summarized by the mean and standard error of the mean.

In Vitro Ag-Presentation Assay. MACS-purified DCs were cocultured together with purified OT-I or OT-II T cells in the presence of a dose range of mAb-OVA (10–0.37 μg/mL, 3-fold dilutions). Proliferation was detected by [^{3}H]thymidine incorporation and data were summarized as described above.

Coupling of Ovalbumin to mAbs. Rat IgG mAb MOMA-1 (specific for Siglec-1), ED31 (specific for MARCO), F4/80, NLDC145 (specific for DEC205), 33D1 (specific for DCIR2), R7D4 (negative control recognizing an idiotypic determinant on a mouse B-cell lymphoma), and Armenian hamster IgG 22D1 (specific for SIGN-R1, generously provided by Dr. C. G. Park, New York) were purified from culture supernatant by protein G. Desalted mAbs (10 mg/mL) were coupled to a No-Weight Sulfo-SMCC linker (Pierce) in phosphate buffer (pH 7.2) at RT for 30 min. LPS-free OVA (10 mg/mL; Seikagaku) was treated with 5 mM DTT for 10 min at RT. After this, Ab-SMCC and OVA-DTT were desalted and incubated in a 1:1 weight ratio in phosphate buffer (pH 7.2) overnight at 4°C. The coupling reaction was stopped by adding 1 mM L-cysteine solution. Noncoupled OVA was removed by protein-G purification.

OVA ELISA. The efficiency of OVA coupling to mAb was determined by ELISA. MaxiSorp plates (Nunc) were coated with 1 μg/mL mAb-OVA or various concentrations of soluble OVA at 37°C for 1 h. After blockade with 10% (wt/vol) newborn calf serum, polyclonal rabbit-αOVA mAb was added followed by goat anti-rabbit-horseradish peroxidase for detection. The efficiency of OVA labeling differed between mAbs. R7D4, 33D1, and NLDC145 bound about 0.19–0.29 μg OVA per 1 μg total protein. Labeling efficiencies of mAbs targeting Mφ subsets were around 0.03 μg OVA per 1 μg total protein. For experiments, we decided to use similar amounts of total mAb-OVA complexes, although this indicated at least six times less OVA in the Mφ-targeted complexes compared to DC-targeted complexes.

Confocal Microscopy. Spleens from mice injected with mAb-OVA (20 μg mAb-OVA + 25 μg αCD40 mAb 1C10) or control B6 mice were embedded in O.C.T. medium (Tissue-Tek; Sakura). Cryosections (7 μm) were fixed in acetone for 2 min, air-dried, and blocked in 5% (vol/vol) mouse serum in PBS for 10 min

before staining with mAbs at RT. Cryosections were incubated with the mAbs SER4, recognizing a noncompeting epitope on Siglec-1, MOMA-1, ED31, 22D1, F4/80, anti-CD11c-bio, anti-hamster IgG, anti-GFP-bio, and rabbit anti-OVA for 30 min, followed by streptavidin-Alexa647, anti-rat IgG, or anti-rabbit IgG. Confocal laser-scanning images were acquired on a Leica TCS-SP2-AOBS system (Leica Microsystems) with 488-, 543-, and 633-nm excitation and Leica confocal software.

1. Schweichel D, et al. (2006) Evaluation of DNA vaccination with recombinant adenoviruses using bioluminescence imaging of antigen expression: Impact of application routes and delivery with dendritic cells. *J Gene Med* 8:1243–1250.
2. Van Rooijen N, Sanders A (1994) Liposome mediated depletion of macrophages: Mechanism of action, preparation of liposomes and applications. *J Immunol Methods* 174:83–93.
3. den Haan JMM, Bevan MJ (2002) Constitutive versus activation-dependent cross-presentation of immune complexes by CD8(+) and CD8(–) dendritic cells in vivo. *J Exp Med* 196:817–827.

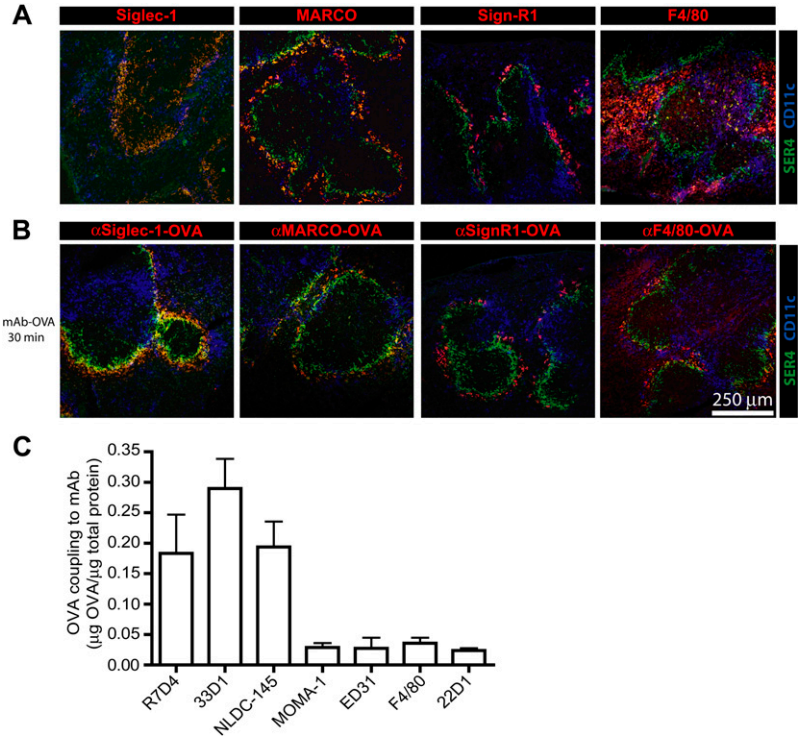

Fig. S1. Specific Mφ subset targeting with mAb-OVA complexes. (*A*) To determine the localization of different Mφ subsets, spleen sections of mice were stained with anti-Siglec-1 (MOMA-1), anti-MARCO (ED31), anti-SIGN-R1 (22D1), and F4/80 Ab (red). Sections were counterstained with anti-CD11c (blue) and SER4 (green) to detect DCs and MMM. Original magnifications: ×20. (*B*) The specificity of OVA-conjugated mAbs was tested by injecting mice i.v. with 20 μg mAb-OVA. Spleens were isolated after 30 min, and the location of indicated mAb-OVA was determined by staining with αrat or αhamster IgG (for 22D1) (red). Sections were counterstained with αCD11c (blue) and SER4 (green). Original magnifications: ×20. (*C*) OVA was covalently conjugated to mAbs by an SMCC linker. Free OVA was removed by protein-G purification and the efficiency of OVA coupling was determined by an ELISA-based assay. Bars indicate the amount of OVA bound to the indicated mAb (μg/μg total protein). R7D4 is a control mAb; NLDC-145 recognizes DEC205. 33D1 is specific for DCIR2. MOMA-1 is specific for Siglec-1. ED31 recognizes MARCO, and 22D1 is specific for SIGN-R1.

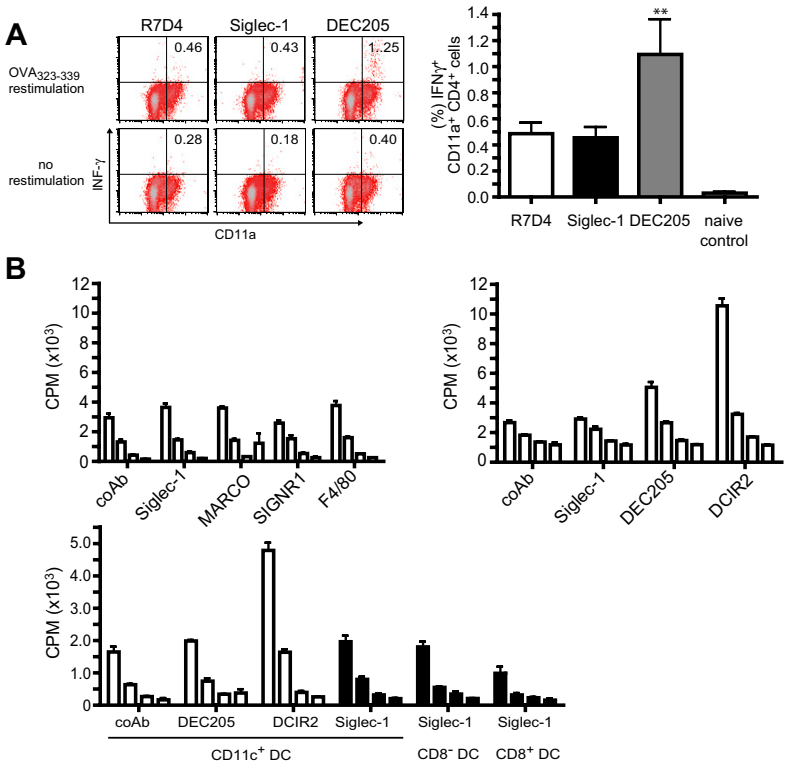

Fig. S2. Targeting to MMM does not result in CD4$^+$ T-cell responses or Ag presentation to CD4$^+$ T cells in vitro. (*A*) Mice were immunized with 1 μg/mouse αSiglec-1-OVA, αDEC205-OVA, or control mAb-OVA. After 7 days, splenocytes were restimulated in vitro with 100 μg/mL MHC class II $OVA_{323–339}$ peptide for 2 days, subsequently followed by 5-h incubation with Brefeldin A and FACS analysis of IFNγ-producing CD4$^+$CD11a$^+$ T cells. (*B*) Mice were injected with 2.5 μg/mouse of the indicated mAb-OVA complexes together with 25 μg αCD40 Ab. CD11c$^+$ DCs were isolated after 16 h. CD11c^{high} CD8$^-$ and CD8$^+$ DCs were FACS-sorted from αSiglec-1-OVA immunized mice and tested for their ability to stimulate naive OT-II cells. After 2 days, cultures were pulsed with 1 μCi [^{3}H]thymidine/well and T-cell proliferation was measured after an additional coculture of 16 h. Different bars indicate titration of DCs in the assay (1×10^5 – till 3700 DCs/well). Error bars indicate SEM of triplicate wells.

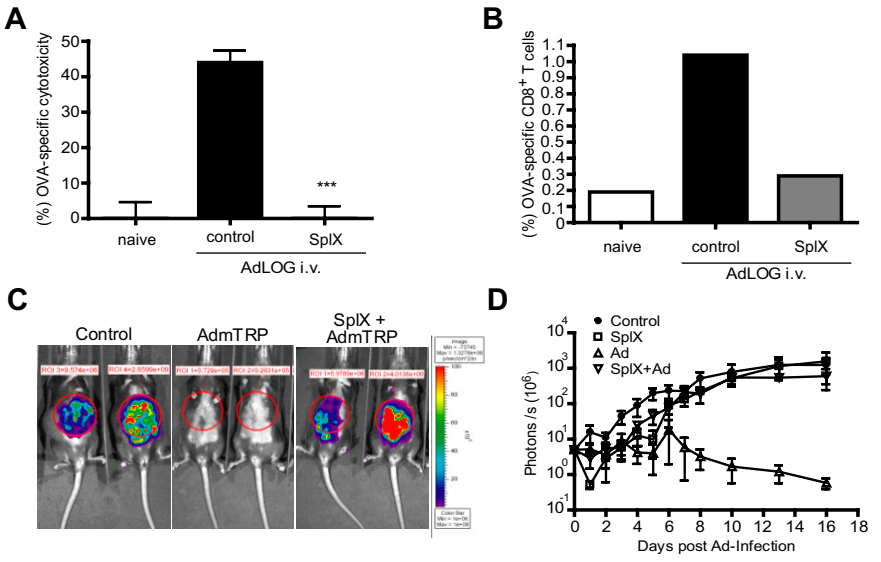

Fig. S3. Antitumor CTL responses induced by adenoviral infection are abrogated in mice lacking the spleen. (*A*) Mice were splenectomized or left untreated. Three days after surgery, mice were infected i.v. with 5×10^7 pfu AdOVA. In vivo cytotoxicity was determined in blood 5 days after infection. (*B*) Quantification of OVA-specific CD8$^+$ T cells. Cells from spleens were isolated and stained for CD8 and MHC pentamer H-2Kb/SIINFEKL 3 days after i.v. infection with AdOVA. Graph shows the percentage of OVA-specific CD8$^+$ T cells. Error bars indicate SEM, $n = 3$ mice per group (SplX $n = 4$). ***$P < 0.01$ versus control, calculated by *t* test (GraphPad Prism 4). (*C* and *D*) Mice were splenectomized or left untreated. Seven days after surgery, mice were injected with B16 melanoma cells expressing luciferase in the portal vein. In addition, mice were infected i.v. with 5×10^9 VP AdmTRP. Tumor growth was monitored every second day by in vivo imaging as depicted in *C* and summarized in *D* (IVIS200; Xenogen).

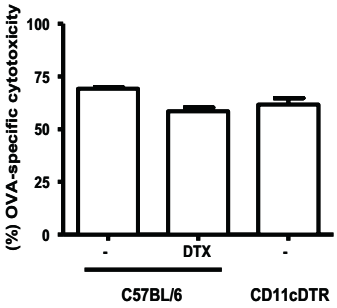

Fig. S4. Diphtheria toxin-treated C57BL/6 mice and nontreated CD11cDTR mice exhibit normal CTL responses after adenoviral infection. C57BL/6 mice were injected with 800 ng diphtheria toxin. After 24 h, mice were infected i.v. with 5×10^9 VP AdLOG. In vivo cytotoxicity was determined 5 days after infection. Error bars indicate SEM, $n = 3$ mice per group.