CD169+ macrophages are sufficient for priming of CTLs with specificities left out by cross-priming dendritic cells

Caroline A. Bernhard*, Christine Ried*, Stefan Kochanekb, and Thomas Brockera,1

*Institute for Immunology, Ludwig-Maximilian University of Munich, 80336 Munich, Germany and bDepartment of Gene Therapy, University of Ulm, 89081 Ulm, Germany

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Dendritic cells (DCs) are considered the most potent antigen-presenting cells (APCs), which directly prime or cross-prime MHC I-restricted cytotoxic T cells (CTLs). However, recent evidence suggests the existence of other, as-yet unidentified APCs also able to prime T cells. To identify those APCs, we used adenoviral (rAd) vectors, which do not infect DCs but selectively accumulate in CD169+ macrophages (MPs). In mice that lack DCs, infection of CD169+ MPs was sufficient to prime CTLs specific for all epitopes tested. In contrast, CTL responses relying exclusively on cross-presenting DCs were biased to selected strong MHC I-binding peptides only. When both DCs and MPs were absent, no CTL responses could be elicited. Therefore, CD169+ MPs can be considered APCs that significantly contribute to CTL responses.

Results

Adenoviral Targeting of CD169+ MPs. To dissect the relative contributions of DCs and MPs to CTL priming, we compared a mouse line that lacks DCs only in secondary lymphoid organs with a mouse line that lacks both DCs and MPs in those organs. As described previously, Δ-DC mice constitutively express diptheria toxin α-chain (DTA) under control of a loxP-flanked stop cassette in the ROSA26 locus, which is removed on breeding to CD11c-Cre mice, leading to DTA-mediated ablation of conventional DCs and plasmacytoid DCs (3). As a consequence, Δ-DC mice lack DCs, but harbor regular MPs, such as marginal zone SIGN-R1+ and CD169+ MPs, and red pulp F4/80+ MPs of the spleen (3) (Fig. 1A). In contrast, CD11c-DTR mice express the receptor for DTA constitutively on all CD11c+ cells and, on DTA treatment, lack CD11c+ DCs and all types of MPs in the spleen, as described previously (10, 11) (Fig. 1A). Subcapsular CD169+ MPs were present in the lymph nodes of Δ-DC mice, whereas CD11c+ DCs and CD11c+CD169+ DCs were not, but all cell types were

Significance

Although we know much about the molecular mechanisms of cross-presentation, its actual contribution to cytotoxic T cell (CTL) immunity under physiological conditions in vivo is still unclear. Cross-presentation is based on the idea that dendritic cells (DCs) are the only professional antigen-presenting cells able to prime naive T cells. If DCs are not directly infected, they must take up antigen and present it indirectly. However, recent evidence suggests that other cells also may be involved in T cell priming, which probably makes cross-presentation less central. This study shows that cross-priming DCs generate highly restricted CTL repertoires, biased to strong MHC I binding epitopes only. Furthermore, the presence of antigen in CD169+ macrophages is sufficient for generation of CTLs with broader repertoires.

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1To whom correspondence should be addressed. E-mail: tbrocker@med.uni-muenchen.de.

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missing in CD11c-DTR mice (Fig. S1A). Thus, these two mouse lines are differentially deprived of APC subsets that are key to pathogen handling and Ag presentation.

To assess the CTL-priming capacities of MPs and DCs without the confounding effects of viral spread and pathogenicity, we injected C57Bl/6 (B6) mice with GFP-expressing recombinant adenovirus particles (rAd-GFP) that are unable to replicate. Histological analyses showed that these particles are selectively trapped by marginal zone CD169+ MPs but not by CD11c+ DCs or any other cells of the spleen (Fig. 1B), just as described previously for WT adenovirus (9, 16). Both CD169+ and SIGN-R1+ MPs of the marginal zone expressed rAd-encoded GFP (Fig. 1B, Lower). Importantly, spleens of Δ-DC mice showed the same distribution of rAd-directed GFP expression as WT mice, and both CD169+ and SIGN-R1+ MPs, but no other cells, expressed GFP (Fig. IC). The analysis of GFP expression kinetics showed decreasing GFP intensity over time, but with localization remaining confined to MPs at later time points as well (Fig. S1B). These data indicated that transcription of rAd-encoded genes occurred exclusively in MPs in the spleen, irrespective of the presence or absence of DCs.

Given that the metallophilic CD169+ MPs of the marginal zone are likely the first cells in the secondary lymphoid organs that get in touch with pathogens and their products, we asked whether they are susceptible to TLR ligands. On LPS injection, DCs increased the surface expression of CD80, CD86, and MHCII, whereas CD169+ MPs selectively up-regulated MHC and costimulatory molecules (Fig. 1A, Upper), with CD80 peculiarly excepted.

This ability to up-regulate MHC and costimulatory molecules, together with the fact that CD169+ MPs, but not DCs, selectively accumulate rAd vectors, suggests the possibility that CD169+ MPs could act as APCs for T cells independent of DCs.

Infection of Marginal Zone CD169+ MPs Leads to Priming of CD8+ T Cells in the Absence of DCs. With the foregoing tools established, we asked whether directly infected CD169+ MPs are able to induce CTL responses in the presence or absence of DCs. To this end, we injected mice with rAd encoding for glycoprotein (GP) from lymphocytic choriomeningitis virus (LCMV) (17) and tested for the priming of CTLs with three different MHC multimers: Kb/GP33–41 (GP33), Dp/GP33, and Dp/GP276–286 (GP276). Interestingly, the GP33 peptide can bind to Kβ and Dp, although it has a 1,000-fold higher binding affinity to Kβ (18–20). The GP276 peptide binds Dp with an intermediate affinity and triggers weaker responses, likely owing to low precursor frequencies (20–22). As expected (2), CD11c-DTR mice did not mount any measurable CTL responses above background, indicating that DCs, MPs, or both are central to CTL priming. In contrast, Δ-DC mice generated CTLs specific for all three epitopes tested despite their lack of DCs (Fig. 2A, Upper). In vitro restimulation with the respective peptides showed that CTLs primed in Δ-DC and WT mice displayed comparable cytokine secretion capacities (Fig. 2A, Lower). These data suggest that rAd-encoded gene products are presented by cells other than DCs, most likely CD169+ MPs.

To extend these analyses to other Ags, we used rAd vectors expressing cytosolic chicken ovalbumin (OVA), which harbors three peptides with immunodominance patterns correlated directly with their binding affinity to Kβ. The immunodominant OVA257–264 (OVA257) binds 12- to 25-fold better to Kβ compared with the subdominant peptide OVA255–62 (OVA55) (23, 24). In addition, a third epitope OVA87–114 (OVA107) with much weaker binding capacities to Kβ is known (25). On rAd-OVA infection, Δ-DC mice primed CTLs specific for Kβ/OVA257 and Kβ/OVA55 as detected with the respective MHC peptide multimers (Fig. 2B, Upper). Intracellular cytokine detection on in vitro restimulation with the three peptides revealed an immunodominance hierarchy similar to that in WT mice (Fig. 2B, Lower). Moreover, CD11c-DTR mice could not mount any responses. Taken together, our data demonstrate that CTLs specific for various
epitopes can be primed in the absence of DCs as well. Considering that CD169+ MPs up-regulate costimulatory markers and are the only cells that also express Ag, they most likely prime these CTLs.

A caveat of the system is that Δ-DC mice have only one-half as many T cells as WT mice, so that numbers of specific CTLs were reduced by one-half. To correct for this, we adoptively transferred identical numbers of allogeneically marked D9/GP33-specific P14 or K9/OVA257-specific OTI TCR-transgenic T cells and analyzed their expansion on rAd-GP or rAd-OVA injection (Fig. 2C). P14 and OTI T cells expanded in Δ-DC mice as they did in WT recipients, whereas neither were activated in CD11c-DTR-mice (Fig. 2C). These findings suggest that despite the absence of DCs, the presence of MPs and infection of CD169+ MPs (Fig. 1C) are sufficient for the priming of CTLs. In contrast, when both DCs and MPs were depleted in CD11c-DTR-mice, no CTL responses could be measured (Fig. 2C). One potential reason for this failure in CD11c-DTR mice could be the lack of primary Ag handling by MPs of the marginal zone (Fig. 1B); however, histological analyses revealed that rAd-directed GFP expression was also present in spleens and lymph nodes of DTA-treated CD11c-DTR mice, but at different localizations than in organs of MP-sufficient mice (Fig. S1D). GFP expression was detected in deeper zones of lymph nodes (Fig. S1D) rather than being confined to few subcapsular CD169+ MPs as observed in WT nodes (Fig. S1 B and C). In the spleens of CD11c-DTR mice, GFP expression was seen in only a few cells of the marginal zone, with the majority found in the red pulp area (Fig. S1D). Therefore, although localized differently, rAd-encoded Ag was present in spleens and lymph nodes of DC- and MP-depleted CD11c-DTR-mice, but could not be presented for CTL priming. The fact that it worked in Δ-DC mice indicated that infection of MPs alone was sufficient for CTL responses.

DCs Selectively Cross-Prime CTLs Specific for Strong Epitopes Only. Our results presented so far show that presence of infected MPs is sufficient to prime CTL responses in the absence of DCs. However, they do not reveal whether CTL priming by MPs and DCs is just redundant or qualitatively different. To determine whether the two APC types prime CTLs of different specificities, we used mice in which only DCs and thymic epithelial cells, and no other cell types, express MHC-I molecules (26). These animals generate a normal CD8+ T-cell repertoire that can be primed only by MHCI+ DCs (27). Using this model, we previously showed that DCs are sufficient to prime different virus-specific CTL responses (27–29) and to cross-present the model Ag OVA to OTI T cells (26). Surprisingly, DC-MHCI mice injected with rAd-GP mounted nearly exclusively K9/OVA257-specific CTL responses, with strongly reduced D9/GP33-specific responses and undetectable D9/GP276-specific responses (Fig. 3A). A comparison of the distribution of CTL specificities as detected by MHC multimers in WT, Δ-DC, and DC-MHCI mice revealed...
that CTL responses induced in that absence of DCs (Δ-DC mice) or presence of DCs and MPs (WT mice) had a very similar composition (Fig. 3A); however, DCs alone (DC-MHCI) preferentially primed CTLs specific for the strongest of the three GP epitopes, K\(^{b}\)/GP33, but were entirely unable to prime D\(^{b}\)/GP33-specific CTLs (Fig. 3A and B). The K\(^{b}\)/GP33-specific CTLs were fully functional, as demonstrated by their capacity to produce IFN-γ. In addition, after an increase in the precursor frequencies of specific CTLs by adoptive transfer of carboxyfluorescein succinimidyl ester (CFSE)-labeled D\(^{b}\)/GP33-specific P14 T cells, no priming by DCs was detected in DC-MHCI recipients (Fig. 3C).

The distribution of rAd-encoded proteins in DC-MHCI mice, as determined by histological analyses of rAd-GFP injection, matched the Ag distribution in WT mice (Fig. 1B) and was restricted to CD169\(^{+}\) MPs of the marginal zone. From this, we concluded that the GP33 peptide was most likely transferred from CD169\(^{+}\) MPs to DCs for cross-presentation. Apparently, via this route the GP33 peptide is presented only by K\(^{b}\), to which it has a much higher affinity compared with D\(^{b}\). Our findings support the possibility that DCs cross-present only peptides of the highest affinity when Ag is acquired from MPs.

To put this hypothesis to a test, we analyzed CTL responses to rAd-OVA with its OVA257 peptide, which binds to K\(^{b}\) with more than 10-fold higher affinity than OVA55 does (23). We detected CTLs specific for both epitopes in WT mice, whereas DC-MHCI mice mounted only CTLs specific for K\(^{b}\)/OVA257, and not those specific for K\(^{b}\)/OVA55 (Fig. 3D and Fig. S2 A and B). These experiments also revealed that rAd-OVA–immunized WT mice primed functional CTLs to the very weakly binding OVA107 peptide, which were not induced by cross-priming in DC-MHCI mice (Fig. 3D and Fig. S2B).

Our data thus extend the evidence supporting the hypothesis that DCs present only high-affinity peptides from Ags handed over from MPs. This conclusion is based on the assumption that in DC-MHCI mice, the directly infected MPs are negative for MHCI and are unable to directly prime CTLs. Indeed, CD169\(^{+}\) MPs of DC-MHCI mice did express very low levels of MHCI, probably too low for CTL priming, whereas DCs of DC-MHCI mice expressed WT MHCI surface levels (Fig. 3E). Taken together, our results indicate that the repertoire induced by cross-presenting DCs are very narrow and biased toward high-affinity peptides from Ags handed over from MPs; however, this gap in the repertoire is filled, and those CTLs spared by DCs are primed, when MPs accumulate Ags and express MHCI class I (Fig. 3 A and D).

**Spleen DCs Cannot Cross-Present D\(^{b}\)/GP33 in Vitro.** To further analyze the Ag-presenting capacities of DCs, we isolated DCs from spleens of rAd-injected mice and tested them in vitro for their capacities to prime specific T cells. DCs from rAd-OVA-injected B6 and DC-MHCI mice could prime OVA257-specific CD8\(^{+}\) OTI T cells equally well, as when loaded with synthetic pOVA257 directly (Fig. 4 A, Left). In marked contrast, DCs from rAd-GP33–immunized mice did not stimulate P14 T cells in vitro even if the DCs originated from either strain, whereas peptide loading worked for both (Fig. 4 A, Right). These results suggest that DCs are able to cross-present K\(^{b}\)/OVA257 following OVA acquisition from CD169\(^{+}\) MPs, but not D\(^{b}\)/GP33.

A simple explanation for this finding is that DCs are not able to generate the complex at all or destroy it proteolytically. Therefore, we tested whether DCs can be infected in vitro with rAd-GFP, and detected a low but reproducible infection rate of ~1% (Fig. 4B, Upper). In addition, rAd-GP33–infected DCs could efficiently trigger proliferation of P14 T cells (Fig. 4B, Lower). These results confirm our in vivo findings that DCs do not cross-present the D\(^{b}\)/GP33 epitope on rAd-GP33 injection (Fig. 3 C and D), although they do so when provided as preprocessed peptide (Fig. 4 A) or when infected directly with rAd-GP33 (Fig. 4B).

To test whether infection or external loading of DCs would also lead to the priming of P14 T cells in vivo, we infected mice with LCMV, which infects spleen DCs directly (30). Indeed, adoptively transferred P14 T cells were primed with similar efficiency in both B6 and DC-MHCI mice, corroborating our previous results (28) (Fig. 4C). Moreover, when mice were immunized with GP33 and LPS, CFSE-labeled P14 T cells proliferated similarly in the two strains (Fig. 4 D). Taken together, these data suggest that DCs are able to prime D\(^{b}\)/GP33-specific CTLs when directly infected, but not by cross-presentation from external sources.

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**Fig. 3.** DCs cross-prime CTLs against high-affinity MHC I/peptide complexes only. (A) Spleens of rAd-GP–infected mice were analyzed as described in Fig. 2 with respective MHC multimers. Pie charts show the frequency of CTLs with the respective specificities in the different mice. Data are representative of more than three independent experiments with three mice per group. (B) Analysis of D\(^{b}\)/GP33- and K\(^{b}\)/GP33-specific T cells in WT and DC-MHCI mice at 8 d after i.v. injection of rAd-GP33. Bar graphs indicate frequencies of specific CTLs as well as total numbers. (C) Adoptive transfer of 2 × 10\(^{6}\) CFSE-labeled P14 T cells into mice and immunization with rAd-GP33. Three days later, the CFSE profiles of CD8\(^{+}\)CD25\(^{-}\) P14 T cells were monitored; numbers represent SE. Data show percentages from three mice per group and are representative of two independent experiments. Data were analyzed using the unpaired two-tailed Student t test. (D) Pie charts displaying the distribution of CTLs with the indicated specificities at 8 d after immunization with rAd-OVA. Data are representative of more than three independent experiments with three mice per group. (E) Analysis of MHCI expression on DCs and CD169\(^{+}\) MPs from spleens of untreated B6 mice, DC-MHCI mice, and MHCI KO mice. Data are from one of three independent experiments with similar results.
In summary, although infection of CD169+ MPs may be important, nonredundant APCs of similar significance as DCs. The necessity for cross-presentation is based on the idea that DCs are the only professional APCs able to prime naïve T cells (1). If Ag is not available to DCs directly, then DCs must take it up and present it indirectly. However, recent evidence suggests that other, as-yet unidentified cells might be involved in CD8 T-cell priming as well (11). Such priming by other cells would possibly make cross-presentation by DCs less important. Our findings support this view. We demonstrate that CTL repertoire induced by cross-priming DCs have only very restricted specificities for a few strong epitopes. Normal CTL responses can be mounted despite a complete lack of DCs. When MHCI+ CD169+ MPs of the marginal zone do capture Ag, CTLs specific for all epitopes tested can be primed, as well as those spared out by cross-presenting DCs. Therefore, we identified CD169+ MPs as important, nonredundant APCs of similar significance as DCs. In summary, although infection of CD169+ MPs leads to generation of CTLs specific for all epitopes tested for, cross-presenting DCs additionally strengthen only a few CTL specificities from a broad repertoire.

Owing to its deficiency for DCs, the Δ-DC mouse model is a valuable tool for analyzing the contribution of MPs to CTL priming, given the impossibility of isolating viable marginal zone MPs for functional T-cell stimulation assays in vitro (12). MPs in general have received much attention recently. Initially considered scavenger cells and pathogen filters, they were thought to have little or no T-cell priming capability; however, they were found to modulate immunity via cytokine production and recruitment. Their Ag-handling capacities remained restricted to Ag redistribution to DCs and B cells. Although it is not possible to isolate marginal zone MPs for a direct demonstration of their APC capacities in vitro, our findings indirectly support the notion that they also might be able to prime CTLs directly, for several reasons. First, we found that virus-encoded Ag colocalizes selectively in CD169+ MPs. Although Ag detection by microscopy most likely is not sensitive enough to exclude the presence of low levels of DC infection, this is insufficient for priming of CTLs specific for weaker epitopes, such as CD14 T cells. Second, in theory CD169+ MP-borne Ag also could reach other cells via the exogenous cross-presentation route, as reported previously for CD8+ DCs (9); however, DCs are generally considered the most potent cross-presenting APCs in vivo (4, 31) and a cross-presenting non-DC able to more potentyl prime CTLs also specific for weak epitopes is currently unknown. Third, we show that exclusive cross-presentation by DCs generates CTL-responses specific for just two of the six epitopes studied, whereas in Δ-DC mice the presence of infected MPs is sufficient for priming of CTLs to all epitopes derived from OVA and LCMV-GP. Taken together, our results support the interpretation that CD169+ MPs are sufficient to prime CTLs also specific for weaker epitopes.

CD169+ MPs are rather sessile in the splenic marginal zone or subcapsular sinus of lymph nodes; therefore, it is conceivable that CD8+ T-cell priming by MPs requires T-cell migration to the marginal zone or lymph node sinus areas. Although such interactions have been observed during memory responses in vivo (32), whether interactions with naive CD8+ T cells may also occur there is unclear. Blood-borne Ag is first trapped by CD169+ marginal zone MPs, making a role for CD169+ MPs in early CTL-priming events rather likely. Thereafter, additional cross-priming may occur once Ag reaches CD8+ DCs in T-cell areas.

Previous studies showed that APC functions of CD169+ MPs are limited to the immunodominant epitope Kb/OVA257 (12, 32), which also can be cross-presented efficiently, but redundantly, by DCs. In contrast, we demonstrate that CD169+ MPs also prime CTLs specific for weaker epitopes that cannot be cross-primed by DCs. Such a division of labor might occur selectively in situations where DCs either are not directly infected or do not receive sufficient amounts of Ag necessary for cross-presentation (33). This is the case with the rAd vectors used in the present study, but might be different for other infectious agents that preferentially target DCs.
Accordingly, we did not observe direct priming of $K\beta$/OVA257-specific CTLs in $\Delta$-DC mice immunized with MVA-OVA (3), a vaccine that depends on infection of DCs for CTL immunity (34).

Cross-presentation depends on high doses of Ag (33). In particular, LCMV-GP is a relatively instable protein with a short half-life (30). Furthermore, GP33-41 is located in the signal sequence of the protein, which might further reduce epitope stability (35), explaining why this peptide is notoriously inefficiently cross-presented. Initial studies using cells expressing high doses of GP or stable variants of peptides as a source for cross-presenting Ag were able to trigger GP33 cross-presentation (36, 37). Most of those studies did not differentiate between $D\beta$/GP33- and $K\beta$/GP33-specific CTLs (reviewed in refs. 14, 15), however, and thus $K\beta$/GP33-specific CTLs might have been overlooked in some cases.

The observed difference in cross-presentation efficiency of GP33 compared with OVA257 stimulated a debate more than a decade ago on the relative importance vs. the very restricted contribution of $D\beta$- and $K\beta$-peptide presentation. The most likely explanation for the cross-presentation preferences of GP33 via $K\beta$ but not $D\beta$ is the machinery of cross-presenting DCs. The most likely explanation for the observed difference in cross-presentation efficiency of GP33 vs $K\beta$ but not $D\beta$ is the higher affinity under conditions where the Ag quantity is limiting. This might also explain why $K\beta$/OVA257 is cross-presented efficiently compared with $K\beta$/OVA55 and $K\beta$/OVA107. In general, weakly binding peptides are poorly cross-presented (38).

Our findings also raise the question of whether similar rules apply for peripheral tolerance induction by cross-presentation. We previously showed that DCs are sufficient to cross-present self-Ag for tolerance induction of CD8+ T cells (26, 39). It is possible that such a bias for strong epitopes also might restrict peripheral cross-tolerance induction.

Although we were able to show that CTLs primed by DCs and CD169+ MPs have different specificities, it remains to be clarified whether those CTLs induced by cross-priming vs. direct priming or by DCs vs. MPs are qualitatively equal with respect to longevity and memory formation. Thus, the precise analysis of CTL responses initiated by different APC types might greatly advance our knowledge for vaccine improvement.

Materials and Methods

All mice were bred and maintained at the animal facility of the Institute for Immunology, Ludwig-Maximilian University of Munich. Unless stated otherwise, mice were immunized i.v. with the respective rAd vectors (1 x 10^9 particles). Flow cytometry was performed with a FACS Canto flow cytometer (BD Biosciences), and data analysis was conducted using FlowJo software (Tree Star).

More detailed information is provided in SI Materials and Methods.

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Supporting Information

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SI Materials and Methods

Mouse Strains. All mice were bred and maintained at the animal facility of the Institute for Immunology, Ludwig-Maximilian University of Munich. C57BL/6 and P14-mice [Tg(TcrLCMV)327Sdz] were obtained from The Jackson Laboratory. DC-MHCI mice (1, 2), Δ-DC mice (3), and CD11c-DTR mice (4) have been described previously. Δ-DC mice were bred to the C57BL/6 background for more than 12 generations and did not develop autoimmune symptoms before age 12 wk. Recipient mice were used for experiments at age 6–8 wk. T-cell donor mice were used at age 6–12 wk and were sex- and age- matched within experiments. Animal experiment were performed in accordance with the guidelines of the local Ethical Committee.

Adenovirus Vectors. The replication deficient adenovirus vectors used in this study were derived from human adenovirus type 5 (HAdV-5) and were deleted in early regions 1 and 3 (Dealta E1/ E3). All cDNAs coding for the different proteins/antigens were under transcriptional control of the hCMV promoter. rAd-GP expresses the LCMV-GP from LCMV strain WE, rAd-OVA expresses chicken ovalbumin in the cytoplasm, rAd-GFP expresses EGFP, and rAd-GP33 expresses the gp33-41 peptide of LCMV embedded in the Env protein of MoMVL.

mAbs and Flow Cytometry. mAbs were purchased from BD Biosciences or eBioscience. Violet Fluorescent Reactive Dye (Invitrogen) was used for staining of live cells. MHC multimers were purchased from Beckman Coulter or TCMetrix. Flow cytometry was performed on a FACSCanto flow cytometer (BD Biosciences), and data analysis was conducted using FlowJo software (Tree Star).

Adaptive Cell Transfer, CFSE Labeling, and in Vivo Cytotoxic Activity Assay. These assays were performed as described previously (5).

Immunizations. Mice were immunized i.v. with 10 μg of rpGP33-41 (NeoMPS) in conjunction with 20 μg of LPS (Sigma-Aldrich), or with replication-deficient recombinant rAd-GP33, rAd-GP, rAd-OVA (6), or rAd-GFP (7) (1 × 106 particles). Ag-specific T cells were restimulated with 1 μg/mL of the indicated peptide in conjunction with anti-CD107a for 1 h, followed by a 3-h incubation in the presence of Brefeldin A (10 μg/mL; Sigma-Aldrich). Cells were surface-stained for 30 min at 4 °C. Intracellular staining for IFN-γ was performed using a Cytotox/Cytoperm Kit (BD Biosciences) according to the manufacturer’s protocol.

Bone Marrow DC Cultures and Infection. Generation of DCs from bone marrow cultures was performed according to standard procedures in the presence of 20 ng/mL GM-CSF. Cells were infected with rAd on day 3 of the culture with a multiplicity of infection of 2 or 5.

Ex Vivo Proliferation Assay. At 24 h after rAd injection, the mice were killed, and DCs from spleens were isolated with CD11c MACS beads (Miltenyi Biotec). Then 2.5 × 107 DCs were cocultured with 5 × 105 P14 or OTI T cells for 3 h at 37 °C in 5% CO2 in the presence of IL-2 (17 × 103 U/mL; ImmunoTools). To determine the absolute number of live cells per culture, a known number of CompBeads (BD Biosciences) was added directly to cell cultures before harvesting for flow cytometry analysis. The proportion of cells in each division was calculated from live cells and bead counts.

Histology. Spleens or skin-draining lymph nodes were embedded in OCT medium (Miles) and snap-frozen. Then 6-μm sections were cut with a cryostat (Jung Frigocut 2800 E; Leica). For the detection of GFP expression, organs were fixed in 2% PFA for 2 h, washed overnight in PBS, embedded in OCT medium, snap-frozen, and cut with a cryostat. Amplification of eGFP was performed with the Tyramide Signal Amplification Kit (PerkinElmer) according to the manufacturer’s recommendations. Sections were analyzed with a Leica DMXA-RF8 microscope (Leica acquisition program QFISH) equipped with a Sensys CCD camera (Photometrix). Images shown are representative of three different experiments with three animals each.

Statistical Analysis. Data were analyzed using the unpaired two-tailed Student t test. Differences in mean values were considered significant at a P < 0.05.

Fig. S1. Immunofluorescence analysis of spleen sections from C57BL/6 and CD11c-DTR mice infected with rAd-GFP. (A) (Left) Representative micrographs of inguinal lymph nodes from C57BL/6 control, Δ-DC, and DTA–treated CD11c-DTR mice analyzed for CD11c, B220, and CD169. (Right) Flow cytometry analysis of skin-draining (inguinal and axillary) lymph nodes from WT control (C57BL/6) and Δ-DC mice. Gates in FACS blots identify CD11c−CD169−DCs, CD11c+CD169+DCs, and CD11c+CD169+MPs. Bar graphs indicate frequencies and total cell numbers of the indicated population in the respective gates. (B) Representative micrographs of spleens from C57BL/6 mice at 48 h after rAd-GFP infection. CD169+SIGN-R1 (blue) or CD169+ marginal zone macrophages and marginal metallophilic macrophages of the marginal zone. (C) Representative micrographs of lymph nodes from C57BL/6 mice infected 4 d (Left) or 7 d (Right) earlier with rAd-GFP (i.v.). At all time points, GFP expression (green) colocalizes with SIGN-R1 (blue) or CD169 (red) marginal zone macrophages and marginal metallophilic macrophages of the marginal zone. (C) Representative micrographs of lymph nodes from C57BL/6 mice at 48 h after rAd-GFP infection. CD169+ subcapsular macrophages (red) are the primary targets for rAd-GFP and express GFP. (D) Representative micrographs of lymph nodes (Left) and spleens (Right) from C57BL/6 and CD11c-DTR mice at 48 h after immunization with rAd-GFP.
Fig. S2. (A) C57BL/6 and DC-MHCI mice were immunized with rAd-OVA i.v., and at 8 d after infection, Ag-specific T cells were identified from spleen cell suspensions with the corresponding MHC multimers as CD19− (not shown), CD8+ Kb-OVA257+, or CD8+ Kb-OVA55+ cells. Nonimmunized C57BL/6 mice served as controls. Results are shown as percentage of multimer-specific CD8+ T cells and total cell numbers. Data are representative of more than three independent experiments with three mice per group. (B) For functional analyses, spleen cells were cultured in vitro with the indicated peptides in the presence of anti-CD107a, and cells were stained for CD8 and IFN-γ. Bar graphs indicate frequencies or total numbers of CD107a+IFN-γ+CD8+ T cells. Data are representative of more than three independent experiments with similar results with three mice per group.