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Priming of CD8 T Cells by Adenoviral Vectors Is Critically Dependent on B7 and Dendritic Cells but Only Partially Dependent on CD28 Ligation on CD8 T Cells

Karen N. Nielsen, Maria A. Steffensen, Jan P. Christensen, and Allan R. Thomsen

Adenoviral vectors have long been forerunners in the development of effective CD8 T cell–based vaccines; therefore, it is imperative that we understand the factors controlling the induction of robust and long-lasting transgene-specific immune responses by these vectors. In this study, we investigated the organ sites, molecules, and cell subsets that play a critical role in the priming of transgene-specific CD8 T cells after vaccination with a replication-deficient adenoviral vector. Using a human adenovirus serotype 5 (Ad5) vector and genetically engineered mice, we found that CD80 and/or CD103 dendritic cells in the draining lymph node played a critical role in the priming of Ad5-induced CD8 T cell responses. Moreover, we found that CD80/86, but not CD28, was essential for efficient generation of both primary effectors and memory CD8 T cells. Interestingly, the lack of CD28 expression resulted in a delayed primary response, whereas memory CD8 T cells generated in CD28-deficient mice appeared almost normal in terms of both phenotype and effector cytokine profile, but they exhibited a significantly reduced proliferative capacity upon secondary challenge while retaining immediate in vivo effector capabilities: in vivo cytotoxicity and short-term in vivo protective capacity. Overall, our data point to an absolute requirement for professional APCs and the expression of the costimulatory molecules CD80/86 for efficient CD8 T cell priming by adenoviral vectors. Additionally, our results suggest the existence of an alternative receptor for CD80/86, which may substitute, in part, for CD28.

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The online version of this article contains supplemental material.

Abbreviations used in this article: Ad5, adenovirus serotype 5; B6, C57BL/6; chAd3, chimpanzee adenovirus serotype 3 (chAd3), and chimpanzee adenovirus serotype 63 (chAd63) (1–5). Therefore, it is of vital importance that we understand the factors leading to the induction of a robust and long-lasting transgene-specific immune response by these vectors. In general, adenoviral vectors induce substantial numbers of primary effector cells, which peak between days 14 and 25 after vaccination, depending on the inserted transgene, the type of adenovirus, and the route of vaccination (2, 6, 7) and contract into a memory population that is maintained, in part, by a sustained low-level Ag expression in vector-transduced cells (8–10). In terms of memory cell quality, a number of studies point to the fact that low doses of Ad5 induce high-quality memory cells, whereas high doses induce poor-quality memory cells (7); however, the initial activation of primary effectors appears to be similar, regardless of vector dose (11–13). Dendritic cells (DCs) are thought to be essential for the induction of antiviral CD8 T cell responses, and the CD8 and CD103 DC subsets were shown to be particularly adept in Ag presentation to naive CD8 T cells, with both subsets having the ability to cross-present extracellular Ag on MHC class I molecules. CD103 and CD8 DCs share origin and function; however, although CD8 DCs are found mainly in lymphoid organs, such as thymus, lymph nodes (LN), and spleen, CD103 DCs are found in both peripheral organs (e.g., skin, intestine) and secondary lymphoid organs, probably because they serve to transport Ag from the peripheral sites of Ag encounter to the lymphoid organs (14–16). The CD8 DC subset was shown to be the main spleen-derived subset driving adenovirus-induced CD8 T cell proliferation in vitro (17); however, it has not yet been shown whether this is also true in vivo. Surprisingly, a recent study using bone marrow chimeras indicated that, although the absence of hematopoietic APCs delayed primary CD8 T cell expansion, nonhematopoietic APCs were sufficient to induce normal numbers of transgene-specific memory CD8 T cells (18). However, these results are quite novel and need to be verified through the use of other experimental approaches. Upon activation, professional APCs, such as DCs, express the costimulatory molecules CD80/86 that bind to CD28 on the surface of T cells, and this costimulatory pathway was shown to be essential for CD8 T cell priming in several infection/vaccination settings (19–21). Interestingly, some pathogens may elicit strong immune responses, even in the absence of CD28 expression (22–25), a finding initially interpreted to mean that these responses functioned independently of CD28 costimulation as a result of prolonged TCR signaling (26–28). However, it was later shown that, for some of these pathogens, the independence...
of CD28 did not necessarily translate into an independence of CD80/86, pointing to the existence of another stimulatory receptor for CD80/86 on T cells that is either equally expressed or may take over in the absence of CD28 (19, 29–32). The relative importance of CD80/86 and CD28 expression on DCs and T cells, respectively, for adenoviral vector–induced CD8 T cell responses is not known.

The draining LNs (DLNs) are thought to be the main site for Ag presentation to naive T cells; indeed, Bassett et al. (18) recently showed that, by surgically removing the DLNs shortly after vaccination, they could prevent the induction of an immune response by a recombinant adenoviral vector injected i.m. This was taken to indicate that the DLN was essential for priming. However, this experimental approach is seriously flawed, because physical removal of the DLN is likely to disrupt the normal trafficking of Ag-loaded APCs and could cause the elimination of relevant T cells, events that together would substantially reduce the likelihood of productive APC/T cell interactions in alternative priming sites (e.g., spleen or bone marrow) (33).

In the current study, we investigated the organ sites, molecules, and cell subsets involved in the priming of transgene-specific CD8 T cells after vaccination with a replication-deficient adenoviral vector. We chose the human Ad5 vector as a model vector because it represents a highly immunogenic class of adenoviral vectors, resembling both chAd3 and chAd63 with regard to magnitude, kinetics, and protective capacity of the induced CD8 T cell response (2, 5). We found that impaired LN homing prevented efficient priming after s.c. Ad5 vaccination. In terms of cell subsets and molecular components, we found that CD8 and/or CD103+ DCs play a critical role in the priming of Ad5-induced CD8 T cell responses and, moreover, that CD80/86, but not CD28, was essential for efficient generation of both primary effectors and memory CD8 T cells. Interestingly, the lack of CD28 expression caused a delayed primary response, whereas memory CD8 T cells generated in CD28−/− mice appeared almost normal in terms of both phenotype and effector cytokine profile; however, they exhibited a significantly reduced proliferative capacity upon secondary challenge while retaining both cytotoxicity and immediate in vivo protective capacity. CD28, although redundant for the generation of memory cells specific for the immunodominant epitope, seemed to be critical for the functionality of subdominant responses, as shown by a lack of protection in vaccinated CD28−/− mice upon challenge with a pathogen that did not express the dominant epitope. Overall, our data point to an absolute requirement of CD80/86 and CD28 expression on DCs and T cells for productive APC/T cell interactions in alternative priming sites (e.g., spleen or bone marrow) (33).

Materials and Methods

Mice

Female C57BL/6 (B6; CD45.2) mice were obtained from Taconic Farms (Ry, Denmark), and B6.SJL (CD45.1), CD28 deficient (CD28−/−), L-selectin deficient (L-sel−/−) and CD80/86 deficient (CD80/86−/−) deficient mice, all on a B6 background, were bred locally from breeding pairs originally obtained from The Jackson Laboratory (Bar Harbor, ME). BATF3-deficient (BATF3−/−) and OX40-deficient (OX40−/−) mice came directly from The Jackson Laboratory, whereas MHC class II−deficient (MHCIId−/−) mice on a B6 background were obtained from Taconic Farms. All mice were housed in a specific pathogen–free facility, and animals from external sources were always allowed to acclimatize to the local environment for at least a week before entering into experiments, by which time the animals were typically between 7 and 9 wk old. All experiments were approved by the local animal ethics council and performed in accordance with national guidelines on animal experiments.

Adenoviral vectors and vaccination

Replication-deficient Ad5 expressing the nucleoprotein (NP) of lymphoctic choriomeningitis virus (LCMV) was produced as described previously (34). Adenoviral particles were purified using standard methods, aliquotted, and frozen at −80°C in 10% glycerol. The NP insert was verified by sequencing. Infectivity of the adenovirus stock was determined with the Adeno-X Rapid Titer Kit (Clontech Laboratories, Mountain View, CA). Mice to be vaccinated were anesthetized and injected s.c. with 2 × 108 infectious units (IFU) in 30 μl PBS in the hind footpad.

Viruses

LCMV Armstrong strain (LCMV Arm) and LCMV Arm NP396 nil were originally provided by M.B.A. Oldstone (The Scripps Research Institute, La Jolla, CA) and further propagated in-house using BHK cells. For infection of mice, an i.p. dose of 2 × 106 PFU or an intracerebral (i.c.) dose of 20–100 PFU was used. For i.c. infection, mice were anesthetized before inoculation and followed closely until they exhibited signs of irreversible disease, at which time they were euthanized. Recombinant vaccinia virus expressing NP of LCMV originally was obtained from Dr. D.H.L. Bishop (Oxford University, Oxford, U.K.) via Annette Oxenuks (ETH Zürich, Zürich, Switzerland) and grown on CV-1 cells at low multiplicity of infection; quantification was performed as described previously. Mice to be infected with vaccinia virus were injected i.p. with 2 × 106 PFU in 300 μl PBS.

Organ virus titers

To determine virus titers in organs, they were homogenized in PBS to yield 10% organ suspensions, and viral titers were determined using an immune focus assay, as previously described (35).

 Bone marrow chimeras

Mixed bone marrow chimeras were produced using CD28−/− and wild-type (WT) donor mice. Recipients were lethally irradiated (9 Gy) in the afternoon and the following day were transplanted i.v. with 10 × 106 femur cells mixed from WT and CD28−/− donors in a 1:1 ratio. Eight weeks later, the mice were vaccinated with Ad5-NP, as described above.

Splenocyte preparation

Splenocytes from mice were removed aseptically and transferred to HBSS. Single-cell suspensions were obtained by pressing the spleens through a fine steel mesh (70 μm), followed by centrifugation and two washes in HBSS before resuspension in RPMI 1640 cell culture medium containing 10% FCS supplemented with NaHCO3, 2-ME, l-glutamine, and penicillin-streptomycin.

Flow cytometry

Frequencies of epitope specific CD8+ T cells were determined by intracellular cytokine staining (ICS) or surface staining using NP-specific tetramers. For surface staining, cells were stained with Abs for PerCP-Cy5.5–CD8 and FITC–CD44, FTTC–CD27, PE–KLRL1, PE–Cy7–CD127, permeabilized, and stained using Abs for intracellular cytokines (allophycocyanin–IFN-γ, PE–IL-2, PE–Cy7–TNF-α), for evaluation of degranulation, Alexa Fluor 488–CD107a/b was present throughout the 5-h incubation period. Samples were run on an LSR II flow cytometer (BD Biosciences) and analyzed using FlowJo software (TreeStar). On the flow cytometer, live lymphocytes were initially identified by size and granularity in the forward/side scatter, followed by subgating for single cells and CD8 expression. IFN-γ+ CD8 T cells were sub gated further for analysis of phenotypic markers or additional cytokines (Supplemental Fig. 1).

In vivo cytotoxicity assay

Splenocytes from naive B6.SJL mice were incubated with NP396 or irrelevant peptide for control (10 μg/spleen). Following incubation for 30 min at 37°C, the cells were washed and stained with 2 μM CFSE (NP396) or left unstained (control). Following another washing step, the labeled cells were mixed in a 1:1 ratio, and 2 × 105 cells were injected i.v. into Ad5-NP–vaccinated WT or CD28−/− recipient mice (C57BL/6 background); 16 h later, recipient splenocytes were isolated, and target cells were identified in the flow cytometer by the expression of CD45.1 and...
The percentage of killing was calculated using the following equation: 100 − [(percentage of LCMV peptide–labeled cells in infected mice/percentage of cells labeled with irrelevant peptide in infected mice)−(percentage of LCMV peptide–labeled cells in uninfected mice/percentage of cells labeled with irrelevant peptide in uninfected mice)] × 100.

**Statistical evaluation**

A nonparametric Mann–Whitney U test was used to compare quantitative data. GraphPad Prism version 6 was used for statistical analysis.

**Results**

**Lack of CD28 expression delays the primary CD8 T cell response toward adenovirus-encoded Ag but does not affect numbers of memory cells generated**

Based on prior findings obtained in the context of i.v. infection with live replicating adenovirus (37), we hypothesized that the primary immune response induced by replication-deficient adenovirus would be diminished or delayed in mice lacking CD28 expression; however, we did not know whether the lack of CD28 would cause a reduction in the number of memory T cells generated. To investigate this, we vaccinated CD28−/− mice and WT mice with Ad5-NP and enumerated Ag-specific (NP396) IFN-γ-producing CD8 T cells in the spleen at various time points after vaccination. Fourteen days after vaccination (Fig. 1A, 1B), numbers of NP-specific primary effector CD8 T cells in CD28−/− mice were significantly reduced compared with those in matched WT mice. However, at 60 d after vaccination, memory cell numbers were comparable in WT and CD28−/− mice (Fig. 1A, 1B). Detailed analysis revealed that the reduction in numbers of primary effectors found in CD28−/− mice on day 14 postvaccination reflected a delayed primary response (with a peak in CD28−/− mice around days 20–27 as opposed to days 11–14 in WT mice), which was followed by contraction of the NP-specific CD8 T cell pool, leaving roughly similar numbers of splenic memory CD8 T cells in both genotypes 2 mo after vaccination (Fig. 2A). To exclude that the delayed response in CD28−/− mice simply reflected deficient effector cell differentiation rather than delayed T cell stimulation and expansion, we also enumerated Ag-specific CD8 T cells through tetramer staining on day 14 after vaccination; however, matching results were obtained whether Ag-specific CD8 T cells were enumerated using ICS or tetramer staining, indicating that T cell expansion was truly delayed in CD28−/− mice (Fig. 1C).

**Memory cells generated in CD28−/− mice after adenoviral vaccination retain short-term effector functions but have a diminished recall capacity**

Although numbers of memory CD8 T cells generated after adenoviral vaccination did not appear to be affected by the lack of CD28 expression, we speculated that there might be phenotypic differences and/or functional impairments in the memory compartment as a result of a lack of CD28-B7 ligation during priming, as previously reported for infection with live replicating LCMV (19). Consequently, additional analysis was performed to compare the phenotype of Ag-specific cells generated in CD28−/− and WT mice.

Regarding our choice of phenotypic markers, we based our analysis on the model introduced by Joshi et al. (38), in which Ag-specific CD8 T cells can be separated into four subsets defined by their expression of IL-7R and KLRG1: very early double negative effector cells (IL7R−/KLRG1+), short-lived effector cells (IL7R+ / KLRG1+), memory precursor effector cells (IL7R+/KLRG1−), and double-positive cells (IL7R+/KLRG1+) that represent a transitional subset between short-lived effector cells and memory precursor effector cells and are particularly prominent under conditions of prolonged Ag stimulation. When early (day-14) cells and late (day-60) cells were analyzed (Fig. 2B), we found a slight reduction in the frequency of memory precursor effector cells generated in CD28−/− mice compared with WT mice, combined with an increase in the frequency of short-lived effector cells, whereas the remaining two compartments seemed unaltered. This could indicate that the lack of CD28 expression drove CD8 T cells in a more effector-like direction, similar to what was seen previously in LCMV-infected mice (19). Supporting this interpretation, we also observed a significantly reduced expression of CD27 on memory CD8 T cells from CD28−/− mice (Supplemental Fig. 2).

We also evaluated the ability of the induced late cells to produce more than one cytokine (i.e., IFN-γ in conjunction with TNF-α and/or IL-2), because the potential to produce multiple cytokines has been correlated with a more competent memory cell type and protection in certain infectious models. Interestingly, we found that lack of CD28 resulted in fewer polyfunctional CD8 T cells, whereas a larger percentage of CD8 T cells from CD28−/− mice coproduced IFN-γ and TNF-α. Similar to the phenotypic analysis, this shift suggests a higher degree of effector cell differentiation in CD28−/− mice and supports the idea that insufficient costimulation (during priming or subsequently) tends to impair the maturation into a polyfunctional central memory CD8 T cell (Fig. 2C).

For further analysis of short-term functionality, Ag-induced degranulation was evaluated by incubation with anti-CD107a/b Abs during the 5 h of in vitro peptide stimulation. As can be seen in Fig. 3A, memory cells generated in CD28−/− mice did not seem to be impaired in their ability to degranulate upon in vitro peptide stimulation. Matching results were obtained when we directly evaluated the cytotoxic capacity of the generated memory cells in vivo (Fig. 3B).

Finally, to assess the proliferative capacity of the induced memory cells, we challenged WT or CD28−/− mice vaccinated with Ad5-NP 60 d earlier with NP-expressing vaccinia virus. Five days after challenge, Ag-specific CD8 T cells in the spleen were enumerated. Vaccinated, unchallenged mice and mice challenged only with vaccinia virus served as controls. As can be seen in Fig. 3C, Ag-specific memory cells generated in CD28−/− mice proliferated poorly compared with those generated in WT mice, translating into an average expansion of 4-fold versus 20-fold, respectively, indicating a prominent role for CD28 in memory cell priming, during recall, or both, because this experimental set-up does not allow distinction among these three possibilities.

**Memory CD8 T cells generated in CD28−/− mice after Ad5-NP vaccination retain short-term protective capacity, but protection is reduced under more limiting conditions**

Having established that all major short-term effector functions were largely intact in memory cells generated in CD28−/− mice, we were interested in investigating whether this would translate into in vivo protection similar to that seen with WT memory cells. To assess this, we vaccinated CD28−/− and WT mice with Ad5-NP, waited 60 d, and challenged the mice with 2 × 107 IFU LCMV Arm i.p.; unvaccinated mice served as controls. Three days after viral challenge, splenic virus loads were assessed and, as is evident from Fig. 3D, CD28−/− mice harbored significantly lower viral loads in the spleen compared with nonvaccinated WT mice. However, viral loads in CD28−/− mice tended to be higher than those found in vaccinated WT mice, possibly indicating that the memory cells generated in the absence of CD28 were not quite as efficient at controlling the infection at this early stage as were those generated in the presence of CD28. Next, we evaluated protection against an i.c. challenge with LCMV. In this situation, it
Vaccinated WT and CD28−/− mice were challenged with lethal doses (20–100 IFU) of LCMV Arm i.c. 60 d after vaccination, and the mice were monitored daily for the next 14 d. As can be seen in Fig. 3E, most vaccinated animals challenged with the WT strain of LCMV Arm survived the challenge, with the notable exception of a few CD28−/− mice. However, if we challenged the vaccinated WT and CD28−/− mice i.c. with a variant of LCMV Arm that does not express the dominant NP epitope (NP396 nil), almost all of the vaccinated CD28−/− mice succumbed to the infection, whereas nearly all WT mice survived, indicating that, under limiting conditions, the impaired recall capacity of CD8 T cells from CD28−/− mice represents a critical bottleneck (Fig. 3F). Alternatively, priming toward subdominant epitopes may be more dependent on CD28 costimulation.

The delayed appearance of acute effectors seen in CD28−/− mice after adenoviral vaccination is not caused by a lack of CD4 T cell help

Many WT and recombinant viruses have been assessed for their ability to induce CD8 T cell responses independently of CD4 T cell help; depending on conditions and virus infection, immune responses were found to vary in their dependence on CD4 T cell help (40). Previously, our laboratory showed that the CD8 T cell response induced by a recombinant adenoviral vector expressing the glycoprotein of LCMV is critically dependent upon CD4 T cell help (41). However, we previously observed that different rules may apply for different transgenic inserts (41); therefore, we wanted to confirm that an adenoviral vector encoding NP of LCMV would be dependent on CD4 T cell help for the induction of a transgene-specific immune response. To this end, we vaccinated WT and MHCII−/− mice with Ad5-NP and measured the Ag-specific CD8 T cell response in the spleen 14 and 60 d after vaccination (Fig. 4A). At both time points, we found a markedly reduced number of Ag-specific CD8 T cells in the spleen of MHCII−/− mice, indicating that the transgene-specific CD8 T cell response induced by the Ad5-NP vector was helper dependent. Having established this, we speculated whether the delayed response seen in CD28−/− mice could be caused, in part, by reduced CD4 T cell help, because the CD4 T cells in these mice also would lack CD28 expression and, therefore, might be impaired with regard to their activation and helper function. To test this, we generated mixed bone marrow chimeras: WT mice were irradiated and reconstituted with CD28−/− (CD45.2) and WT (CD45.1) bone marrow cells in a 1:1 ratio. Eight weeks after reconstitution, mice were vaccinated with Ad5-NP; 14 d later, the percentage of Ag-specific (IFN-γ-producing) CD8 T cells present in the splenic CD45.1+ or CD45.2+ CD8 T cell populations was assessed. As can be seen in Fig. 4B, the presence of WT Th cells did not rescue the delayed response of CD28−/− CD8 T cells, indicating that a lack of CD28 expression directly on the responding CD8 T cells, and not simply reduced CD4 T cell help, is the cause of the delayed response.

FIGURE 1. Lack of CD28 expression delays the primary CD8 T cell response toward adenovirus-encoded Ag but does not affect numbers of memory cells generated. (A) CD28−/− mice and WT mice were vaccinated with Ad5-NP, and Ag-specific (NP396) IFN-γ–producing CD8 T cells were enumerated in the spleen on days 14 and 60 after vaccination. (B) Representative dot plots of gated IFN-γ+ CD8 T cells from vaccinated WT and CD28−/− mice. (C) Numbers of Ag-specific CD8 T cells, as determined by tetramers on day 14 after vaccination. (D) Representative dot plots of gated tetramer+ CD8 T cells from vaccinated WT and CD28−/− mice. Symbols represent individual mice; the data shown are representative of two independent experiments with five mice/group. **p < 0.01.
The transgene-specific immune response induced by replication-deficient adenoviral vectors is dependent on CD80/86 costimulation

Having established that the CD8 T cell response induced by adenoviral vectors was only partially dependent on CD28, we found it pertinent to investigate whether this would translate into a partial dependency on CD80/86 (B7-1/2) as well. This was relevant because earlier studies found inconsistencies in the relative dependence on CD28 and CD80/86 for the induction of CD8 T cell responses, pointing to the existence of alternative ligand(s) for CD80/86 (19, 29–31). To investigate this, we vaccinated WT and CD80/86<sup>−/−</sup> mice with Ad5-NP and assessed the NP396-specific CD8 T cell response in the spleen on days 14 and 60 after vaccination. As can be seen in Fig. 5A, the CD8 T cell response was essentially absent in CD80/86<sup>−/−</sup> mice on day 14, whereas 60 d after vaccination, Ag-specific CD8 T cells could be detected in some of these mice, albeit always in lower numbers than found in WT mice and with a large fraction of cells producing only IFN-γ (Supplemental Fig. 3). It should be added that we observed considerable interindividual variation in the size of the memory pool in CD80/86<sup>−/−</sup> mice at day 60 after vaccination, ranging from undetectable to small populations of Ag-specific cells (Fig. 5A, data not shown). However, our data clearly show that the CD8 T cell response induced by adenoviral vectors is critically dependent on CD80/86 costimulation, and, based on a comparison with the results from CD28<sup>−/−</sup> mice, this clearly adds to the evidence pointing to the existence of an additional stimulatory receptor for CD80/86 on CD8 T cells (Fig. 1). To exclude the possibility that we might have missed a vaccine-induced expansion of transgene-specific cells that fail to undergo substantial effector differentiation by using ICS, we also performed tetramer staining of splenocytes from CD80/86<sup>−/−</sup> mice 14 and 60 d after vaccination; however, no NP-specific CD8 T cells were found in either case (data not shown).

Having established the dependence on one costimulatory pathway, we speculated whether other costimulatory molecules could be essential for adenoviral vector–induced CD8 T cell responses. Although the role of CD40/CD40L during adenoviral priming was nicely delineated recently (42), the role of OX40 and its binding partner, OX40 ligand, as a costimulatory pathway for adenovirus vaccination is unclear, although one earlier study suggested that OX40<sup>−/−</sup> CD8 T cells expanded normally initially, but their accumulation and survival at later time points were reduced (43). Perhaps of interest to adenovector-induced T cell priming, which also involves prolonged Ag presentation/stimulation (8, 44), OX40 signaling regulates the accumulation of CD8 T cells reactive to inflationary epitopes during chronic murine CMV infection (45). Therefore, we found it pertinent to assess whether adenoviral vector–induced CD8 T cell responses would be dependent on the presence of OX40. To investigate this, we vaccinated WT and OX40<sup>−/−</sup> mice with Ad5-NP and measured the Ag-specific CD8 T cell response in the spleens on days 14 and 60 after vaccination. As can be seen in Fig. 5B, there was no difference in the numbers of NP396-specific CD8 T cells generated in the spleens of WT and OX40<sup>−/−</sup> mice early, as suggested by the literature (43), or in...
terms of memory cell accumulation, thus suggesting that this
costimulatory pathway is nonessential for adenoviral vector–
duced CD8 T cell responses.

The priming of Ag-specific CD8 T cells by adenoviral vectors is
dependent on CD8+ and/or CD103+ DCs and takes place in
the DLNs

Costimulatory molecules are expressed exclusively on professional
APCs, and it was shown in numerous models that DCs are the most
important APCs involved in the priming of CD8 T cells. Therefore,
the above results suggest that CD8 T cell responses induced by
adenoviral vectors would be critically dependent on the presence of
DCs. CD8+ and CD103+ DC subsets were shown to be particularly
adept in Ag presentation to naive CD8 T cells, with both subsets
having the ability to cross-present extracellular Ag on MHC class
I molecules (14–16). Mice deficient in BATF3 exhibit severe
defects in the development of CD8+ and CD103+ DCs; therefore,
these mice may be used to assess the dependence of CD8 T cell
activation on these DC subsets after vaccination with recombinant
adenovirus. WT and BATF3−/− mice were vaccinated with Ad5-
NP, and numbers of Ag-specific CD8 T cells were assessed in the
spleen on days 14 and 60 after vaccination. As expected, we found
that the generation of primary effectors was substantially reduced,
but not completely aborted, in BATF3−/− mice (Fig. 6). This
raised the question about the quality of these cells; because
a sufficient number (>10^5) of primary CD8 T cells was generated
to allow phenotypic subtyping in this situation, the composition of
this primary population was compared with that in matched WT
mice. Applying the same cell subset–defining markers as above
(CD127 and KLRG1), we found that, although numbers of Ag-
specific CD8 T cells generated in BATF3−/− mice are reduced, the
initial quality of these cells appears to match those in WT mice
(Supplemental Fig. 4). In line with this, we found that, although
BATF3−/− mice contained a reduced number of memory CD8

FIGURE 3. Memory cells generated
in CD28−/− mice after adenoviral
vaccination retain short-term effector
functions but have a diminished recall
capacity. CD28−/− mice and WT mice
were vaccinated with Ad5-NP; 60 d
after vaccination, memory CD8 T cells
were analyzed for ability to undergo
degranulation (A), in vivo cytotoxicity
(B), in vivo proliferative capacity upon
secondary stimulation (C), in vivo pro-
tective capacity after i.p. challenge with
2 × 10^7 IFU LCMV Arm (D), i.c.
challenge with 20–100 IFU LCMV Arm
(E), and i.c. challenge with 20–100 IFU
LCMV Arm NP396 nil (F). The data
shown in (A), (B), and (D) are represen-
tative of two independent experi-
ments with five mice/group. The data
shown in (E) and (F) are pooled from
two independent experiments with
five mice/group. The data shown in (C)
are from a single experiment with five
mice/group. **p < 0.01, ****p <
0.0001.
T cells 60 d after vaccination compared with WT mice (Fig. 6), the relative contraction (day 14 → day 60) appeared to be of a similar magnitude in BATF3−/− and WT mice. Taken together, these observations indicate that, although Ag presentation by CD8+ and/or CD103+ DCs represents a critical bottleneck in the induction of a potent CD8 T cell response, other APCs may also contribute; however, the latter are less frequently involved in productive presentation.

It is a well-established fact that, upon deposition in peripheral tissues, Ag is taken up by local DCs and transported to the DLNs, where Ag presentation to naive T cells takes place. Based on the above results pointing to a pivotal role for professional APCs in the stimulation and expansion of CD8 T cells following adenoviral vaccination subcutaneously in the foot pad, we were interested in assessing to what extent DLNs are crucial in the induction of the transgene-specific CD8 T cell response. Our group previously found that, after s.c. adenoviral vaccination, the proliferation of Ag-specific T cells can be detected first in the DLNs (46), but it remains to be elucidated whether alternative organ sites, such as the spleen, could play a similar role (33). Consequently, we vaccinated L-selectin−/− mice and WT mice with Ad5-NP and waited either 14 or 60 d before we assessed the Ag-specific CD8 T cell response in the spleen. As can be seen in Fig. 5B, virtually no transgene-specific CD8 T cells were detected in the spleens of L-selectin−/− mice 14 or 60 d after vaccination as opposed to a strong response in WT mice, indicating that priming of CD8 T cells by adenoviral vectors primarily takes place in the DLNs after vaccination in the foot pad.

**Discussion**

Adenoviral vectors have long been forerunners in the development of effective CD8 T cell–based vaccines, and after the emergence of possibly critical issues regarding the use of Ad5 in humans, alternative serotypes and species of adenovirus have received increased attention in recent years. In this respect, chimpanzee vectors have proved highly promising, and in terms of immunogenicity, chAd3 and chAd63 seem to match the human Ad5 vector (2, 5). As development of adenoviral vector–based vaccines proceeds, it is vital that we understand the mechanisms regulating the induction of protective CD8 T cell responses by adenoviral vectors, because this will enable further improvement and customization of existing constructs. In the current study, we used human Ad5 as a model vector to investigate the organ sites, molecules, and cell subsets involved in the priming of transgene-specific CD8

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**FIGURE 4.** The delayed appearance of Ag-specific CD8 T cells in CD28−/− mice after adenoviral vaccination is not caused by a lack of CD4 T cell help. (A) WT and MHCII−/− mice were vaccinated with Ad5-NP, and Ag-specific (NP396) IFN-γ-producing CD8 T cells were enumerated in the spleen on days 14 and 60 after vaccination. The data shown are representative of two independent experiments with five mice/group. (B) Mixed bone marrow chimeras were generated by irradiating WT mice and reconstituting them with CD28−/− (CD45.2) and WT (CD45.1) bone marrow cells in a 1:1 ratio. Eight weeks after reconstitution, mice were vaccinated with Ad5-NP; 14 d later, the percentage of Ag-specific (IFN-γ–producing) CD8 T cells present in the splenic CD45.1+ or CD45.2+ CD8 T cell populations was assessed. Results are pooled from two independent experiments with five mice in each experiment. *p < 0.05, **p < 0.01, ****p < 0.0001.

**FIGURE 5.** The transgene-specific immune response induced by replication-deficient adenoviral vectors is dependent on CD80/86 costimulation. WT and CD80/86−/− mice (A) or WT and OX40−/− mice (B) were vaccinated with Ad5-NP, and Ag-specific (NP396) IFN-γ-producing CD8 T cells were enumerated in the spleen on days 14 and 60 after vaccination. The data shown are representative of two independent experiments with five mice/group. **p < 0.01.
and producing multiple cytokines. Importantly, memory cells
migration within the memory pool, with fewer cells expressing CD127
examination revealed a shift toward a more effector-like differen-
tmost vaccinated CD28
challenge. The biological relevance of this was revealed when
impaired in their capacity to proliferate in response to secondary
significant short-term protective capability, while being markedly
generated in the absence of CD28 retained cytotoxicity and sig-
absence of CD28 after live viral infection were described previously, and
although memory cells generated in CD28<sup>−/−</sup> mice postinfection
with LCMV, vaccinia, and murid herpesvirus 68 appear normal at
first sight, they exhibit certain unfavorable characteristics (19,
29–31). Thus, in terms of the number of IFN-γ-producing cells and
protective capacity under optimal conditions, the cells gener-
generated in CD28<sup>−/−</sup> mice after viral infection are comparable to
those generated in WT mice, as we also showed in this study for
a replication-deficient virus. However, upon detailed analysis,
subtle phenotypic differences, with an inclination toward more
effector-like memory cells (decreased expression of CD27 and
CD127) and decreased IL-2 production, were revealed, charac-
teristics that we also found using replication-deficient adenovirus.
Most importantly, the lack of CD28 costimulation during both
viral infection and vaccination with a replication-deficient viral
vector generated memory cells with a severely reduced recall
potential, a characteristic that can have devastating effects on the
ability to clear an infection. Therefore, as opposed to CD80/86,
CD28 seems to be redundant in some aspects of memory cell
generation and differentiation, whereas in other aspects it is
clearly nonredundant. Therefore, our results and those obtained
earlier (19, 29–31) suggest that at least one alternative receptor for
CD80/86 on CD8 T cells exists, but this receptor cannot com-
pletely make up for a lack of CD28. Thus, memory CD8 T cells
generated in CD28<sup>−/−</sup> mice might perform satisfactorily in a
long range of challenge settings, but they would underperform
in challenge settings in which recall capacity and/or multi-
functionality are vital for efficient host protection. These observa-
tions seem to apply in both infectious settings and noninfectious
settings, such as adenoviral vectors.

A requirement for CD80/86 costimulation would normally
translate into a requirement for professional APCs; indeed, we also
found that the transgene-specific CD8 T cell response induced by
our adenoviral vector was substantially reduced in the absence of
highly specialized CD8<sup>+</sup> and CD103<sup>+</sup> DCs. Both the acute effector
phase and the generation of memory cells were significantly im-
paired in the deficiency of costimulatory molecules CD80/86 and
CD103/CD8<sup>+</sup> DCs. This is surprising and, at first glance, is not in
(complete) agreement with the results recently published by
Bassett et al. (18). Using an adenoviral vector given i.m., these
investigators found that, although the initial priming of effector
cells by an adenoviral vector was significantly reduced in the absence
of hematopoietic APCs, nonhematopoietic APCs completely sufficed for the generation and maintenance of a memory
cell population of normal magnitude. In contrast, in our model of
foot pad injection, we noted severe deficiencies in both the acute
and memory phases, with few Ag-specific cells generated in the
absence of professional APCs or costimulatory molecules. The
discrepancies between our study and the study by Bassett et al.
(18) might reflect differences in the models used, such as virus
dose and route of virus injection. Subcutaneous injection of a
physiologically relevant virus dose (in relation to human vac-
infection and vaccination with a replication-deficient viral
vector generated memory cells with a severely reduced recall
potential, a characteristic that can have devastating effects on the
ability to clear an infection. Therefore, as opposed to CD80/86,
CD28 seems to be redundant in some aspects of memory cell

tting within the LNs, such as CD103<sup>+</sup> and CD8<sup>+</sup> DCs, were the primary

Using genetically engineered mice, we found that the draining
lymph was absolutely essential for initial priming of transgene-
specific CD8 T cells and that cross-presenting DC subsets
within the LNs, such as CD103<sup>+</sup> and CD8<sup>+</sup> DCs, were the primary
presenters of adenoviral vector–delivered transgene. The initial
priming also was dependent on expression of the costimulatory
molecules CD80/86 on the surface of presenting DCs, whereas
CD28 expression on the surface of Ag-specific CD8 T cells did
not seem to be absolutely essential, suggesting the existence of
other ligands for CD80/86 and CD103<sup>+</sup> DCs. Although memory cell
generation seemed unimpaired in CD28<sup>−/−</sup> mice, a closer in-
spection revealed a shift toward a more effector-like differen-
tion within the memory pool, with fewer cells expressing CD127
and producing multiple cytokines. Importantly, memory cells
generated in the absence of CD28 retained cytotoxicity and sig-
ificant short-term protective capability, while being markedly
impaired in their capacity to proliferate in response to secondary
challenge. The biological relevance of this was revealed when
vaccinated mice were challenged under suboptimal conditions,
such as with a virus lacking a dominant epitope. In this case,
most vaccinated CD28<sup>−/−</sup> mice failed to control the infection,
from this study strongly indicate that the initial, most efficient priming of Ag-specific CD8 T cells takes place almost exclusively by professional APCs in the DNLs after vaccination with adenoviral vectors. Whether delayed memory generation can be accomplished in the complete absence of professional APCs is less clear, although we found low numbers of memory cells in some CD80/86−/− mice at 60 d after vaccination. Indeed, if one assumes that local interaction with professional APCs represents the bottleneck for initial priming of naive cells and that non-hematopoietic cells can only efficiently expand already-primed CD8 T cells, then the difference between our observations and those of Bassett et al. (18) simply becomes a matter of degree: if low numbers of recipient APCs survive the radiation, these could provide the essential initial priming signals that, on the other hand, might be much more efficiently eliminated in genetically engineered CD80/86−/− mice. In this context, it is interesting to note the striking similarity between the memory cells generated in the absence of hematopoietic APCs (18) and those found in mice with deficient costimulation (this study).

In conclusion, the current study provides important knowledge about how Ag presentation from adenoviral vectors is accomplished and may impact memory cell number and quality. Our findings emphasize the importance of professional APCs and costimulatory molecules as key factors for induction of a high-quality transgene-encoded CD80/86−/− transgenic mouse. In this context, it is interesting to note the striking similarity between the memory cells generated in the absence of hematopoietic APCs (18) and those found in mice with deficient costimulation (this study).

Disclosures

The authors have no financial conflicts of interest.

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In conclusion, the current study provides important knowledge about how Ag presentation from adenoviral vectors is accomplished and may impact memory cell number and quality. Our findings emphasize the importance of professional APCs and costimulatory molecules as key factors for induction of a high-quality transgene-specific CD8 T-cell response by adenoviral vectors. Furthermore, our study adds to the growing evidence for the existence of an unidentified alternative ligand for CD80/86 on CD8 T cells that is capable of taking over some of the functions of CD28.


Suppl. figure 1

Suppl. Fig S1: Gating strategy for flow cytometry experiments.
Suppl. Figure 2

Suppl. Fig S2: CD27 expression on memory CD8 T cells. CD28−/− and WT mice were vaccinated with Ad5-NP, and 60 days after vaccination memory CD8 T cells were analyzed for CD27 expression. The data shown are representative of two independent experiments with 5 mice per group.
Suppl. Fig S3: Cytokine production by memory CD8 T cells. CD80/86−/−, CD28−/− and WT mice were vaccinated with Ad5-NP, and 60 days after vaccination memory cells were analyzed for cytokine production. The data shown represent a single experiment with 5 mice per group. Data from CD28−/− and WT mice are the same as previously shown in fig. 2B.
Suppl. Fig S4: Numbers and phenotypes of effector CD8 T cells. BATF3 and WT mice were vaccinated with Ad5-NP, and 14 days after vaccination, effector cells were analyzed for expression of phenotypic markers. The data shown represent a single experiment with 5 mice per group.