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CD40-CD40 Ligand Interaction between Dendritic Cells and CD8\(^+\) T Cells Is Needed to Stimulate Maximal T Cell Responses in the Absence of CD4\(^+\) T Cell Help

Maria Genevive H. Hernandez,*,† Lianjun Shen,* and Kenneth L. Rock²*

Stimulation of CD40 on APCs through CD40L expressed on helper CD4\(^+\) T cells activates and “licenses” the APCs to prime CD8\(^+\) T cell responses. Although other stimuli, such as TLR agonists, can also activate APCs, it is unclear to what extent they can replace the signals provided by CD40-CD40L interactions. In this study, we used an adoptive transfer system to re-examine the role of CD40 in the priming of naïve CD8\(^+\) T cells. We find an ~50% reduction in expansion and cytokine production in TCR-transgenic T cells in the absence of CD40 on all APCs, and on dendritic cells in particular. Moreover, CD40-deficient and CD40L-deficient mice fail to develop endogenous CTL responses after immunization. Surprisingly, the role for CD40 and CD40L are observed even in the absence of CD4\(^+\) T cells; in this situation, the CD8\(^+\) T cell itself provides CD40L. Furthermore, we show that although TLR stimulation improves T cell responses, it cannot fully substitute for CD40. Altogether, these results reveal a direct and unique role for CD40L on CD8\(^+\) T cells interacting with CD40 on APCs that affects the magnitude and quality of CD8\(^+\) T cell responses. The Journal of Immunology, 2007, 178: 2844–2852.

Naïve T cells require contact with appropriately activated APCs in order to be primed (1–3). CD40-CD40L (CD154) interactions mediate one of the most effective APC-activating signals. CD40 is a TNFR family member that is constitutively expressed on all APCs and is up-regulated upon infection or inflammation (4–6). It binds to CD40L, a member of the TNF family, which is expressed mainly on activated CD4\(^+\) T cells (4–6). Stimulation of CD40 on dendritic cells (DCs), through either activated CD4\(^+\) T cells, soluble CD40L, or activating anti-CD40 Ab, up-regulates expression of costimulatory molecules CD80 and CD86, enhances production of cytokines (most notably IL-12), and promotes cross-priming to exogenous Ags (2, 7, 8). In vivo, CD40 stimulation increases the magnitude of CD4\(^+\) and CD8\(^+\) T cell expansion, leading to enhanced tumor protection and conversion of steady-state tolerance into immunity (9–15). Conversely, CD40-signaling blockade, mainly through anti-CD40L Ab, inhibits T cell activation and results in tolerance, e.g., to transplants, and control of some autoimmune diseases (16, 17).

The pivotal role of CD40-CD40L interactions in the generation of productive immune responses is highlighted by the phenotype of CD40-deficient and CD40L-deficient mice, which exhibit defects in both humoral and cellular immunity (4, 18–21). The reduced CD8\(^+\) T cell responses are thought to be largely due to impaired “licensing” of APCs. This process is considered to be the mechanism through which CD4\(^+\) T cells provide help for the generation of primary CD8\(^+\) T cell responses, especially to non-inflammatory Ags (22). There are two models for how CD4\(^+\) T cell help occurs. One model involves a sequential two-cell interaction, first, between CD40L-expressing CD4\(^+\) T cells and CD40-expressing DCs leading to DC activation, and then between the activated DCs and CD8\(^+\) T cells (8, 23, 24). An alternative model involves a direct interaction between CD40L-expressing CD4\(^+\) T cells and CD40-expressing CD8\(^+\) T cells (25). In either case, CD4\(^+\) T cell help and CD40 activity are often considered to be identical because CD40 stimulation can restore CD8\(^+\) T cell responses primed in the absence of CD4\(^+\) T cell help (8, 23, 24).

Nonetheless, primary CD8\(^+\) T cell responses against some pathogens including lymphocytic choriomeningitis virus (LCMV), vesicular stomatitis virus, and Listeria monocytogenes are unimpaired in the absence of CD4\(^+\) T cells or CD40 (15, 26–28). These pathogens are thought to bypass the need for CD4\(^+\) T cell help in part because of their ability to directly activate DCs. Recent studies show that DCs and other APCs express TLRs that are able to bind microbial components such as LPS, CpG, dsRNA, and some viral proteins, e.g., respiratory syncytial virus fusion protein (29–31). Ligation of TLRs with these pathogen-associated molecular patterns induces similar effects as that of CD40 stimulation, e.g., activation of NF-κB, up-regulation of costimulatory molecules, production of cytokines, and promotion of cross-priming (29, 32, 33). It has even been shown recently that TLR agonists can abrogate tolerance induced by CD40L blockade (34). However, it is still not clear whether CD40-stimulated or TLR-stimulated DCs have identical CD8\(^+\) T cell priming capability in vivo.

Given the different pathways by which DCs can be activated, we re-examined the requirement for CD40-CD40L interaction in vivo. We asked what is its natural role in the priming of naïve CD8\(^+\) T cell responses in the absence or presence of microbial pathogen-associated molecular patterns. Using an adoptive transfer system of TCR-transgenic (Tg) CD8\(^+\) T cells into wild-type (WT) or CD40\(^{-/-}\) hosts, we find that CD40 signaling on DCs, as well as other APCs, has an important function in inducing maximal T cell responses.
proliferation and effector function. Surprisingly, this effect is observed even in the absence of CD4+ T cells. In this situation, CD40L expression by the responding CD8+ T cells contributes to the maximum response. Finally, we find that TLR stimulation cannot fully compensate for CD40 activity. Therefore, CD40 provides a unique and nonredundant signal for APC activation that impacts the ensuing naive CD8+ T cell responses.

Materials and Methods

**Mice and cell lines**

C57BL/6J, B6.129P2-Cd40tm1Kik/J (CD40−/−), and B6.129S2-Cd40l−/− (CD40L−/−) mice were purchased from The Jackson Laboratory and used from 5 to 10 wk of age. P-14 TCR-Tg and OT-I TCR-Tg breeders were originally obtained from Dr. R. Welsh (University of Massachusetts Medical School, Worcester, MA) and Dr. S. Jameson (University of Minnesota, Minneapolis, MN), respectively. These were bred with C57BL/6-Igh-thy1Gpil (The Jackson Laboratory) to yield Thy1.1+ T cells. Additionally, P-14 mice were bred with B6.SJL-Ptprc−/− Pep3/Boy mice to yield CD45.1+ T cells. Lastly, P-14/Thy1.1+ mice were bred with CD40L−/− mice to yield CD40L-deficient, Thy1.1+ P-14 T cells. All mice were bred and housed in specific pathogen-free conditions at the University of Massachusetts Medical School animal facility.

The fibroblast cell line L cell (DAP) transfected with full-length OVA and TIR-OVA fusion constructs has been described previously (35). B16 tumor cells expressing Flt3 ligand (B16-F1t3L) (36) were obtained from Dr. U. von Andrian (Center for Blood Research, Harvard Medical School, Boston, MA).

**Dendritic cells**

Bone marrow-derived DCs (BMDC) were generated by flushing cells from femurs and tibias of mice and culturing them in complete medium containing 10 ng/ml GM-CSF and 5 ng/ml IL-4 (Corixa). Fresh cytokines were added on day 4, and on day 7 both adherent and nonadherent cells were harvested. Splenic DCs were collected from mice that were injected s.c. with B16-F1t3L tumor cells 10–14 days previously. At the time of harvest, the spleen cellularity had increased up to 7-fold and contained 25–50% CD11c+ cells (clone N418; BD Pharmingen) by flow cytometry, with all DC subsets having expanded. DCs were pulsed with the minimal MHC class I epitope from LCMV glycoprotein (KAVYNFATC; gp33 peptide) or chicken OVA (SIINFEKL; OVA peptide) at a concentration of 1 μg per 5 x 10^6 cells for 2–4 h at 37°C. When WT and CD40-deficient DCs were pulsed with SIINFEKL under these conditions, they had the same levels of SIINFEKL-Kb complexes as quantified with the 25D1 mAb. In some experiments, 200 μg/ml LPS (obtained from L. Latz and T. Thorn, University of Massachusetts Medical School, Worcester, MA), 5 μg of CpG 2395 (Coley Pharmaceutical Group), or 5 μg of poly I:C (Amersham) per 5 x 10^6 cells was added during peptide pulsing. The cells were washed once with complete medium and twice with HBSS (Invitrogen Life Technologies) before immunization.

**Adoptive transfer and immunizations**

Spleen and lymph node cells from P-14/Thy1.1+, P-14/CD45.1+, P-14/Thy1.1+/CD40L−/−, or OT-I/Thy1.1+ mice were depleted of RBC and labeled with 1 μM CFSE (Molecular Probes) for 10–20 min at 37°C. After two washes with HBSS, 2 x 10^6 total cells containing 30–50% TCR-Tg T cells were injected i.v. into hosts. In experiments comparing WT and CD40L-deficient P-14 T cells, the proportion of TCR-Tg T cells was determined by FACs before adoptive transfer and identical numbers of TCR-Tg cells (clone N418; BD Pharmingen) were injected into hosts. One day later, the hosts were immunized i.v. with one of the following: a 13-mer peptide containing the LCMV gp33 epitope (KAVYNFATC; LCMV 13-mer), peptide-pulsed DCs, or OVA-transfected cells. The number of DCs and/or amount of peptide injected into mice were ones that gave reproducibly strong but not maximal responses. In some experiments, 30–50 μg of LPS, 100 μg of CpG 2395, 50 μg of poly I:C, or 25–100 μg of agonistic anti-CD40 mAb (FGK45; Bioexpress) was injected i.p. per mouse at the time of peptide immunization. In some experiments, hosts were depleted of CD4+ T cells by giving anti-CD4 mAb (GK1.5; Taconic Farms or Bioexpress) i.p. at the time of peptide injection. Two consecutive days (50 μg/dose) before adoptive transfer, CD4+ T cell depletion was consistently >99% as verified by flow cytometry. Except when indicated, spleens and lymph nodes were harvested on day 4 since the peak of the responses occurred at this time point. The cells were then stained with PerCP-anti-CD8 (53-6.7; BD Pharmingen) and allophycocyanin-anti-Thy1.1 (HS51; eBioscience) or allophycocyanin-anti-CD45.1 (A20; eBioscience) in the presence of 2.4G2 supernatant to block FcRs. Flow cytometry was done with a FACSCalibur (BD Biosciences) and data were analyzed using FlowJo software (Tree Star).

**Assessment of endogenous CD8+ T cell responses**

In one set of experiments, WT, CD40−/−, and CD40L−/− mice were immunized s.c. on one flank with 4–5 x 10^6 TIR-OVA cells. One week later, the mice were given peptide-pulsed target cells for an in vivo CTL assay. In a second set of experiments, WT mice were immunized i.v. with 1 x 10^6 WT or CD40−/− DCs pulsed with OVA peptide. On day 7, some mice received targets for an in vivo CTL assay while some mice were sacrificed for in vitro restimulation of spleen cells. In some of the experiments, CD4+ T cell-depleted hosts were used.

**In vivo CTL assay**

The in vivo CTL assay was performed as described previously (37). Briefly, splenocyte targets were pulsed with relevant or irrelevant peptide and labeled with different concentrations of CFSE. The targets were then mixed at a 1:1 ratio and injected i.v. into immunized and unimmunized control mice. After 2–20 h, spleen or blood was collected and the percentage of target cell killing was calculated using the formula: 100 – (((% relevant peptide-pulsed in immunized/%) irrelevant peptide-pulsed in immunized)/(% relevant peptide-peptide pulsed in control/%) irrelevant peptide-pulsed in control)) x 100).

**In vitro restimulation**

Splenocytes from DC-immunized mice were depleted of RBC and plated at 5 x 10^6 cells/well in a 24-well plate and stimulated with 1 μg of OVA peptide. After 4 to 6 days, OVA-specific CD8+ T cell responses were evaluated by intracellular IFN-γ staining.

**Intracellular cytokine staining**

Spleen and lymph node cells were incubated with the indicated peptide in the presence of brefeldin A (Golgi Plug; BD Pharmingen) and RIL-2 for 3 h at 37°C. The samples were stained with anti-CD8 and anti-Thy1.1 or anti-CD45.1 Ab, fixed, and permeabilized using Cytofix/Cytoperm buffer (BD Pharmingen), and stained with anti-cytokine Ab diluted in Perm/Wash buffer (BD Pharmingen) according to manufacturer’s instructions. Anti-IFN-γ (XMG1.2), anti-TNF-α (MP6-XT22), and anti-IL-2 (JES6-5H4) were purchased from BD Pharmingen or eBioscience. The samples were washed twice with Perm/Wash buffer and analyzed by flow cytometry.

**Statistical analysis**

Data were analyzed for statistical significance with a two-tailed Student’s t test using Microsoft Excel software. Differences in T cell responses were considered significant when a probability value of p < 0.05 was obtained.

**Results**

**Reduced CD8+ T cell expansion and effector function in CD40−/− hosts**

Most of the previous studies showing a role for CD40 in APC activation and CD8+ T cell responses in vivo relied on exogenous stimulation of the receptor with agonistic Ab (9–12, 38). Two major limitations of these studies are the nonphysiologic nature of Ab-mediated stimulation and the possibility of nonspecific effects because of the numerous cell types that can express CD40. We therefore took the opposite approach and examined the priming of TCR-Tg CD8+ T cells upon adoptive transfer into CD40-deficient animals. In this system, all APCs lack CD40 while the responding T cells express both CD40 and CD40L. In addition, we used peptide or transfected cells as Ag instead of viruses or bacteria; this was to avoid the potential complication of TLR stimulation, which might bypass a CD40 requirement.

We injected WT B6 and CD40−/− hosts with CFSE-labeled P-14 T cells, which recognize the LCMV gp33 peptide bound to H-2Dd. One day later, we immunized the hosts i.v. with a 13-mer peptide containing the minimal MHC class I epitope from LCMV gp33 (LCMV 13-mer). This Ag requires cross-presentation by host APCs to stimulate naive T cells even without adjuvant (39). On day 4 postimmunization, >95% of the P-14 T cells in both the WT and CD40−/− hosts had divided, as evidenced by the dilution of
CFSE (Fig. 1A). The P-14 T cells in the WT hosts made up ~24% and ~15% of the total CD8+ T cells in the spleen and lymph nodes, respectively (Fig. 1B). In contrast, the P-14 T cells in the CD40-/- hosts only comprised ~10% of the splenic and ~5% of the lymph node CD8+ T cells. When compared with the unimmunized control mice, there was 12- vs 5-fold expansion of T cells in the spleens, and 7- vs 3-fold expansion in the lymph nodes of WT and CD40-/- hosts, respectively.

FIGURE 1. Reduced P-14 T cell response in CD40-/- hosts. WT and CD40-/- hosts containing adoptively transferred CFSE-labeled Thy1.1+ P-14 T cells were immunized i.v. with 5 μg of LCMV 13-mer peptide or left unimmunized. Four days later, spleens and lymph nodes were harvested, stained with anti-CD8 and anti-Thy1.1 Ab, and analyzed by flow cytometry. A. CFSE profiles of transferred T cells from representative mice. The shaded histogram represents P-14 T cells in an unimmunized mouse. B. Overall accumulation of P-14 T cells was determined by calculating the percentage of Thy1.1+ cells in the total CD8+ T cell population. The data are presented as mean + SD. C. Effector function was assayed by looking at IFN-γ production after a 5-h incubation with gp33 peptide in the presence of brefeldin A (Golgi Plug). Representative FACS plots gated on Thy1.1+ P-14 T cells are shown. The numbers above indicate the percentage of IFN-γ-secreting P-14 T cells in the total CD8+ T cell population while the numbers in parentheses indicate the percentage of P-14 T cells secreting IFN-γ. In unimmunized mice, the frequency of IFN-γ-producing P-14 T cells was typically <0.1%. The results shown are representative of three independent experiments with two to three mice per group. *, p < 0.05.

We next examined whether the Tg T cells became functional effectors by assaying for cytokine secretion. The number of P-14 T cells making IFN-γ in the spleen and lymph nodes of CD40-/- hosts is ~10-fold less than in the WT hosts (Fig. 1C). The same difference was observed for TNF-α and IL-2 production (data not shown).

We also tested the response of adoptively transferred OT-I TCR-Tg T cells, which are specific for the OVA peptide SIINFEKL bound to H-2Kb, to make sure that the observed effects were not confined to the P-14 TCR-Tg T cells. Instead of peptide, we immunized the hosts i.v. with a stable OVA-transfected cell line that also gets cross-presented by host DCs (35). On day 4 of the response, almost all of the OT-I T cells in both the WT and CD40-/- hosts had divided more than eight times (data not shown). However, the OT-I T cells accumulated to a lesser extent in the spleens and lymph nodes of CD40-/- compared with the WT hosts (Fig. 2A). The proportion, as well as the absolute number of IFN-γ-secreting cells, was also reduced by as much as 50% in the CD40-/- hosts (Fig. 2B). Furthermore, the killing of SIINFEKL-pulsed target cells was reduced in the CD40-/- hosts compared with the WT hosts (94% vs 56%; Fig. 2C). We also obtained similar results after s.c. immunization of the OVA transfectants (data not shown).

Altogether, these results demonstrate that although CD40 on APCs is not absolutely required to initiate naive CD8+ T cell priming, it is important in inducing T cells to undergo maximum expansion and differentiation into effectors.

**CD40-/- DC induce suboptimal T cell responses**

The preceding experiments examined T cell responses in which all APCs in the host are either expressing or not expressing CD40. Because DCs are considered to be the most potent APC (1), we analyzed T cell responses stimulated by CD40-deficient DCs. To do this, we immunized WT mice containing adoptively transferred P-14 T cells with WT or CD40-/- BMDCs pulsed with LCMV gp33 peptide. In this situation, the only cell that lacks CD40 is the immunizing DC; at the time of immunization, WT and CD40-deficient DCs had the same activation phenotype (data not shown). Compared with the previous immunizations with peptide or cell-associated Ag, immunization with peptide-pulsed DCs generally resulted in lower CD8+ T cell responses. Nevertheless, WT DCs induced proliferation of P-14 T cells in the spleen as early as day 2, and reached a peak at day 4 postimmunization (Fig. 3A). In

![Graph A](image1.png) **OT-I-C57BL/6 T cells (%)**  
- **Spleen**  
  - OT-I-C57BL/6, WT, 100  
  - OT-I-C57BL/6, CD40-/-, 20  
- **Lymph node**  
  - OT-I-C57BL/6, WT, 80  
  - OT-I-C57BL/6, CD40-/-, 20

![Graph B](image2.png) **IFN-γ (x 10^4)**  
- **Spleen**  
  - (1), (15)  
  - (15), (4)  
- **Lymph node**  
  - (37), (32)  
  - (37), (32)

![Graph C](image3.png) **% Target cell killing**  
- **WT**  
  - 120  
- **CD40-/-**  
  - 120

**CD40-/- DC induce suboptimal T cell responses**

The preceding experiments examined T cell responses in which all APCs in the host are either expressing or not expressing CD40. Because DCs are considered to be the most potent APC (1), we analyzed T cell responses stimulated by CD40-deficient DCs. To do this, we immunized WT mice containing adoptively transferred P-14 T cells with WT or CD40-/- BMDCs pulsed with LCMV gp33 peptide. In this situation, the only cell that lacks CD40 is the immunizing DC; at the time of immunization, WT and CD40-deficient DCs had the same activation phenotype (data not shown). Compared with the previous immunizations with peptide or cell-associated Ag, immunization with peptide-pulsed DCs generally resulted in lower CD8+ T cell responses. Nevertheless, WT DCs induced proliferation of P-14 T cells in the spleen as early as day 2, and reached a peak at day 4 postimmunization (Fig. 3A). In
CD40-deficient DCs induced significantly weaker T cell expansion as well as IFN-γ production at all time points (Fig. 3, A and B). The reduced T cell response induced by the CD40−/− DCs led to a corresponding decrease in the ability of the hosts to eliminate peptide-pulsed targets during the peak of the response (Fig. 3C). By day 12, however, there is very little CTL activity left regardless of the immunizing DC. This is most probably due to the low numbers of effector cells that remain at this point. We also analyzed CD8+ T cell responses induced by DCs generated in vivo. Because there is only a small population of DCs in the spleen and they are difficult to isolate in large numbers, we first injected WT and CD40−/− mice with B16 tumor cells that secrete Flt-3L. Flt-3L has previously been shown to induce the differentiation and expansion of functionally mature DC subsets in vivo (36, 40). In our case, injection of B16-Flt3L cells resulted in up to 50-fold expansion of CD11c+ cells in the spleen, with no difference in DC subsets between WT and CD40−/− mice (data not shown). We pulsed the cells with LCMV gp33 peptide and used them to stimulate P-14 T cells in vivo. Similar to in vitro-derived BMDCs, CD40−/− splenic DCs induced much less T cell proliferation and cytokine production on day 4 of the response (Fig. 3D and data not shown).

The above results are concordant with the data obtained using CD40-deficient hosts. Moreover, they directly demonstrate a key role for CD40 on DCs in inducing maximal naive CD8+ T cell responses because they are the only cells lacking this receptor in these experiments.

**Peptide-pulsed DC induce CD4+ T cell-independent CD8+ T cell responses**

CD8+ T cell responses to noninflammatory Ags such as peptides, soluble proteins, particulate Ags, and cell-associated Ags, including peptide-pulsed DCs are largely dependent on CD4+ T cells (15, 41). It is believed that CD40L on activated CD4+ T cells is needed to stimulate CD40 on APCs and trigger licensing, and that this is the mechanism by which CD4+ T cells provide help for CD8+ T cell responses. In our system, CD4+ T cell responses might be generated to bovine serum proteins presented by cultured BMDCs or if the LCMV 13mer peptide contained a MHC class II epitope. To determine whether CD4+ T cells are responsible for activating CD40 on the DCs in our experiments, we examined P-14 T cell responses induced by peptide-pulsed DCs in CD4−/− T cell-deficient hosts. We chose to acutely deplete CD4+ T cells with GK1.5 Ab because this gives the most complete elimination of these cells. Surprisingly, we found that WT DCs stimulated an equivalent P-14 T cell response with or without CD4+ T cells (Fig. 4). This indicates that the CD8+ T cell response to peptide-pulsed DCs can occur independently of CD4+ T cell help. Depletion of CD4+ T cells resulted in a slight increase in the absolute number of IFN-γ-producing cells, which perhaps suggests a possible effect of regulatory T cells. However, this difference was not statistically significant. Interestingly, CD40−/− DCs still induced weaker P-14 T cell priming in the absence of CD4+ T cells. This implies that CD4+ T cell and CD40 activity are not always equivalent. Moreover, it suggests that CD4+ T cells are not the only cells capable of activating CD40 on APCs during an immune response. To investigate this further, we did most of our subsequent experiments in CD4−/− T cell-depleted hosts.

**Pivotal role of CD40-CD40L interaction in endogenous CD8+ T cell response**

We next sought to extend our analysis of naive CD8+ T cell priming to a non-Tg system, in which host mice have normal T cell frequencies. In the first set of experiments, we analyzed in vivo
CTL activity in WT, CD40−/−, and CD40L−/− mice 1 wk after immunization with OVA-transfected cells. As expected, unimmu-
nized WT, CD40−/−, and CD40L−/− mice were unable to lyse OVA peptide-pulsed targets (data not shown). Immunized WT
mice exhibited strong CTL responses, being able to lyse as much as 80% of targets (Fig. 5A). In contrast, both the immunized
CD40−/− and CD40L−/− mice showed much weaker CD8+ T cell priming as target cell killing in these mice was reduced to ~20% (Fig. 5A). Depletion of CD4+ T cells had no effect on the ability
of WT animals to mount a primary CTL response (Fig. 5B).

In the second set of experiments, we examined endogenous CD8+ T cell responses in WT hosts upon immunization with WT
or CD40−/− DCs pulsed with OVA peptide. WT DCs induced a
very strong CTL response, resulting in almost complete elimina-
tion of target cells (Fig. 5C). In contrast, CD40−/− DCs induced
more modest CTL activity (Fig. 5C) and this weaker response was
observed whether or not CD4+ T cells were present. We could not
detect CD8+ T cell cytokine production directly ex vivo in either
WT DC or CD40−/− DC immunized mice (data not shown). Upon
in vitro restimulation, we found that only CD8+ T cells from mice
immunized with WT DCs were able to secrete IFN-γ (Fig. 5D).
Interestingly, we observed a reduction in the number of IFN-γ-
producing cells in the absence of CD4+ T cells. This appears to be a
difference between the TCR-Tg vs the endogenous (polyclonal) T cell
response and could be due to differences in T cell affinity and/or pre-
cursor frequency. However, it is important to note that despite the
reduced number of endogenous IFN-γ-producing CD8+ T cells in
CD4-depleted hosts, the response remains CD40-dependent.

Overall, these results indicate that the CD40-CD40L interaction
is required to generate a highly effective primary response from an
endogenous polyclonal CD8+ T cell population and this require-
ment is observed even in the absence of CD4+ T cell help.

CD40L expression by CD8+ T cells contributes to maximal
response

We hypothesized that in the absence of CD4+ T cells, CD40L
might be provided to DCs by the CD8+ T cells themselves. To test
this hypothesis, we examined the response of CD40L-deficient
P-14 T cells in WT hosts after immunization with the LCMV 13-
mer peptide. In this system all the host APCs express CD40 and all
the host T cells express CD40L; only the adoptively transferred T
cells are unable to express CD40L. P-14/CD40L−/− T cells prolif-
erated 2- to 3-fold less compared with WT P-14 T cells in the
presence or absence of CD4+ T cells (Fig. 6A). The proportion
and absolute number of P-14/CD40L−/− T cells secreting IFN-γ
was also significantly decreased (Fig. 6B). The reduced P-14/
CD40L−/− T cell response parallels that of WT P-14 T cells stimu-
lated by CD40L+ DCs in CD4+ T cell-deficient hosts.

We next investigated whether the reduced CD8+ T cell response
was due to deficient activation of the CD40-positive host APCs
and not to an inherent defect of the CD40L-deficient T cells.
Injection of agonistic anti-CD40 Ab along with the LCMV 13-mer
increased the P-14/CD40L−/− T cell response to WT levels (Fig.
6C). This increased response was not observed when CD4−/−
hosts were used (data not shown) ruling out the possibility of the
Ab directly activating the transferred T cells, which can express
CD40. Furthermore, adoptive transfer of a 1:1 mixture of WT and
CD40L-deficient P-14 T cells resulted in equal expansion and

**FIGURE 5.** Endogenous CD8+ T cell responses are compromised in the absence of CD40 or CD40L. A, WT, CD40−/−, and CD40L−/− mice were immunized s.c. with 5 × 106 OVA-transfected cells or left unimmunized. One week later, OVA peptide-specific CTL responses were assessed using an in vivo CTL assay. Specific target cell killing from the different mice are presented as mean ± SD. The results shown are representative of three independent experiments with three to five mice per group. B, WT mice were immunized with 1 × 106 gp33 peptide-pulsed or unpulsed DCs from the indicated mice. In vivo CTL activity against OVA peptide-pulsed targets was assessed 1 wk later and the data are presented as mean ± SD. The results shown are repre-
sentative of three independent experiments. C, CD4-depleted or undepleted WT mice were immunized i.v. with 1 × 106 OVA peptide-pulsed or unpulsed DCs from the indicated mice. In vivo CTL activity against OVA peptide-pulsed targets was determined 1 wk later and presented as mean ± SD of specific target killing. D, CD4-depleted or undepleted mice were immunized as in C. On day 7, splenocytes were harvested and restimulated in vitro with 1 μg/ml OVA peptide. After 6 days, intracellular IFN-γ production was assessed following an additional 5-h incubation with OVA peptide. Representative plots are shown, with the numbers indicating the percentage of CD8+ T cells producing IFN-γ. The frequency of IFN-γ+ cells in mice im-
unized with unpulsed DCs was ~0.5%. The results shown are representative of two independent experiments with three mice per group.
IFN-γ production of both T cell populations (Fig. 6D and data not shown). Injection of the anti-CD40L blocking Ab MR1 inhibited the ability of the WT CD8+ T cells to rescue the response of the CD40L-deficient T cells (Fig. 6D). Therefore, the CD40L-deficient T cells were fully functional when the WT CD8+ T cells provided the CD40L signal, presumably to the CD40-expressing APCs.

Our data so far do not indicate whether Ag-specific CD40L-expressing CD8+ T cells from the host might be providing “help” to the adoptively transferred T cells. To address this issue, we used CD40L−/− mice, which are completely unable to express CD40L, as hosts. The magnitude of T cell expansion was very similar in WT and CD40L−/− hosts, but more importantly, the CD40L-deficient P-14 T cells still exhibited ~2-fold reduction in proliferation compared with the WT P-14 T cells (data not shown). In preliminary experiments, we also obtained similar data using RAG−/− hosts, which lack both endogenous CD4+ and CD8+ T cells (data not shown).

All of these results show that there is no inherent defect in the ability of the CD40L-deficient CD8+ T cells to be primed and their reduced response can be attributed to their inability to activate APCs. These data provide functional evidence that CD8+ T cells are expressing CD40L. We verified that this is the case by stimulating the TCR-Tg CD8+ T cells with peptide or PMA and ionomycin and detecting low levels of CD40L on the surface of WT cells but not CD40L−/− cells (data not shown); this is consistent with some other reports that CD8+ T cells can express CD40L (42–45). Therefore, CD40L expression by the responding CD8+ T cells contributes to the generation of a maximal primary response.

TLR stimulation does not compensate for CD40 or CD40L deficiency

TLR ligands stimulate APCs to mature and enhance their ability to induce T cell activation and differentiation (29, 32). To determine whether TLR stimulation can substitute for CD40 signaling, we...
first examined P-14 T cell responses in CD4-depleted WT hosts immunized with peptide-pulsed WT or CD40−/− DCs that were incubated in vitro with representative TLR agonists LPS (TLR4), CpG (TLR9), or poly I:C (TLR3). Consistent with our previous results, CD40−/− DCs pulsed with peptide alone stimulated less P-14 T cell proliferation compared with WT DCs (Fig. 7A). As expected, activation of DCs with any one of the TLR ligands augmented T cell expansion. However, whereas TLR-activated WT DCs induced a tremendous increase in T cell numbers (3- to 4-fold greater compared with peptide alone), TLR-activated CD40−/− DCs only induced a more modest increase (2-fold greater compared with peptide alone). More strikingly, CD40−/− DCs induced consistently lower P-14 T cell expansion and IFN-γ production compared with WT DCs (~50% less) despite TLR stimulation (Fig. 7). In other words, in the presence of TLR ligands, CD40-deficient DCs were still inferior to CD40-sufficient DCs in stimulating naive CD8+ T cells. In the presence of MR1 Ab, the responses induced by TLR-activated WT DCs became equivalent to that induced by TLR-activated CD40−/− DCs (data not shown). This indicates further that DCs are unable to provide a complete costimulatory repertoire to naive CD8+ T cells in the absence of CD40 signaling.

In similar experiments, we analyzed the response of WT and CD40L-deficient P-14 T cells in CD4-depleted WT hosts immunized with LCMV 13-mer peptide and TLR ligands. Again, the P-14/CD40L−/− T cell response paralleled that of WT P-14 stimulated with CD40−/− DCs. Even after injection of LPS, CpG, and poly I:C, the P-14/CD40L−/− T cells accumulated to a lesser extent compared with WT P-14 T cells (Fig. 8A). The absolute number of IFN-γ-producing cells was also reduced (Fig. 8B). Taken together, these results establish that TLR stimulation cannot completely compensate for CD40 or CD40L deficiency. Therefore, CD40-CD40L signaling has a unique function in inducing maximal primary CD8+ T cell responses.

Discussion

CD8+ T cells play a critical role in protective immunity against viruses and tumors. Therefore, it is important to understand the signals that are necessary for inducing maximum CD8+ T cell responses. In the present study, our goal was to examine the role of CD40-CD40L interactions in the priming of naive CD8+ T cells. We found that CD40 was not absolutely required to prime naive CD8+ T cells. However, the proliferation, cytokine production, and cytotoxic activity of both adaptively transferred TCR-Tg and endogenous (non-Tg) CD8+ T cells were consistently reduced by as much as 50% in the absence of CD40 or CD40L. We have not determined the exact mechanism(s) for the lower overall accumulation of CD8+ T cells in the CD40−/− hosts. CD40 may affect the number of T cells initially recruited to proliferate, the number of divisions a cell undergoes, and/or the survival of activated cells. However, regardless of the underlying mechanism, our results, combined with previous studies showing augmented T cell responses upon CD40 stimulation by exogenous anti-CD40 Ab, point to an important role for CD40 signaling in maximizing primary CD8+ T cell responses. This is in contrast with the more stringent requirement for CD40 in the priming of naive CD4+ T cells (20, 46).

CD40 is expressed not only by professional APC but also by hemopoietic precursors, epithelial cells, endothelial cells, and even activated T cells (4–6). Because CD40 plays a role in the generation of CD8+ T cell responses, it was important to determine on which cells CD40 was acting. This issue has not been resolved in the many previous studies that have explored CD40 function using agonistic anti-CD40 Ab, which bind to all CD40-expressing cells. Our adoptive transfer experiments map the key role of CD40 to APCs in the host. Moreover, we demonstrate that when DCs are the only APC lacking CD40, the reduction in T cell responses is similar to that observed when all host APCs were CD40 deficient. Therefore, CD40 is working at least in part on DCs. Our findings do not rule out the possibility that CD40 might also have the same function on other APCs. We have previously shown that macrophages can stimulate primary CD8+ T cell responses (47). In addition, recent reports show that activation of B cells through CD40 converts them into efficient stimulators of both CD8+ and CD4+ T cells (48, 49). It remains to be tested whether or not CD40-deficient macrophages and B cells show a reduced capacity to stimulate CD8+ T cells in our system.

Another important question was what cell was the source of CD40L that was needed to stimulate APCs in vivo. It has generally been thought that Th cells are the principal source of CD40L in CD40-dependent responses. However, our finding that the absence of helper CD4+ T cells did not affect the CD40 dependence of the CD8+ T cell responses indicated that some other cells provided CD40L for APC activation. Because CD8+ T cells can express CD40L (42–46) and they are able to directly interact with APCs, we therefore reasoned that they might be able to provide their own help. This possibility has been hinted at by some earlier studies (26, 50, 51); however, the experiments in which we use CD40L-deficient CD8+ TCR-Tg T cells provided the first direct test of this hypothesis. These mutant cells exhibited the same defective responses that were observed when WT T cells were stimulated with CD40-deficient APCs in the presence or absence of CD4+ T cells. Moreover, WT P-14 T cells were able to rescue the P-14/
CD40L−/− T cell response, formally showing that CD8+ T cells can provide help in “trans.” NK cells, NKT cells, and platelets also express CD40L (5). Moreover, NKT cells have been shown to directly activate DCs in a CD40-CD40L-dependent manner (52–54). However, we find that depletion of NK cells, NKT cells, and platelets had no effect on the generation of primary CD8+ T cell responses in our experimental systems (data not shown). We conclude that Ag-specific CD8+ T cells can directly activate APCs through CD40L and thereby provide their own help in the absence of CD4+ T cells.

It has been shown in other systems that CD4+ T cells provide help through CD40L (8, 23, 24). Given our results, it is likely that CD40L expressed by both CD4+ and CD8+ T cells can activate APCs and contribute to the amplification of a normal immune response. It is remarkable that we observed CD8+ T cell responses in the absence of help in light of the general requirement for CD4+ T cells in CD8+ T cell responses against noninflammatory Ags. The precursor frequency and affinity of responding CD8+ T cells have been shown to affect helper dependence (55, 56). However, titrating the number of adoptively transferred T cells still resulted in detectable CD40-dependent responses in the presence or absence of CD4+ T cells (data not shown). Moreover, we were able to detect endogenous primary CTL responses; aside from having a low frequency, the responders in this case also consisted of a spectrum of affinities. In the APC-licensing model, CD40 signaling is usually equated with CD4+ T cell help. The fact that we observed CD40-dependent responses despite the absence of helper CD4+ T cells indicates that the two are not always equivalent.

The final issue that our studies address is how CD40 stimulation compares to microbial (TLR) stimulation for licensing APCs to support CD8+ T cell responses. Although it is known that APCs must be activated to stimulate naïve T cell responses, it has been unclear whether all activating stimuli are similarly effective in this process, particularly for responses in vivo. By incubating WT and CD40−/− DCs with TLR ligands before immunization, we were able to directly show that the two signals have nonredundant effects on the stimulatory property of DCs. Although TLR ligands were able to amplify CD8+ T cell responses in the absence of CD40 signaling, these responses never reached the levels that were induced when CD40 signaling was present. In other words, maximum CD8+ T cell proliferation could only be achieved when both CD40 and TLR are stimulated. This result is consistent with reports that TLR stimulation can influence CD40 responses for cytokine production and amplification of CD8+ T cell responses (57, 58).

CD40 stimulation of DCs has been shown to be important for production of IL-12, which promotes CD8+ T cell expansion and differentiation (7, 59). However, we found that WT and CD40-deficient DCs made similar levels of IL-12 upon incubation with either WT or CD40L-deficient CD8+ T cells (data not shown). Moreover, we found that with or without TLR ligation, there was no difference in MHC-peptide levels as well as costimulatory molecule expression (CD80 and CD86) and IL-12 production between WT and CD40−/− DCs (data not shown). Therefore, the different responses induced by CD40- and TLR-stimulated DCs are not due to differences in conventional “costimulatory repertoire” and the underlying molecular mechanism(s) remain to be determined. This is especially important in light of recent efforts that establish the need to distinguish between phenotypically and functionally mature DCs (3). CD40-matured DCs have been reported to be more phenotypically stable compared with TLR-matured DCs (60). In addition, CD40 stimulation has been shown to increase the lifespan of DCs (61). It has also been shown that CD40 induces higher levels of CD70 (CD27L) on DCs compared with TLRs and this correlates with increased immunogenicity even in the absence of helper CD4+ T cells (62, 63). Whether any of these previously reported mechanisms and/or other ones account for the effects we observed will require further studies.

In conclusion, our data support a new model of CD8+ T cell-mediated APC “licensing,” in which CD40L expressed by Ag-specific CD8+ T cells interacts with CD40 on APCs, leading to maximal CD8+ T cell responses that can be primed in the absence of CD4+ T cell help. Our findings also reveal a unique role for CD40 signaling on APC activation that cannot be fully replaced by TLR stimulation. It will be interesting to examine further whether the CD40 dependence of the CD8+ T cell response extends to noninflammatory Ags considering that some pathogen-specific responses occur in the absence of CD40 ligation. It is also important to study whether CD40-CD40L interactions are needed not just during primary but also during other phases of the immune response. One of the major issues that still needs to be resolved is determining what is the ultimate fate of CD8+ T cells primed in the absence of CD40. One study has shown that systemic administration of Ag-loaded CD40−/− DCs failed to sustain the activation and led to deletional tolerance of CD4+ T cells (64). There is also evidence that CD40L is involved in the generation but not maintenance of LCMV-specific memory CD8+ T cells (65). In light of these findings, we are currently investigating whether CD40-CD40L interaction also plays a role in the generation, maintenance, and response of memory CD8+ T cells.

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Disclosures

The authors have no financial conflict of interest.

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