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# Selected Toll-like Receptor Ligands and Viruses Promote Helper-Independent Cytotoxic T Cell Priming by Upregulating CD40L on Dendritic Cells

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## SUMMARY

**CD40L (CD154) on $CD4^+$ T cells has been shown to license dendritic cells (DCs) via CD40 to prime cytotoxic T lymphocyte (CTL) responses. We found that the converse (CD40L on DCs) was also important. Anti-CD40L treatment decreased endogenous CTL responses to both ovalbumin and influenza infection even in the absence of $CD4^+$ T cells. DCs expressed CD40L upon stimulation with agonists to Toll-like receptor 3 (TLR3) and TLR9. Moreover, influenza infection, which stimulates CTLs without help, upregulated CD40L on DCs, but herpes simplex infection, which elicits CTLs through help, did not. CD40L-deficient ($Cd40lg^{-/-}$) DCs are suboptimal both in vivo in bone marrow chimera experiments and in vitro in mixed lymphocyte reactions. In contrast, $Cd40lg^{-/-}$ $CD8^+$ T cells killed as effectively as wild-type cells. Thus, CD40L upregulation on DCs promoted optimal priming of $CD8^+$ T cells without $CD4^+$ T cells, providing a mechanism by which pathogens may elicit helper-independent CTL immunity.**

## INTRODUCTION

CD40L is a member of the tumor necrosis factor (TNF) superfamily whose expression is tightly regulated (Armitage et al., 1992; Cayabyab et al., 1994; Grewal et al., 1996). Surface expression on $CD4^+$ T cells is detectable within 2 hr of activation by peptide-pulsed antigen-presenting cells (APCs), peaks at 6 hr and drops by 24 hr (Lee et al., 2002). Activated $CD4^+$ T cells are the predominant CD40L-bearing population. Modest expression can be found on activated $CD8^+$ T cells, B cells, NK cells, monocytes, Langerhans cells, human thrombocytes, and activated dendritic cells (DCs) (Pinchuk et al., 1996; Salgado et al., 1999; Schonbeck and Libby, 2001). However, low or absent surface staining for CD40L could be misleading for several reasons. First, like FasL, much of the CD40L is stored in secretory lysosomes and is only released to the cell surface upon activation (Koguchi et al., 2007). Second, surface CD40L is rapidly endocytosed upon binding to CD40 expressed on cells such as B cells and DCs, and third, detection of CD40L can be masked by soluble CD40 (van Kooten et al., 1994; Yellin et al., 1994). CD40L on $CD4^+$ T cells is critical in effecting isotype switching by B cells (Gray et al., 1994), "licensing" DCs to prime CTLs (Bennett et al., 1998; Ridge et al., 1998; Schoenberger et al., 1998; Smith et al., 2004) and generating memory CTLs (Borrow et al., 1996; Sun and Bevan, 2003). Despite the vast literature on the function of CD40L on $CD4^+$ T cells (and hence CD40 on DCs and B cells), less attention has been devoted to CD40L on the other cells that express it, perhaps because of the modest expression.

Although $CD4^+$ T cell help is important, helper-independent primary CTL responses are generated against certain infections, including viral infections with ectromelia, vesicular stomatitis virus, human immunodeficiency virus, Epstein-Barr virus, influenza, and cytomegalovirus (Andreasen et al., 2000; Buller et al., 1987; Ruedl et al., 1999; Tripp et al., 1995; Zimmerli et al., 2005). Likewise, helper-independent CTLs can be generated against Listeria (Hamilton et al., 2001). The ascribed mechanism by which helper-independent CTLs were elicited was activation of DCs by the microbial infection (Ruedl et al., 1999; Sun and Bevan, 2004) or self help by $CD8^+$ T cells (Hamilton et al., 2001; Wang et al., 2001), although the latter mechanism fails to explain how such cells were stimulated to begin with. Although many microbial pathogens directly activate DCs, the basis of why only some pathogens can elicit helper-independent CTLs remains unclear.

The use of a blocking CD40L mAb is also of translational interest. The mAb can prolong graft survival dramatically in both mouse and primate transplantation studies (Bucher et al., 2005; Kirk et al., 1999). It can promote nonmyeloablative conditioning for the establishment of chimerism (Ito et al., 2006) and it can ameliorate autoimmunity (Bagenstose et al., 2005; Hanninen et al., 2002; Komura et al., 2007). Most such studies have presumed that the anti-CD40L acts on $CD4^+$ T cells alone. We have previously shown that in the absence of $CD4^+$ T cells, treatment with CD40L Ab still reduced CTL response to alloantigens (Zhan et al., 2000). Another report has also shown that in the absence of $CD4^+$ T cells CD40L is still important for the priming of transgenic $CD8^+$ T cells (Hernandez et al., 2007) and suggested a role for CD40L on $CD8^+$ T cells.

No previous studies have explored the converse phenomenon in which CD40L on DCs may trigger CD40 on T cells. We found that endogenous in vivo CTL responses to cell-associated

ovalbumin (OVA) were sensitive to CD40L blockade even in the absence of $CD4^+$ T cell help. We then set out to understand the mechanism of anti-CD40L treatment and to determine the role of CD40L in the absence of $CD4^+$ T cells, including its expression on $CD8^+$ T cells and DCs. Results from both in vitro mixed lymphocyte reactions and in vivo mixed bone marrow (BM) chimera experiments directed us to focus on the role of CD40L being expressed on DCs and the correlation between CD40L upregulation by selected TLR agonists and viruses. We found that TLR3 and TLR9 agonists and influenza could upregulate CD40L expression on DCs and that this was critical in effecting helper-independent CTL responses. It should also be noted that our studies primarily relate to primary CTL induction; the parameters for the generation of memory may be different.

## RESULTS

### Anti-CD40L Treatment Decreases the Endogenous In Vivo CTL Response

We initially used the endogenous CTL response to OVA as our model system. Anti-CD40L treatment was administered intraperitoneally on the day of immunization with cell-associated OVA (namely, OVA-coated spleen cells). Seven days later, we assessed in vivo CTL activity by comparing the numbers of peptide-loaded targets versus nonloaded targets. As expected, we found that anti-CD40L or anti-CD4 treatment decreased the endogenous CTL response (Figure 1A). Moreover, in the absence of $CD4^+$ T cells, CD40 antibody could substitute for help and restore CTL killing (Figure 1A) as had been shown previously (Bennett et al., 1998; Schoenberger et al., 1998). The agonistic anti-CD40 (FGK45) is thought to simulate $CD4^+$ T cell help to license DCs for CTL priming.

Initially, we thought that anti-CD40L acted by interfering with $CD4^+$ T cell licensing of DCs and thus had assumed that anti-CD40L would have no effect in any model that induced CTLs without $CD4^+$ T cells. To investigate this, we used two different mouse models that lack $CD4^+$ T cells. In the first model, we used GK mice, which secrete CD4 (GK1.5) antibody transgenically, effectively depleting the mouse of all peripheral $CD4^+$ T cells (Zhan et al., 2004). In the second model, we analyzed MHC class II-deficient mice, which are unable to positively select for $CD4^+$ T cells (Cosgrove et al., 1991). Again, we used anti-CD40 to facilitate priming of CTL responses to OVA-coated spleen cells. In both models, our results showed that even in the absence of $CD4^+$ T cells, the anti-CD40L Ab treatment was still able to suppress the CTL response (Figures 1B and 1C). This indicated that the antibody treatment played another role, apart from blocking $CD4^+$ T cell help, and led us to investigate what other cell type is being targeted by this treatment.

### CD40L Is Expressed on DCs

The expression of CD40L on $CD4^+$ T cells has been well documented (Grewal et al., 1996). However, the expression of CD40L on other cells has been less well characterized. CD40L expression on DCs has been reported for human blood DCs (Pinchuk et al., 1996), mouse Langerhans cells (Salgado et al., 1999), mouse lung DCs (Masten et al., 1997), and plasmacytoid DCs (Kuwajima et al., 2006). In preliminary studies, we found that surface staining of CD40L on splenic $CD8^+$ T cells and DCs (both resting and activated) was unconvincing. To further explore CD40L expression, we next used intracellular staining because we surmised that accumulation of CD40L after the addition of monensin during the procedure might avail easier detection. Lymph node cells obtained from naive wild-type C57BL/6 (B6) or CD40L-deficient (*Cd40lg*$^{-/-}$) mice were cultured for 4 hr in the presence of PMA and ionomycin, permeabilized, and stained for intracellular stores of CD40L. DCs were cultured overnight in media, which is known to induce activation (Wilson et al.,

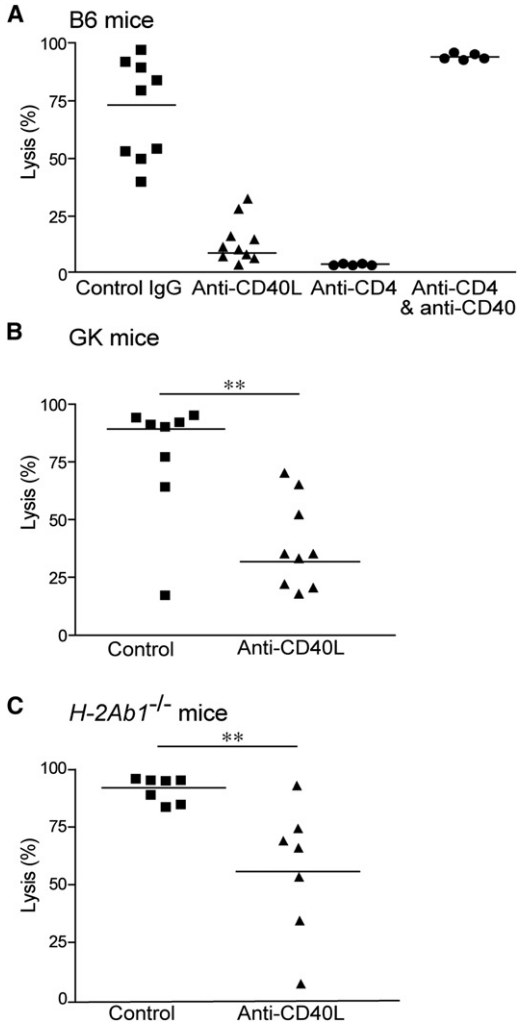


**Figure 1. Treatment with Anti-CD40L Decreases the CTL Response Even in the Absence of $CD4^+$ T Cells**

(A) Wild-type, (B) GK transgenic mice and (C) MHC class II-deficient (*H-2Ab1*$^{-/-}$) mice were immunized with $2 \times 10^7$ irradiated OVA-coated spleen cells with 1 μg LPS I.V. On the same day as immunization, 400 μg of anti-CD40L (MR1) or control hamster IgG i.p. was administered. After 7–8 days, mice were given i.v. $2 \times 10^7$ $CFSE^{hi}$-labeled peptide-pulsed cells and $CFSE^{lo}$-labeled control unpulsed cells in equal numbers. After 24 hr, spleens were analyzed by flow cytometry. The percentage lysis was calculated by the reduction in the $CFSE^{hi}$-labeled peptide-pulsed target cells compared with unprimed mice. Each dot represents a mouse and the bar represents the mean. CD4-deficient mice (B and C) were treated with 100 μg of anti-CD40 (FGK45) i.p. on the day of immunization. **p = 0.0076 and 0.0072, respectively, Mann-Whitney U Test.

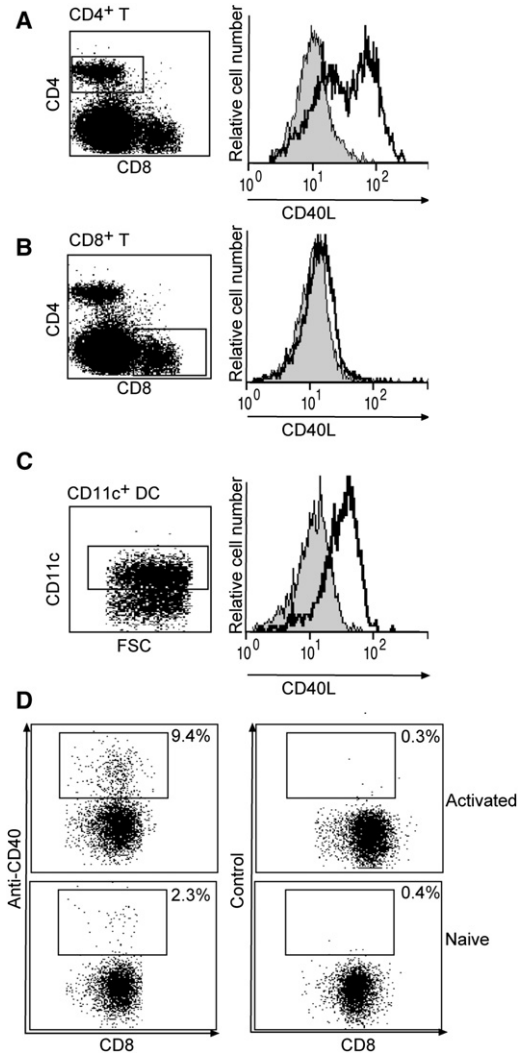


**Figure 2. CD40L Is Expressed on DCs and CD8$^+$ T Cells Modestly Express CD40**

Lymph node cells from naive C57BL/6 (black line) or *Cd40lg*$^{-/-}$ mice (gray, filled) were stimulated in vitro for 4 hr with PMA and ionomycin. Cells were stained for CD4 and CD8 and intracellular CD40L. The histogram in (A) is gated on CD4$^+$ cells and the one in (B) gated on CD8$^+$ cells. As shown in (C), DCs were purified from spleens of naive C57BL/6 (black line) or *Cd40lg*$^{-/-}$ mice (gray, filled) and cultured overnight prior to intracellular staining. All of the above experiments were independently performed at least three times.

As shown in (D), lymph node cells from C57BL/6 mice were analyzed immediately or were activated with PMA and ionomycin. They were then stained with CD8 and CD40 and analyzed with flow cytometry. Two independent experiments were performed.

2006). CD40L, as expected, stained strongly on CD4$^+$ T cells (Figure 2A), compared with either *Cd40lg*$^{-/-}$ controls (Figure 2A) or with control antibody staining (data not shown). CD8$^+$ T cells showed virtually no staining of CD40L (Figure 2B). We found that CD40L was also expressed quite strongly on cultured splenic DCs (Figure 2C). Given that CD40 is also found on activated wild-type CD8$^+$ T cells (Bourgeois et al., 2002; Sun and Bevan, 2004; Figure 2D) and DCs, it was feasible that CD40L expressed by DCs may interact with CD40 on T cells, in addition to the previously described interaction of CD40L on T cells interact with CD40 on DCs.

## *Cd40lg*$^{-/-}$ APCs Have Reduced Function in Priming CD8$^+$ T Cells

To explore further the role of CD40L being expressed by APCs, we tested whether this CD40L was required for priming CD8$^+$ T cells in an endogenous CTL assay. To discriminate between the role of CD40L on APCs and that of CD8$^+$ T cells, we generated mixed BM chimeras that would allow us to examine independently the different cell types expressing CD40L. Irradiated mice were reconstituted with equal parts of bm1 and *Cd40lg*$^{-/-}$ BM. Bm1 mice differ from B6 mice in that H-2K$^{bm1}$ has three mutations in the H-2K$^b$ molecule that disallows presentation of the OVA peptide SIINFEKL (Nikolic-Zugic and Carbone, 1990). The resultant mice will have two sets of APCs. One set can present OVA but is CD40L-deficient. The other set cannot present SIINFEKL but is capable of expressing CD40L. In this situation, any CTL priming must be due to the CD40L-deficient APCs. For examination of CTL priming in the absence of CD4$^+$ T cell help, reconstituted mice were CD4-depleted and treated with agonistic CD40 Ab. To exclude the possibility that the bm1 T cells are unable to kill, we generated mixed chimeras of bm1 and *Rag1*$^{-/-}$ BM (1:1 ratio). Such mice will have bm1 T cells and a set of wild-type B6 APCs (that can present OVA and express CD40L). Our results (Figure 3A) showed that there was a significant decrease in killing in bm1 *Cd40lg*$^{-/-}$ chimeric mice compared with the chimeric bm1 *Rag1*$^{-/-}$ controls. Thus, the *Cd40lg*$^{-/-}$ APCs are less capable of eliciting CTL priming.

## CD40L on DCs and CD40 on CD8 T Cells Were Important for CTL Induction

To show that DCs were critical in this CD40L-dependent antigen presentation, we generated chimeric mice with CD11cDTR (Probst et al., 2005) and *Cd40lg*$^{-/-}$ BM. The CD11cDTR mice express a fusion protein of the grivet monkey diphtheria toxin receptor and green fluorescent protein (GFP) under the control of the CD11c promoter. Therefore, all mice are generated similarly and the reconstitution of DCs with *Cd40lg*$^{-/-}$ versus CD11cDTR *Cd40lg*$^{+/+}$ genotype can easily be tracked by GFP and CD11c expression (CD11c$^+$GFP$^-$ versus CD11c$^+$GFP$^+$). Diphtheria toxin (DT) was administered to one group (100 ng per 20 g mouse on day −1, 0, and 1 relative to immunization; n = 5) and compared to untreated mice. All mice were depleted of CD4$^+$ T cells with 0.5 mg GK1.5 prior to immunizing with $2 \times 10^7$ irradiated OVA-coated spleen cells i.v. and 0.1 mg anti-CD40 (FGK45) i.p. After 7 days, $2 \times 10^7$ CFSE$^{hi}$-labeled peptide pulsed cells and CFSE$^{lo}$-labeled control cells in equal numbers were injected i.v. Spleens were analyzed 24 hr later in this in vivo CTL assay. As shown in Figure 3B, the DCs in these mixed BM chimeras comprise 32%–40% CD11cDTR and 60%–68% *Cd40lg*$^{-/-}$. Thus the majority of DCs were of the *Cd40lg*$^{-/-}$ genotype, and therefore there was a bias to show presentation function by the *Cd40lg*$^{-/-}$ DCs. The depletion of GFP$^+$ CD11cDTR DCs (*Cd40lg*$^{+/+}$ genotype) upon administration of DT (Figure 3B) resulted in a substantial reduction (>50%) in CTL killing (Figure 3C; $p = 0.021$). This confirms that DCs expressing CD40L is a critical component of CTL priming in the absence of CD4$^+$ T cells.

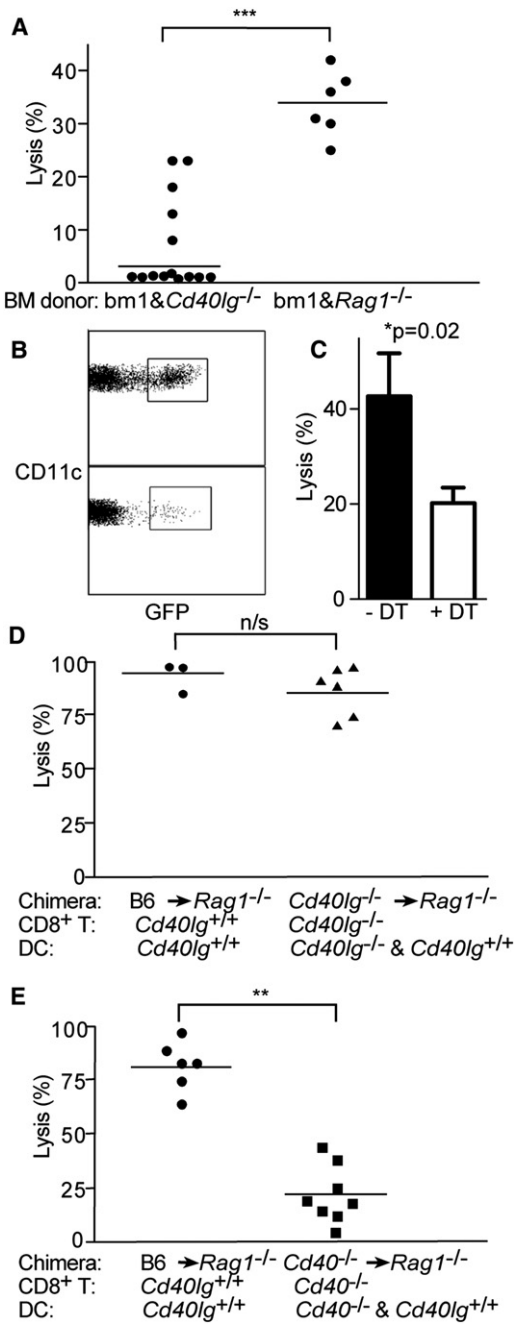


**Figure 3. *Cd40lg*$^{-/-}$ DCs Have Reduced Function in Priming Helper-Independent CD8$^+$ T Cells**

(A, D, and E) Either (A) bm1 reconstituted or (D and E) *Rag1*$^{-/-}$ reconstituted mice were treated with 0.5 mg GK1.5 prior to immunizing with $2 \times 10^7$ irradiated OVA-coated spleen cells with 1 μg LPS i.v. and 0.1 mg anti-CD40 (FGK45). Staining with a noncompeting RM4-4 Ab had shown that the CD4 depletion was complete. After 7–8 days, $2 \times 10^7$ CFSE$^{hi}$-labeled peptide-pulsed cells and CFSE$^{lo}$-labeled control unpulsed cells in equal numbers. After 24 hr, spleens analyzed by flow cytometry. The percentage lysis is calculated by the reduction in the CFSE$^{hi}$-labeled peptide-pulsed target cells compared with unprimed mice. Error bars represent mean ± SEM. ***p = < 0.0002, Mann-Whitney U Test.

(B and C) Elimination of *Cd40lg*$^{+/+}$ DCs by diphtheria toxin (DT) in *Cd40lg*$^{-/-}$ and CD11cDTR mixed BM chimeras. Chimeric mice were generated with CD11cDTR BM and *Cd40lg*$^{-/-}$ BM. DT was administered to one group (n = 5) prior to immunization and compared to untreated mice (n = 5).

(B) Enumeration of DC reconstitution in chimeric mice. DCs were enriched with a nycodenz gradient and stained for CD11c. DCs from CD11cDTR mice express GFP. The top panel shows that the DCs comprise 35% CD11cDTR and 65% *Cd40lg*$^{-/-}$, and the bottom panel shows the reduced GFP expression (i.e., the GFP$^+$ CD11cDTR DCs are depleted) after DT administration.

(C) Mice were treated with 0.5 mg GK1.5 prior to immunizing with $2 \times 10^7$ irradiated OVA-coated spleen cells I.V. and 0.1 mg anti-CD40 (FGK45). After 7 days, $2 \times 10^7$ CFSE$^{hi}$-labeled peptide-pulsed cells and CFSE$^{lo}$-labeled control cells in equal numbers were injected i.v. Spleens were analyzed 24 hr later. Percentage lysis was calculated by the reduction in the peptide-pulsed target cells compared to unprimed mice.

To show that *Cd40*$^{-/-}$ but not *Cd40lg*$^{-/-}$ CD8$^+$ T cells have reduced in vivo killing, we sublethally irradiated *Rag1*$^{-/-}$ mice (300 cGy) and reconstituted them with either *Cd40lg*$^{-/-}$ (Figure 3D) or *Cd40*$^{-/-}$ (Figure 3E) BM. Mice were depleted of CD4$^+$ T cells prior to immunizing with OVA-coated spleen cells and CD40 Ab. Using CFSE-labeled peptide-coated cells to detect differential in vivo lysis, we showed that *Cd40lg*$^{-/-}$ CD8$^+$ T cells but not *Cd40*$^{-/-}$ CD8$^+$ T cells could be primed to become CTLs (Figures 3D and 3E).

## *Cd40lg*$^{-/-}$ DCs Are Inefficient at T Cell Priming, but *Cd40lg*$^{-/-}$ T Cells Are Able to be Stimulated In Vitro

To further test the role of CD40L expression by DCs on T cell priming, we investigated an in vitro system, namely mixed lymphocyte reactions (MLRs) between BALB/c and B6 cells, by using naive CD8$^+$ T cells as responders. Purified (97%–99% purity) CD11c$^+$ cells were used as APCs and the proliferation of mismatched purified CD8$^+$ T cells was measured by thymidine incorporation after 3 days. The addition of anti-CD40L to the MLR caused a significant decrease in proliferation of CD8$^+$ T cells (Figure 4A), supporting our earlier findings of its effect on CTL generation in vivo. Whereas *Cd40lg*$^{-/-}$ CD8$^+$ T cells behaved like wild-type CD8$^+$ T cells (Figure 4B), *Cd40lg*$^{-/-}$ DCs were poor stimulators compared to wild-type DCs (Figure 4C). Reciprocally, *Cd40*$^{-/-}$ CD8$^+$ T cells showed decreased proliferation, consistent with a role of CD40L on DCs (Figure 4D). Hence, for CD8$^+$ T cell responders in MLR, it is important for DCs to have CD40L and for CD8$^+$ T cell responders to have CD40.

## CD40L on DCs Is Upregulated upon Activation with TLR3 and TLR9 Agonists

Our results above indicated that CD40L on DCs was important in priming anti-CD40 potentiated CTL responses. This led us to investigate whether anti-CD40 might upregulate CD40L on DCs. We cultured DCs for 8 hr with CD40 Ab. This shortened time point was necessary because longer incubation of DCs (e.g., overnight) resulted in their activation (Wilson et al., 2006) and CD40L upregulation (Figure 2C). Indeed, we found that CD40L on DCs was upregulated by CD40 Ab (Figure 5A).

This raised the question whether other DCs-activating agents like TLR agonists (mimicking infection) could also upregulate CD40L on DCs. To test this, we used a panel of TLR agonists, including pam2cys (TLR2), poly I:C (TLR3), LPS (TLR4), Loxoribine (TLR7), and CpG (TLR9). We observed expression of CD40L when adding TLR3 agonists (poly I:C) or TLR9 agonists for an 8 hr time point (Figure 5B). In contrast, agonists against

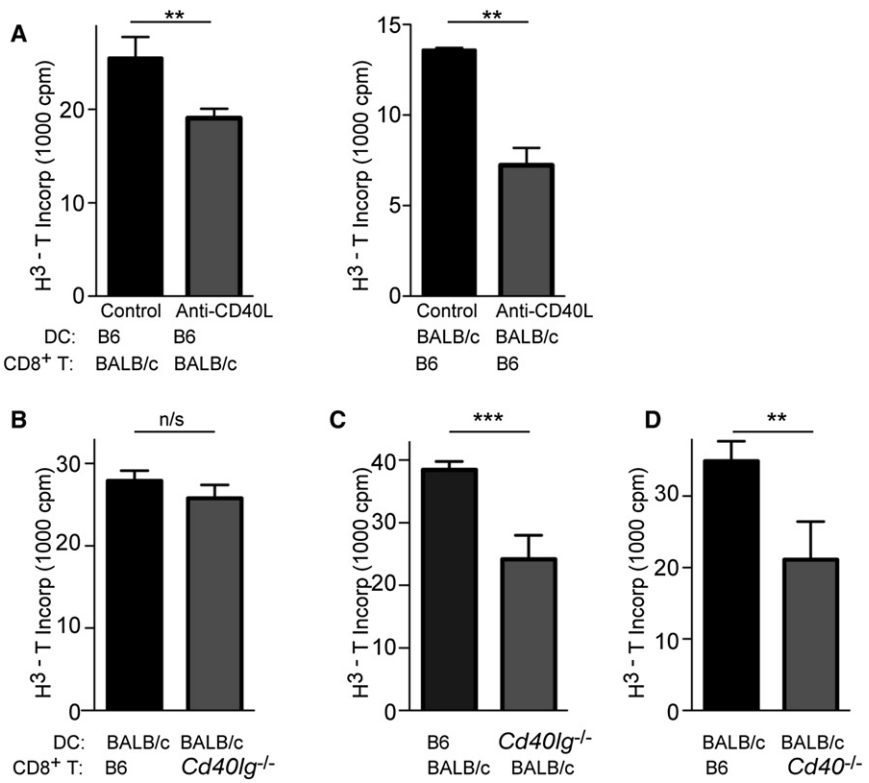


**Figure 4. *Cd40lg*$^{-/-}$ DCs Are Inefficient at Eliciting MLR**

A total of $2 \times 10^5$ purified $CD8^+$ T cells were cultured with allogeneic purified $CD11c^+$ DCs for 3 days prior to the addition of [$H^3$] thymidine. Proliferation was measured by the incorporation of [$H^3$] thymidine above that of syngeneic cultures. (A) shows MLR using B6 T cells with BALB/c DCs or BALB/c T cells with B6 DCs in the presence of anti-CD40L or control hamster IgG. (B) shows *Cd40lg*$^{-/-}$ compared with wild-type B6 T cells reacting to BALB/c DCs. (C) shows *Cd40lg*$^{-/-}$ compared with wild-type B6 DCs as presenters to BALB/c T cells. (D) shows *Cd40*$^{-/-}$ compared with wild-type B6 T cells reacting to BALB/c DCs. **$p < 0.005$, Mann Whitney U Test. Mean + SEM are shown.

TLR2 (Pam2cys), TLR4 (LPS), and TLR7 (Loxoribine) despite activating DCs as shown by CD86 expression (Figure S1 available online), did not upregulate CD40L (Figure 5B).

### The Potentiation of Helper-Independent CTL Responses by TLR3 and TLR9 Agonists Is Dependent on CD40L Upregulation

As shown above, anti-CD40 can upregulate CD40L on DCs and facilitate CTL priming in the absence of $CD4^+$ T cells. Because TLR3 and TLR9 agonists could also upregulate CD40L on DCs just like anti-CD40 (Figure 5B), we wanted to determine whether these agonists could also potentiate helper-independent CTL responses. We showed that in CD4 T cell-deficient mice (GK mice), the only TLR ligands to allow helper-independent CTL induction were those that upregulated CD40L, namely poly I:C and CpG (black columns; Figure 5C). Moreover, this induction was dependent on CD40L, given that anti-CD40L dramatically reduced the CTL response (white columns; Figure 5C).

### Helper-Independent Pathogens May License APCs by Upregulating CD40L

Because agonists to TLR3 and TLR9 can activate APC to potentiate helper-independent CTL responses, we investigated whether a pathogen itself is able to have the same function. It has been known that some pathogens are able to elicit immune responses in the absence of $CD4^+$ T cells (Behrens et al., 2004; Shedlock and Shen, 2003; Sun and Bevan, 2003) and that this was due to such viruses activating DCs (Wu and Liu, 1994). However, no differential activation between viruses that elicit CTLs without help and those that do not has been forthcoming. Thus, we questioned whether the upregulation of CD40L is required for these helper-independent responses. To investigate this, we chose two viruses: influenza whose primary effector CTL responses are mainly helper independent (Allan et al., 1990; Tripp et al., 1995) and herpes simplex virus (HSV) whose CTL responses are mainly helper dependent (for the intravenous and intradermal route [Smith et al., 2004]; for flank infection, Figure S2).

Mice depleted of $CD4^+$ T cells were infected intranasally with influenza virus and treated with either anti-CD40L or control IgG. The CTL response against the influenza NP peptide-pulsed cells was reduced in the treated mice (Figures 6A and 6B). The number of NP-specific $CD8^+$ T cells was also dramatically reduced as shown with tetramer staining (Figure 6C). Thus, we hypothesized that part of helper independence for influenza-induced CTL immunity is the virus's ability to upregulate CD40L on APC. Indeed, we showed that influenza infection of DCs upregulated CD40L expression in vitro, whereas HSV did not (Figure 6D), even though both viruses upregulated CD86 (Figure 6D). Therefore, we conclude that upregulation of CD40L on APCs is one mechanism by which some pathogens are able to elicit helper-independent effector CTLs.

## DISCUSSION

CD40-CD40L interactions have long been associated with DC maturation (O'Sullivan and Thomas, 2003), although the majority of investigations have approached this from the angle of DCs displaying CD40. Because both CD40L and CD40 are found in $CD4^+$ T cells, DCs (highlighted in this study), and $CD8^+$ T cells, there are many potential permutations for amplifying the response within or between each cell type. Moreover such potential interactions may differ among various situations, e.g., between initial CTL induction and memory generation. In this study, we have provided evidence that CD40L is required on DCs for optimal priming of naive $CD8^+$ T cells to become CTLs. Our results showed that anti-CD40L treatment decreased the endogenous in vivo cytotoxic T cell response to OVA. Anti-CD40L

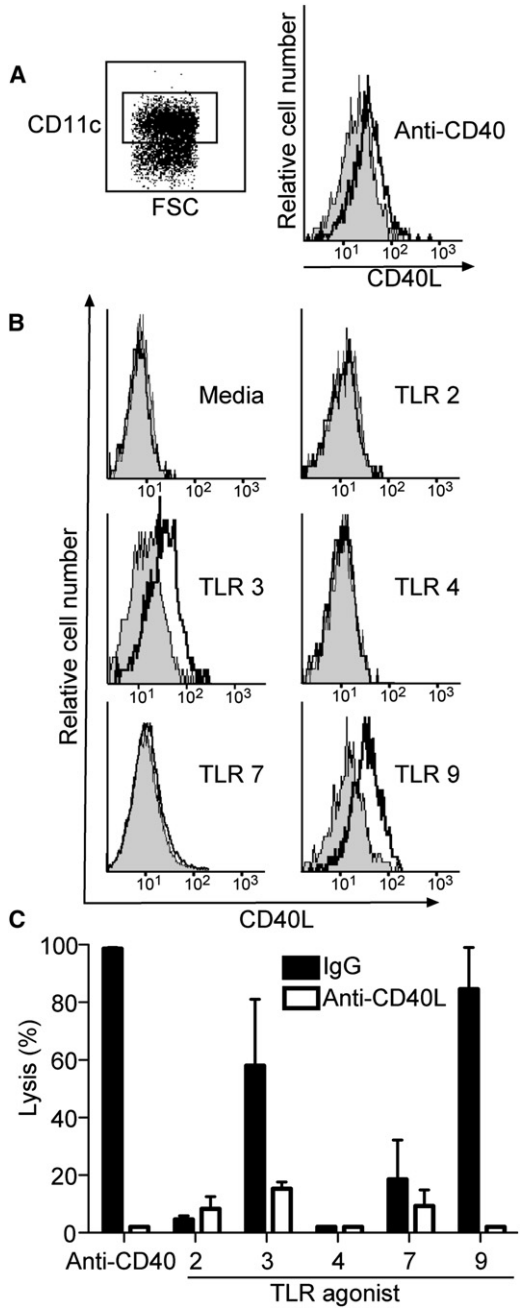


**Figure 5. CD40L Is Upregulated in DCs by TLR3 and TLR9 Agonists and These Agonists Can Potentiate CD40L-Dependent CTL Responses**

DCs were purified ($CD11c^+$) and cultured for 8 hr with (A) CD40 monoclonal antibody (FGK45) or (B) a panel of agonists for TLR2, TLR3, TLR4, TLR7, and TLR9. Histograms show expression of intracellular CD40L on gated $CD11c^+$ cells from C57BL/6 mice (black line) relative to $Cd40lg^{-/-}$ mice (gray filled). As shown in (C), GK (CD4-deficient) mice were immunized with $2 \times 10^7$ irradiated OVA-coated spleen cells with 1 μg LPS i.v. and 0.1 mg anti-CD40, 40 nmols CpG, 20 nmols Pam2Cys, 1 mg Loxoribine, or 100 μg Poly I:C. Anti-CD40L or hamster IgG, 400 μg, was also given on the day of immunization. After 7 days, $2 \times 10^7$ $CFSE^{hi}$-labeled peptide-pulsed cells and $CFSE^{low}$-labeled control unpulsed cells in equal numbers were injected i.v. After 24 hr, spleens were analyzed by flow cytometry. The percentage lysis was calculated by the reduction in the $CFSE^{hi}$-labeled peptide-pulsed target cells compared with unprimed mice. The graph shows mean + SD; n = 3. Two independent experiments were performed.

treatment was most effective when used on the day of immunization, which inferred interference at the priming or expansion phase rather than the effector phase. Although the importance of CD40L expression by $CD4^+$ T cells and CD40 by DCs in licensing DCs for CTL priming is not in dispute, little attention has been drawn to the possible role for CD40L expression by DCs in helper-independent responses. In using two different models of CD4-deficient mice, we have shown that anti-CD40L treatment decreased the CTL response in the absence of $CD4^+$ T cells. Thus, in the absence of $CD4^+$ T cells, anti-CD40L treatment must be targeting another cell type that expresses CD40L. Upon examination of CD40L expression on various cell types, we discovered that CD40L was upregulated on DCs. This CD40L seemed to be functionally required because in vivo CTL assays in BM chimeras showed that when the DCs are $Cd40lg^{-/-}$, killing was reduced. Likewise in vitro, MLRs show that $Cd40lg^{-/-}$ DCs did not prime T cells as effectively as wild-type DCs, whereas $Cd40lg^{-/-}$ $CD8^+$ T cells proliferated just as efficiently as wild-type $CD8^+$ T cells. $Cd40^{-/-}$ $CD8^+$ T cells however, in both in vivo and in vitro assays, were less efficacious than wild-type or $Cd40lg^{-/-}$ $CD8^+$ T cells. CD40 expression by T cells, especially activated ones, has been reported previously (Bourgeois et al., 2002; Munroe and Bishop, 2007), although the role for CD40 on $CD8^+$ T cells remained undefined. It has been shown to have a costimulatory function, augmenting in vitro responses to agonists against CD3 and CD28 (Munroe and Bishop, 2007). Our work may give insight into an undefined pathway in which the CD40 on these $CD8^+$ T cells interact with the CD40L on the DCs. Our hypothesis that CD40L on DCs is important in helper-independent responses does not discredit the notion that CD40 may also play a role in some situations. Hernandez et al., (2007) had ascribed a role of CD40L on $CD8^+$ T cells; much of that work used the P-14 TCR transgenic system (and lymphocytic choriomeningitis virus gp33 peptide on $H\text{-}2D^b$). In contrast, we have found no evidence that CD40L is important on $CD8^+$ T cells in our endogenous models given that $Cd40lg^{-/-}$ $CD8^+$ T cells were able to have normal cytotoxic activity in the presence of $Cd40lg^+$ DCs. We have also looked at TCR-transgenic models; namely, OT-I and found OT-I T cells were not reliant on CD40L for proliferation or killing (data not shown). Given the recent findings that clonal size is important in determining outcomes, it may not be surprising that there may be differences between TCR-transgenic systems and endogenous systems (Ford et al., 2007; Mintern et al., 2002). One of the key components of the Hernandez paper was that the $Cd40lg^{-/-}$ P-14 T cells proliferated poorly compared with wild-type P-14 T cells in vivo. Interestingly, anti-CD40 stimulation availed these $Cd40lg^{-/-}$ T cells to proliferate, and this suggests that $CD8^+$ T cells do not require CD40L to proliferate. Our proposal that CD40 Ab can upregulate CD40L on DCs to prime $CD8^+$ T cells would explain this result.

We therefore propose that CD40L on the DCs interacts with the CD40 on $CD8^+$ T cells and is crucial for helper-independent CTL responses. Given that CD40 is expressed on other immune cells including $CD4^+$ T cells, B cells, endothelial cells, thymic epithelial cells and DCs themselves (Gray et al., 2006; Quezada

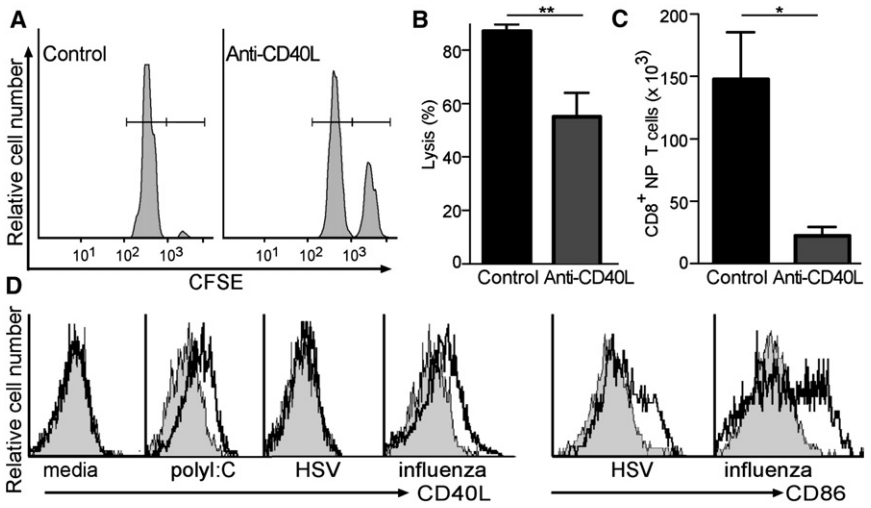


**Figure 6. Influenza Can Upregulate CD40L on the Dendritic Cell and Generate CD40L Partially Dependent CTLs**

Mice treated with GK1.5 (anti-CD4) were infected with 30 pfu intranasal influenza (PR8 strain); on the same day, 400 μg anti-CD40L or IgG treatment was administered. Six days later, mice were injected with $2 \times 10^7$ $CFSE^{hi}$-labeled peptide (influenza $NP_{366-374}$)-pulsed cells and $CFSE^{lo}$-labeled control unpulsed cells. After 24 hr, spleens were analyzed by flow cytometry. In (A), histograms showed the two peaks indicating $CFSE^{hi}$ peptide-pulsed cells versus $CFSE^{lo}$ unpulsed cells in hamster IgG-treated and anti-CD40L-treated mice. As shown in (B), the percentage lysis was calculated by the reduction in the peptide-pulsed target cells compared with uninfected mice. The graph shows mean and SEM. $^{**}p = 0.0027$, Mann-Whitney U Test. As shown in (C), mediastinal lymph nodes were taken from CD4-depleted influenza infected mice at day 5 and stained with NP tetramer. The bar graph shows the total number of $CD8^+$ NP-specific T cells. The graph shows mean ± SEM. $^{*}p = 0.0286$, Mann-Whitney U test. As shown in (D), $CD11c^+$ DCs were purified and infected for 1 hr with 5 pfu per cell of either PR8 influenza or HSV. DCs were then cultured for 6 hr prior to surface staining of CD11c and CD86 and intracellular staining of CD40L. Histograms are gated on $CD11c^+$ cells. CD40L histograms show B6 (black line) overlaid onto *Cd40lg*$^{-/-}$ DCs (gray filled). CD86 histograms show HSV- or influenza-infected (black line) cells overlaid onto uninfected DCs (gray filled). All experiments were performed at least twice.

et al., 2004; van Kooten and Banchereau, 2000), whether CD40L on DCs may interact with CD40 of all these cell types invites further elucidation. The CD40L on DCs could be induced in vitro upon activation with TLR3 and TLR9 agonists. TLR3 and TLR9 agonists could also be used to generate helper-independent CTL responses; this could be dramatically blocked by anti-CD40L. However agonists to TLR2, TLR4, and TLR7 failed to upregulate CD40L and were unable to deliver signals to prime T cells in vivo. Thus so that helper-independent CTL responses could be generated, TLR3 and TLR9 agonists can upregulate CD40L expression on DCs and promote CTL priming. TLR3 and TLR9 agonists (but not TLR2 or TLR4 agonists) have previously been shown to facilitate crosspresentation in vitro (Datta et al., 2003). Moreover, the addition of TLR3 or TLR9 agonists to cationic liposomes can elicit in vivo crosspriming of $CD8^+$ T cells independent of $CD4^+$ T cell help (Zaks et al., 2006). Our findings now offer a mechanistic explanation for their findings, i.e., upregulation of CD40L on DCs by agonists to TLR3 and TLR9 promotes $CD8^+$ T cell priming.

Because TLR agonists mimic microbial infection, we surmised that pathogens that promote CTL priming in a helper-independent fashion may do so by upregulating CD40L on DCs. Viruses can infect both human and murine DCs, leading to activation and upregulation of costimulatory molecules such as CD40 and CD80-CD86 (Harui et al., 2006; Jain et al., 2007; Montoya et al., 2005; Saurwein-Teissl et al., 1998; Wu and Liu, 1994). CD40L as a costimulatory marker on DCs has not been investigated in this capacity, presumably because most workers assume that CD40L is primarily a T cell molecule. We chose two infectious models: intranasal influenza, which is helper independent (Allan et al., 1990; Tripp et al., 1995) and HSV flank infection, which is mostly dependent on CD4 help, just like for the intravenous or footpad route (Smith et al., 2004). CD40L on DCs was upregulated by influenza but not by HSV, whereas both infections upregulated CD86 on DCs. Moreover, the helper-independent CTL responses by influenza were substantially reduced by anti-CD40L treatment.

The mechanism of how viruses like ectromelia, vesicular stomatitis virus, human immunodeficiency virus, Epstein-Barr virus, influenza, and cytomegalovirus can potentiate helper-independent CTL responses in the absence of CD4 help remained unclear despite much exploration (Hamilton et al., 2001; Ruedl et al., 1999; Sun and Bevan, 2004; Wang et al., 2001). Our work provides at least one mechanism by which some viruses can elicit CTL responses in a CD4-deficient environment. Many viruses, such as HIV (Lore et al., 2002; Trimble et al., 2000), MCMV (Mintern et al., 2006), HPV (Ortiz-Sanchez et al., 2007), HCMV (Moutaftsi et al., 2002), and HSV (Mikloska et al., 2001), have developed evasion strategies that downregulate costimulatory markers on both T cells and DCs. This evasion mechanism leads to decreased T cell-mediated immunity to the virus, thereby allowing chronic infections to manifest. Interestingly, HIV has been shown to downregulate CD40L on T cells (Subauste et al., 2007), thus it would be interesting to speculate that CD40L on DCs may also be downregulated. Correcting such a deficiency (e.g., by TLR agonists) could potentially lead to increased immunity in the absence of $CD4^+$ T cells in $HIV^+$ patients. There is ongoing work investigating the creation of viruses or cell lines expressing CD40L (Bereta et al., 2004; Dotti et al., 2001; Mehling et al., 2001; Ostrowski et al., 2000; Tomihara et al., 2008) for either vaccine strategies or eradication of tumors with cell-based therapies. Our findings that CD40L upregulation on DCs is involved in generating CTL responses to influenza virus may lead to increased understanding of how these therapies may be tailored.

In conclusion, we show that CD40L upregulation on DCs allows the generation of helper-independent CTLs. This CD40L upregulation also explains why only certain TLR agonists can promote helper-independent CTLs and provides a mechanism by which certain viruses can stimulate helper-independent CTLs.

## EXPERIMENTAL PROCEDURES

### Mice

Mice aged 6 to 8 weeks were used in all experiments. CD4-deficient mice GK5 (transgenic for anti-CD4 monoclonal antibody [Zhan et al., 2004]), CD40L-deficient mice (CD154-H) (Xu et al., 1994), MHC class II-deficient mice (Cosgrove et al., 1991), CD11cDTR mice (Probst et al., 2005), *Rag1*$^{-/-}$ (Mombaerts et al., 1992) *Cd40*$^{-/-}$, and MHC class I:C.H-2$^{bm1}$ (called bm1) mice were maintained in specific pathogen-free conditions in the animal facilities of Walter and Eliza Hall Institute of Medical Research. All mice were used according to the regulations of the Institute Animal Ethics Committee.

### DCs Preparations

DCs were harvested from spleens as previously described (Vremec et al., 1992). Spleens were finely diced and added to a collagenase-DNase solution. Digestion occurred for 25 min before EDTA was added. After sieving, diluting, and centrifuging, the pellet was then resuspended in 5 mL of Nycodenz/4 spleens and overlaid onto an additional 5 mL Nycodenz in a polypropylene tube. A total of 2 mL of FCS-EDTA was overlaid onto the splenic cell layer. The gradient was then centrifuged at 4000 rpm without brake for 15 min. The low-density cells were collected and washed with EDTA-BSS-FCS before cell counts were performed. A depletion antibody cocktail containing antibodies against CD3, Thy1, GR1, B220, and Ery was added to negatively select for DCs. Dynabeads sheep anti-rat IgG magnetic beads (Dynal Biotech, Oslo, Norway) were washed in EDTA-BSS-FCS and added at five beads per cell for 20 min at 4°C on a slowly rotating mixer. Cells were then depleted via negative selection on a magnet (Miltenyi Biotec, Bergisch Gladbach, Germany), and the supernatant was collected and the depletion was performed twice for extra purity. Cells were washed in EDTA-BSS-FCS, counted, and checked via flow cytometry for yield.

### Intracellular Staining

Cells were stained intracellularly for CD40L. T cells from B6 mice or *Cd40lg*$^{-/-}$ mice were cultured 4 × 10$^6$ cells/ml in a 24-well plate in RPMI with 10% FCS in 5% incubator. For activation, cells were incubated with PMA (25 ng/ml) and Ionomycin (1 μg/ml) for 3 hr. Monensin (Golgistop, Becton Dickinson, Franklin Lakes, NJ, USA) at 0.6 μl/ml was added to the cultures so that transport out of the cells was prevented. Cells were washed and stained with surface markers prior to fixing. Cells were fixed with 100 μl Cytofix (Becton Dickinson) for 20 min at 4°C. Cells were permeabilized in saponin (PermWash, Becton Dickinson) before staining with biotinylated CD40L and allophycocyanin-streptavidin (BDBiosciences, San Jose, CA, USA).

### In Vivo CTL Assays

Mice were immunized with 2 × 10$^7$ irradiated OVA-coated spleen cells from either a B6 or bm1 donor with 1 μg LPS i.v. Seven days later, mice were given i.v. 2 × 10$^7$ CFSE$^{hi}$-labeled SIINFEKL (Mimotopes, Clayton, Australia) peptide-pulsed cells and CFSE$^{lo}$-labeled control unpulsed cells in equal ratios. Spleens were analyzed by flow cytometry 18–24 hr later.

### Generation of Chimeric Mice

Lethal irradiation consisted of two doses of 550 cGy (γ-irradiation; $^{60}$Co source) 2 hr apart. Femoral BM, 1 × 10$^6$ cells were injected i.v. into the recipient mice within hours of irradiation. Neomycin sulfate was added prophylactically to the drinking water for 4 weeks postirradiation. We treated mice with 100 μl of Thy1 antibody (T24) i.p. day 1 postreconstitution to eliminate residual host T cells. Chimeric mice were used experimentally 6–8 weeks later.

### Mixed Lymphocyte Reactions

Spleens and lymph nodes were isolated from C57BL/6, BALB/c, or *Cd40lg*$^{-/-}$ mice. DCs and CD8$^+$ T cells were purified by MACS (Miltenyi Biotech) positive selection. A total of 2 × 10$^5$ T cells, as responders, were cultured with allogeneic DCs, as stimulators, at concentrations of 1:100. Cells were cultured in a 96-well round-bottom plate in MT-RPMI-10% FCS with 55 μM 2-mercapto-ethanol and incubated with 5% CO2. Syngeneic stimulators and responders were used as background controls. [H$^3$] thymidine was added 2 or 3 days later. Cells were harvested 18 hr after the addition of [H$^3$] thymidine, and proliferation was measured as CPM over background.

### Antibodies and TLR Agonists

Anti-CD40L (MR1), anti-CD40 (FGK45), and anti-CD4 (GK1.5) were purified by in-house facilities. Flow cytometry antibodies were purchased from BDBiosciences. Loxoribine and polyinosinic-polycytidilyc acid (Poly I:C) were purchased from Invivogen, San Diego, CA, USA; lipopolysaccharide (LPS) from *E. coli* 0111:B4 was purchased from Sigma, St Louis, MO, USA. D. Jackson, University of Melbourne, generously provided Pam2Cys.

### Viral Infections

All viruses were stored at −70°C in PBS as single-use aliquots. The influenza strain used was influenza A/Puerto Rico/8/34 (Mt Sinai, H1N1) (PR8). Titer of the original influenza virus AF17B = 3.1 × 10$^7$ plaque forming units (pfu)/ml.

B6 mice were infected with 30 pfu per mouse of the above strain of influenza intranasally in a volume of 20 μl. Purified DCs were infected by incubating at 37°C for 45 min with virus at a concentration of 5 pfu/cell. The suspension was swirled periodically. We washed DCs three times in KDS-RPMI-FCS to ensure any excess virus was removed prior to incubation for 5 hr to upregulate surface markers.

HSV flank infections were performed as previously described (Allan et al., 2006; van Lint et al., 2004).

### Tetramer Staining

Mediastinal lymph nodes were removed from mice infected with influenza 5 days prior. Cells were prepared into a single-cell solution and incubated with NP tetramer-PE, 1 in 200, for 40 min at room temperature. Cells on ice were stained for CD8 for 30 min and analyzed via flow cytometry.

### Statistical Methods

Data are shown as mean ± SEM. Nonnormally distributed data were compared with unpaired Mann-Whitney U Tests.

## SUPPLEMENTAL DATA

Supplemental Data include two figures and can be found with this article online at http://www.immunity.com/supplemental/S1074-7613(09)00067-3.

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