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Listeria monocytogenes Infection Overcomes the Requirement for CD40 Ligand in Exogenous Antigen Presentation to $CD8^+$ T Cells[1]

Sara E. Hamilton,* Amy R. Tvinnereim,† and John T. Harty[2]*†

In vivo priming of $CD8^+$ T lymphocytes against exogenously processed model Ags requires $CD4^+$ T cell help, specifically interactions between CD40 ligand (CD40L) expressed by activated $CD4^+$ T cells and CD40, which is present on professional APC such as dendritic cells (DCs). To address this issue in the context of bacterial infection, we examined CD40L-CD40 interactions in $CD8^+$ T cell priming against an exogenously processed, nonsecreted bacterial Ag. CD40L interactions were blocked by in vivo treatment with anti-CD40L mAb MR-1, which inhibited germinal center formation and $CD8^+$ T cell cross-priming against an exogenous model Ag, OVA. In contrast, MR-1 treatment did not interfere with $CD8^+$ T cell priming against a nonsecreted or secreted recombinant Ag expressed by *Listeria monocytogenes*. Memory and secondary responses of $CD8^+$ T cells against nonsecreted and secreted bacterial Ags were also largely unimpaired by transient MR-1 treatment. When MR-1-treated mice were concurrently immunized with *L. monocytogenes* and OVA-loaded splenocytes, cross-priming of OVA-specific naive $CD8^+$ T cells occurred. No significant decline in cross-priming against OVA was measured when either TNF or IFN-γ was neutralized in *L. monocytogenes*-infected animals, demonstrating that multiple signals exist to overcome CD40L blockade of $CD8^+$ T cell cross-priming during bacterial infection. These data support a model in which DCs can be stimulated in vivo through signals other than CD40, becoming APC that can effectively stimulate $CD8^+$ T cell responses against exogenous Ags during infection. ***The Journal of Immunology*, 2001, 167: 5603–5609.**

Induction of $CD8^+$ T cell responses against exogenously processed model Ags requires $CD4^+$ T cell help (1–3). This has been demonstrated in systems using model Ags loaded into splenocytes or expressed in cells which are not APCs. In these systems $CD8^+$ T cells are primed through an Ag-processing mechanism termed cross-presentation (1–4). During cross-presentation, cell-associated Ag is taken up by bone marrow-derived APC and presented via exogenous MHC class I processing pathways (reviewed in Ref. 5). A model for $CD8^+$ T cell cross-priming has emerged in which a $CD4^+$ T cell recognizes peptide-MHC class II complexes on the surface of an APC causing activation of the T cell and up-regulation of CD40 ligand (CD40L)[3] on its surface. This enables the T cell to signal through CD40 on an APC or dendritic cell (DC) to induce its maturation. Ab-mediated ligation of CD40 replaces the need for $CD4^+$ T cell help in vivo for efficient priming of $CD8^+$ T cells against model exogenous Ags (1–3). The factors required for efficient priming of naive $CD8^+$ T cells by DCs are not known, but up-regulation of costimulatory molecules and/or the secretion of various proinflammatory cytokines may be required. CD40 ligation, LPS, and TNF-α can cause the up-regulation of B7.1, B7.2, and MHC class II as well as the secretion of IL-12 by DCs (6, 7).

Several studies have examined $CD8^+$ T cell responses in $CD40L^{-/-}$ mice after viral infection. $CD8^+$ T cell responses appear to be normal in response to many viruses including lymphocytic choriomeningitis virus (LCMV), suggesting that viral infection may in some instances bypass the requirement for CD40L in DC activation (8). Some deficiencies have been observed in primary $CD8^+$ T cell responses after vesicular stomatitis virus (VSV) infection and memory $CD8^+$ T cell responses after LCMV infection (9, 10). Early T cell independent inflammatory responses to infection with the intracellular bacterium *L. monocytogenes* (LM) and T cell-mediated protective immunity appear to be unaffected by the absence of CD40L (11). Recent evidence has also shown a minimal impact on the splenic $CD8^+$ T cell response to secreted LM Ags in $CD40^{-/-}$ mice (12). However, these studies have measured responses generated through the endogenous MHC class I presentation pathway. Thus, it is not known whether CD40L-CD40 interactions are required for naive $CD8^+$ T cell priming against exogenously processed Ags expressed by pathogens.

This study examines the role of CD40L-CD40 interactions in $CD8^+$ T cell priming against LM Ags presented by either the endogenous or exogenous MHC class I presentation pathway. LM is a Gram-positive facultative intracellular bacterial pathogen that can enter and multiply within phagocytic and nonphagocytic cells. LM has the ability to escape from the membrane-bound phagosome after infection and enter the host cell cytoplasm (13). Secreted bacterial Ags can then be processed through the endogenous MHC class I presentation pathway, which involves proteolytic degradation by the proteasome and transport into the endoplasmic reticulum by the TAP complex. Nonsecreted bacterial Ags, although not accessible to the endogenous pathway, may become accessible to exogenous MHC class I presentation pathways after

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[3] Abbreviations used in this paper: CD40L, CD40 ligand; DC, dendritic cell; hIg, hamster Ig; LCMV, lymphocytic choriomeningitis virus; LLO, listeriolysin O; LM, *Listeria monocytogenes*; NP, nucleoprotein.

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destruction of the organism (14). Consistent with this idea, infection of mice with recombinant LM strains expressing an H-2L^d-restricted epitope from the nucleoprotein of LCMV ($NP_{118-126}$) as either a secreted or a nonsecreted fusion protein prime $NP_{118-126}$-specific CD8$^+$ T cell responses that differ in magnitude by only 3- to 4-fold (14).

To examine the role of CD40L-CD40 interactions in CD8$^+$ T cell priming against exogenously processed bacterial Ags, CD8$^+$ T cell responses were measured in mice treated with CD40L-blocking Abs and infected with recombinant LM. Our data demonstrate that although CD40L blocking Ab treatment prevented cross-priming of CD8$^+$ T cells against a model Ag, it does not inhibit priming against LM Ags. In addition, we show that LM infection overcomes CD40L blockade to allow CD8$^+$ T cell cross-priming against a model exogenous Ag.

Materials and Methods

Mice and bacteria

Six- to 8-wk-old female BALB/c (H-2^d MHC) and CB6F1 (H-2^{bxd} MHC) mice were purchased from National Cancer Institute (Frederick, MD). Virulent LM strains used in this study were recombinant strain XFL304 (LM-NPns) expressing a nonsecreted fusion protein containing the LCMV $NP_{118-126}$ epitope and strain XFL303 (LM-NPs) expressing the same fusion protein in secreted form (14). Attenuated LM strain DP-L1942 (ActA$^-$ LM), which carries an in-frame deletion in the *act*A gene (15), and attenuated LM strain DP-L2161 (listeriolysin O (LLO)$^-$ LM), which carries an in-frame deletion in the *hly* gene (16), were also used. Growth and maintenance of all LM strains were described previously (17).

Infection with LM

Age- and sex-matched adult BALB/c or CB6F1 mice were infected by i.v. injection with 2×10^3 LM-NPns or LM-NPs, 1×10^6 ActA$^-$ LM, and 1×10^8 LLO$^-$ LM. Actual numbers of CFU injected were determined for each experiment by plate count.

Cross-priming with OVA-loaded splenocytes

Age- and sex-matched CB6F1 mice were immunized with OVA-loaded BALB/c splenocytes as previously described (18, 19). Briefly, RBC-depleted BALB/c splenocytes were incubated with freshly prepared OVA (10 mg/ml) in hypertonic medium (RPMI 1640, 0.5 M sucrose, 10% polyethylene glycol 1000, and 10 mM HEPES, pH 7.2) for 10 min at 37°C. Splenocytes were then diluted in warm hypotonic medium (60% HBSS and 40% H_2O) and incubated for 2 min at 37°C. Cells were washed twice in HBSS and resuspended at $60–70 \times 10^6$ splenocytes/ml. CB6F1 mice received 0.5 ml/animal i.v. For coimmunization with LM and OVA-loaded splenocytes, i.v. infection with the indicated number of organisms was performed first followed 2–4 h later by injection of OVA-loaded splenocytes.

Cells lines and Abs

Hybridomas producing anti-CD40L mAb MR-1 (20) (a gift from Dr. T. Waldschmidt, University of Iowa, Iowa City, IA) and anti-TNF (XT22 and XT3 used in combination at a mass ratio of 1:1) (21) were grown in DMEM supplemented with 10% FCS, L-glutamine, and antibiotics. Hybridomas producing anti-IFN-γ (XMG1.2) (22) were grown in RPMI 1640 supplemented with 10% FCS, L-glutamine, and antibiotics (RP10) (23). Ab was purified from culture supernatant by affinity chromatography with protein G (Amersham Pharmacia, Piscataway, NJ). Each batch of Ab was concentrated to 2 mg/ml and assessed for endotoxin contamination by *Limulus* amebocyte lysate assay (Associates of Cape Cod, Falmouth, MA). All preparations contained <15 ng/ml endotoxin.

Anti-CD40L Ab MR-1 has been used in multiple studies to block CD40L in vivo (24). To determine whether our purification procedure was sufficient in yielding MR-1 that could effectively block CD40L in vivo, MR-1- or hamster Ig (hIg)-treated BALB/c mice were immunized with SRBC. Immunization results in CD40L-dependent germinal center formation within 4 days in control hIgG (Jackson ImmunoResearch, West Grove, PA)-treated animals or animals receiving no Ab treatment. In animals that received 1 mg MR-1 in total, given on days −2, 0, 2, and 4, surface staining revealed a 70–85% decrease in germinal center B cells (data not shown), a level below that of germinal center B cells found in naive animals housed in our animal facility. Similar data were obtained 7 days after SRBC immunization (data not shown).

Intracellular cytokine staining of splenocytes

The number of CD8$^+$ T cells specific for $OVA_{257-264}$ in the context of H-2K^b, $NP_{118-126}$ in the context of H-2L^d, LLO_{91-99} in the context of H-2K^d, or $p60_{217-225}$ in the context of H-2K^d was determined by intracellular cytokine staining for IFN-γ as previously described (25). RBC-depleted splenocytes from immunized mice were incubated for 5–6 h in 2 μl/ml brefeldin A at 37°C with or without synthetic peptides at 200 nM. For staining, cells were placed on ice, washed, and incubated with Ab directed against the FcγII/III receptors (2.4G2) and FITC-conjugated anti-CD8 mAb (BD PharMingen, San Diego, CA), fixed, permeabilized, and stained with PE-conjugated anti-IFN-γ mAb according to manufacturer's instructions (BD PharMingen).

Data were acquired on FACScan flow cytometer (Becton Dickinson, Mountain View, CA) using Summit software. One hundred thousand events were collected for analysis with FlowJo software (Tree Star, San Carlos, CA). The gate for IFN-γ$^+$ cells was selected so that the percentage of IFN-γ$^+$ cells in the unstimulated sample for each mouse was 0.2% or less of CD8$^+$ splenocytes. This value was subtracted from the peptide-stimulated values to determine the frequency of Ag-specific CD8$^+$ T cells. Total numbers of epitope-specific CD8$^+$ T cells/spleen were calculated using this frequency, the percentage of CD8$^+$ T cells in each sample, and the total number of splenocytes per animal.

IFN-γ ELISPOT

Ag-specific CD8$^+$ T cell responses were measured by IFN-γ ELISPOT analysis as previously described (26). Briefly, splenocytes (10^4–10^5/well) were cultured with medium alone or with 100 nM synthetic peptide (LLO_{91-99} or $NP_{118-126}$) for 36–48 h in flat-bottom 96-well plates that had been previously coated with rat anti-mouse IFN-γ mAb (RA-6A2, BD PharMingen). Following washes with PBS-0.05% Tween, the plates were incubated for 3 h at room temperature with rabbit anti-IFN-γ antiserum (a gift from Dr. J. Cowdery, University of Iowa). After washing, the plates were incubated for 4–5 h with donkey anti-rabbit Ig conjugated to alkaline phosphatase (Jackson ImmunoResearch). Further washing was followed by addition of 1 mg/ml 5-bromo-4-chloro-3-indolylphosphate (Sigma, St. Louis, MO) buffer with 0.75% agarose. The reaction was developed for 0.5–1 h at 37°C and was stopped by storage at 4°C. Spots were counted using a dissecting microscope. The average frequency of responders from triplicate wells was multiplied by the total number of splenocytes in calculating the number of responders per spleen. Background (no peptide stimulation) was subtracted from this value to determine the total number of Ag-specific cells per spleen.

Results

Blocking CD40L in vivo inhibits CD8$^+$ T cell priming against OVA-loaded splenocytes

Disruption of CD40L-CD40 interactions has been shown to prevent CD8$^+$ T cell cross-priming against model Ags (1, 2). We first determined whether our preparation of MR-1 (CD40L blocking Ab) was capable of disrupting CD40L-CD40 interactions in vivo by measuring CD8$^+$ T cell cross-priming against OVA-loaded splenocytes in animals treated with MR-1.

CB6F1 (H-2^{bxd}) animals were treated with MR-1 or hIg and injected i.v. with OVA-loaded BALB/c (H-2^d) splenocytes. Priming of an H-2K^b-restricted CD8$^+$ T cell response to the $OVA_{257-264}$ epitope in this experiment requires cross-presentation, since the Ag is loaded into BALB/c splenocytes, which lack the necessary MHC class I-presenting molecule. $OVA_{257-264}$-specific CD8$^+$ T cell responses were quantitated 7 days after injection using intracellular IFN-γ staining of splenocytes to detect Ag-specific cells (25). Representative mice treated with hIg control Abs or MR-1 are shown in Fig. 1*A*. In the absence of peptide stimulation, few CD8$^+$ T cells make IFN-γ. However, when splenocytes from hIg-treated, OVA-immunized animals were incubated with the $OVA_{257-264}$ peptide, approximately 0.75% of CD8$^+$ T cells produced IFN-γ after subtraction of background. The total number of Ag-specific cells ranged from $0.4–1 \times 10^5$/spleen in this experiment and is representative of 12 independent experiments (Fig. 1*B*). In contrast, CD8$^+$ splenocytes from MR-1-treated, OVA-immunized animals failed to make a detectable IFN-γ response over background after

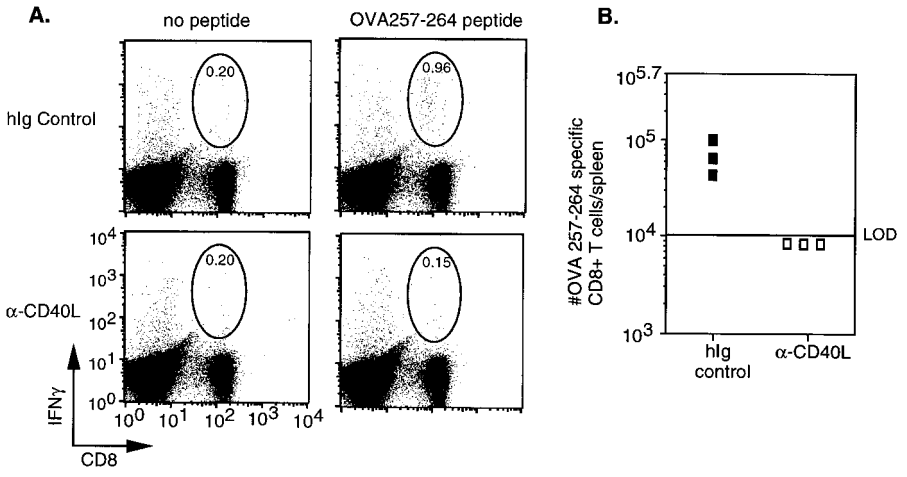

FIGURE 1. Cross-priming of $OVA_{257–264}$-specific $CD8^+$ T cells is inhibited by CD40L-blocking Ab. CB6F1 (H-2^{bxd}) mice were immunized i.v. with $30–35 \times 10^6$ OVA-loaded BALB/c (H-2^d) splenocytes. On days −2, 0, 2, and 4, mice received i.p. injections of 0.25 mg hIg control Ab (■) or CD40L Ab MR-1 (□). On day 7 after OVA immunization intracellular staining for IFN-γ was performed on $OVA_{257–264}$ peptide (H-$2K^b$-restricted)-stimulated splenocytes. *A*, Representative FACS profiles of splenocytes with and without peptide stimulation from both a mouse that received hIg control Ab treatment and a mouse that received MR-1 treatment. Both animals were also immunized with OVA-loaded BALB/c splenocytes. The frequency of Ag-specific $CD8^+$ T cells in the spleen is indicated. α-, Anti-. *B*, Total number of $OVA_{257–264}$-specific $CD8^+$ T cells in the spleen. Data represent individual mice from one experiment and are representative of 12 independent experiments. LOD, limit of detection.

stimulation with synthetic $OVA_{257–264}$ (Fig. 1, *A* and *B*). In total, 4 of 36 MR-1-treated mice and 33 of 36 hIg-treated mice had responses above the limit of detection. Similar results were reported with $CD40L^{-/-}$ and $CD40^{-/-}$ mice (2). Thus, MR-1 treatment effectively prevents detectable cross-priming of $CD8^+$ T cells against a model exogenous Ag.

$CD8^+$ T cell responses against secreted and nonsecreted LM Ags are not inhibited with CD40L blockade

We investigated the role of CD40L-CD40 interactions in $CD8^+$ T cell priming against secreted (endogenously processed) and nonsecreted (exogenously processed) LM Ags. Mice treated with MR-1 or hIg control Abs were infected with 2×10^3 LM-NPns (~0.1 LD_{50}) that expresses a well-characterized $CD8^+$ T cell epitope from the nucleoprotein of LCMV ($NP_{118–126}$) as a nonsecreted fusion protein. As an internal control, $CD8^+$ T cell priming against a secreted, endogenously processed epitope from the LM molecule $LLO_{91–99}$ was also measured. Ag-specific $CD8^+$ T cells were detected by IFN-γ intracellular staining 7 days after infection. Responses of approximately $3.3–3.9 \times 10^5$ $LLO_{91–99}$-specific cells/spleen were detected from both groups of animals (Fig. 2*A*). Similarly, the total number of $NP_{118–126}$-specific $CD8^+$ splenocytes from both MR-1- and hIg-treated animals ranged from 6 to 8×10^4 cells/spleen (Fig. 2*B*). Splenocytes from uninfected mice did not produce IFN-γ after stimulation with either peptide (data not shown). As a control for the course of infection, liver CFU analysis was performed on days 3 and 7, as infections in the spleen and liver are generally parallel (J. Harty, unpublished observations). MR-1- and hIg control Ab-treated animals showed no difference in infection at these time points and did not have CFU in the liver above the limit of detection (~150 organisms/g liver) on day 7 (data not shown). This demonstrates that in contrast to cross-priming against OVA, naive $CD8^+$ T cell priming against nonsecreted or secreted Ags expressed by LM is not inhibited by MR-1 treatment.

Impaired memory $CD8^+$ T cell responses after LCMV infection have been reported in $CD40L^{-/-}$ mice (10). To investigate whether transient blockade of CD40L-CD40 interactions would

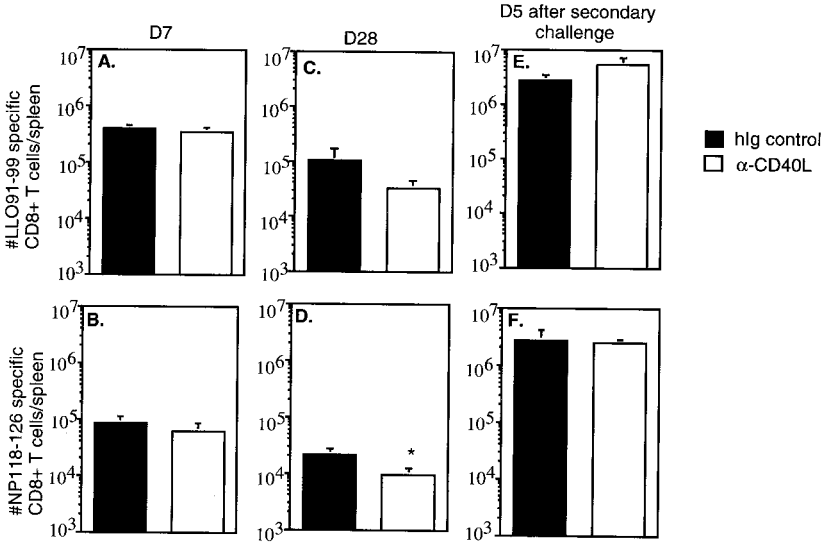

FIGURE 2. Responses of $CD8^+$ T cells to secreted and nonsecreted bacterial Ags under conditions of CD40L blockade. BALB/c mice were immunized i.v. with 2×10^3 CFU LM-NPns. On days −2, 0, 2, and 4 mice received i.p. injections of 0.25 mg hIg control Ab (■) or anti-CD40L Ab MR-1 (□). On day 7 after infection intracellular staining for IFN-γ was performed on peptide-stimulated splenocytes. Total numbers of $LLO_{91–99}$ (*A*)- and $NP_{118–126}$ (*B*)-specific $CD8^+$ T cells in the spleen were calculated. α-, Anti-. Data are the means ± SD of four independent experiments, each with three mice per group. Total numbers of $LLO_{91–99}$ (*C*)- and $NP_{118–126}$ (*D*)-specific $CD8^+$ T cells in the spleen were also determined by IFN-γ ELISPOT at 28 days after infection and by intracellular staining for IFN-γ 5 days after challenge with 2×10^5 CFU LM-NPs (*E* and *F*). A representative experiment of two is shown. The limit of detection is 10^4 for *A*, *B*, *E*, and *F* and 10^3 for *C* and *D*. Data are the means ± SD of three mice per group. ∗, $p < 0.05$.

impact $CD8^+$ T cell memory responses, mice treated with MR-1 or control hIg were infected with 2×10^3 LM-NPns. Twenty-eight days after infection splenocytes were stimulated for 36 h with LLO_{91-99} or $NP_{118-126}$ peptide, and Ag-specific cells were quantitated by IFN-γ ELISPOT (Fig. 2, *C* and *D*). In a preliminary experiment memory $CD8^+$ T cell responses to $NP_{118-126}$ were at or below the level of detection using intracellular cytokine staining for IFN-γ. Therefore, the data shown in Fig. 2, *C* and *D*, were determined using the ELISPOT assay, which allows detection of low frequencies of Ag-specific $CD8^+$ T cells. A 2- to 3-fold decrease in the number of Ag-specific cells was observed in MR-1-treated mice compared with control animals, but this revealed statistical significance only for $NP_{118-126}$-specific cells. However, since both LLO_{91-99}- and $NP_{118-126}$-specific cells were reduced in number, and a slightly larger spleen size was seen in the hIg control group, it is unlikely that this difference is biologically significant.

$CD8^+$ T cell recall responses were also examined in animals that received transient CD40L blockade during primary infection with LM-NPns. For challenge, the mice were infected with LM-NPs, which is isogenic with LM-NPns except that LM-NPs secretes the $NP_{118-126}$ fusion protein and primes a 3- to 4-fold higher $NP_{118-126}$-specific response (14). On day 30 after primary infection, mice were challenged with 2×10^5 LM-NPs (~$10LD_{50}$ for naive mice; Fig. 2, *E* and *F*). Both MR-1-treated and hIg-treated animals contained 3–5 $\times 10^6$ LLO_{91-99}-specific $CD8^+$ T cells (Fig. 2*E*) and 2.4–2.7 $\times 10^6$ $NP_{118-126}$-specific $CD8^+$ T cells (Fig. 2*F*) in the spleen as determined by IFN-γ intracellular staining five days after infection. Challenge of immune mice with LM-NPns also resulted in comparable levels of secondary Ag-specific $CD8^+$ T cell responses between MR-1- and hIg-treated animals (data not shown). These experiments illustrate that MR-1 treatment during the primary exposure to Ag does not impact the ability of $CD8^+$ T cells to respond to either secreted or nonsecreted Ags (Fig. 2). Memory responses to $NP_{118-126}$ were significantly decreased 2- to 3-fold compared with control animals, but this did not result in any deficiency in $CD8^+$ T cell responses to a secondary challenge.

Infection with rLM overcomes CD40L blockade in cross-priming

We next asked whether LM infection could overcome the capacity of MR-1 to block $CD8^+$ T cell cross-priming against OVA-loaded splenocytes. MR-1 treatment blocked the $OVA_{257-264}$-specific $CD8^+$ T cell response in CB6F1 animals immunized with OVA-loaded splenocytes (Figs. 1 and 3). In contrast, animals treated with MR-1, infected with 2×10^3 LM-NPns that lacks the $OVA_{257-264}$ epitope, and immunized with OVA-loaded splenocytes, made a substantial $OVA_{257-264}$ response of 3.3×10^4 $CD8^+$ T cells/spleen (Fig. 3). This response was not significantly different from OVA-immunized, uninfected animals treated with hIg control Abs. There was a significant increase in the number of $OVA_{257-264}$-specific $CD8^+$ T cells (~6-fold) under conditions of infection and hIg treatment, suggesting that infection results in an enhanced environment for $CD8^+$ T cell cross-priming. The experiment in Fig. 3 demonstrates that LM infection is able to overcome CD40L blockade in cross-priming of naive $CD8^+$ T cells against OVA-loaded splenocytes.

Attenuated LM strains also overcome CD40L blockade of cross-priming

The mechanism by which LM infection overcomes CD40L blockade of cross-priming is unknown, but may relate to specific properties of the organism or the host response to LM. To address this issue, we asked whether infection with the attenuated LM strain DP-L1942 ($ActA^-$ LM) would also be able to overcome MR-1 treatment. $ActA^-$ LM has an in-frame deletion in the *act*A gene, which is required for intracellular spread of LM to neighboring host cells (15). Since this strain is highly attenuated, mice in this experiment were infected with 1×10^6 $ActA^-$ LM (~0.1 LD_{50}). CB6F1 animals were treated with MR-1 or hIg as described, infected with $ActA^-$ LM, and immunized with OVA-loaded splenocytes. Similar to the experiments with virulent LM, $OVA_{257-264}$-specific $CD8^+$ T cell responses were only found in MR-1-treated animals when they were also infected with LM (Figs. 3 and 4). The magnitude of the response in MR-1-treated, $ActA^-$ LM-infected animals was approximately 10^5 Ag specific cells/spleen and was equivalent to that seen in uninfected hIg-treated animals. These data show that infection with either virulent or attenuated LM enhances $CD8^+$ T cell cross-priming against OVA-loaded splenocytes, overcoming CD40L blockade in MR-1-treated animals and increasing the Ag-specific response in hIg-treated animals.

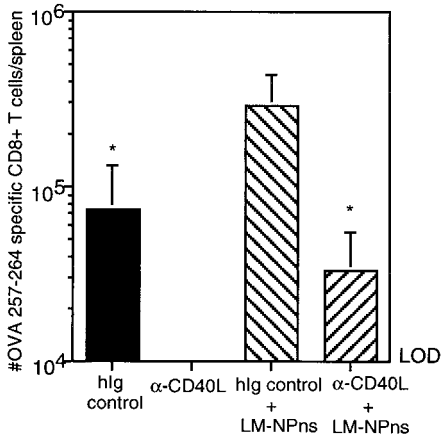

FIGURE 3. Infection with virulent LM overcomes CD40L blockade of $CD8^+$ T cell cross-priming against OVA-loaded splenocytes. CB6F1 mice were given i.p. injections of 0.25 mg hIg control Ab (■ and ▨) or MR-1 (below level of detection and ▨) on days −2, 0, 2, and 4. On day 0 mice were infected i.v. with 2×10^3 CFU LM-NPns (▨ and ▧) and injected with $30–35 \times 10^6$ OVA-loaded BALB/c splenocytes (all groups). Intracellular IFN-γ staining for peptide-specific $CD8^+$ T cells was performed on splenocytes 7 days later. The total number of $OVA_{257-264}$-specific $CD8^+$ T cells in the spleen was calculated. Data are means ± SD of three independent experiments, each with three mice per group. α-, Anti-. LOD, limit of detection. *, $p < 0.025$ vs hIg control Ab plus LM-NPns.

When infection with ActA- LM was titrated, injection of as few as 1×10^4 $ActA^-$ LM (0.001 LD_{50}) overcame CD40L blockade of cross-priming (Fig. 4*B*). A lower dose of 1×10^3 $ActA^-$ LM resulted in measurable priming against an endogenous, secreted LM epitope ($p60_{217-225}$) that primes similar numbers of Ag-specific cells to OVA during LM infection (27). However, this dose of infection failed to overcome CD40L blockade of $OVA_{257-264}$-specific $CD8^+$ T cell priming. Immunization with 1×10^6 heat-killed $ActA^-$ LM also did not result in measurable $CD8^+$ T cell priming against OVA or LM Ags. This result demonstrates that bacterial components alone, at least at the level tested here, are not sufficient to overcome CD40L blockade of $CD8^+$ T cell cross-priming and that active infection must take place in the host.

A second attenuated strain, DP-L2161 (LLO^- LM), was also used to infect CB6F1 animals treated with MR-1 or hIg and immunized with OVA-loaded splenocytes (Fig. 4*C*). LLO^- LM has an in-frame deletion in the *hly* gene, which encodes the LLO virulence factor (16). Loss of functional LLO results in organisms that are highly attenuated due to an inability to escape from the

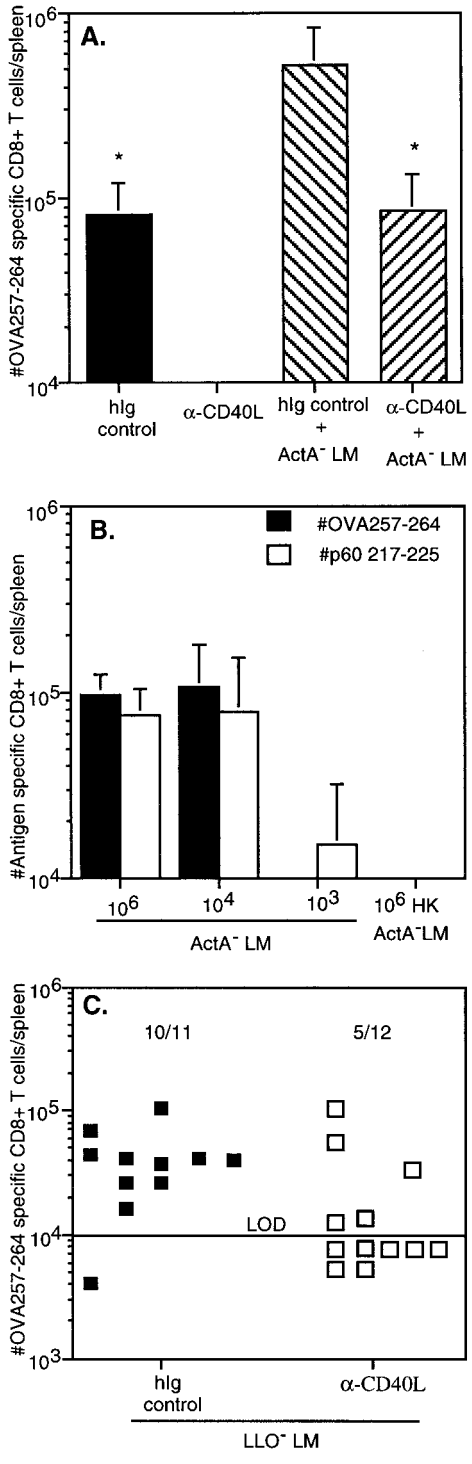

FIGURE 4. Attenuated LM overcomes MR-1 blockade of $CD8^+$ T cell cross-priming. *A*, CB6F1 mice were given i.p. injections of 0.25 mg hIg control Ab (■ and ▧) or MR-1 (below level of detection and ▨) or on days −2, 0, 2, and 4. On day 0 mice were infected i.v. with 1×10^6 CFU $ActA^-$ LM (▨ and ▧). Mice were also immunized i.v. with $30–35 \times 10^6$ OVA-loaded BALB/c splenocytes. Intracellular IFN-γ staining was performed on splenocytes on day 7. The total number of $OVA_{257–264}$-specific $CD8^+$ T cells in the spleen was calculated. Data are the means ± SD of three independent experiments, each with three mice per group. *B*, The total numbers of $OVA_{257–264}$- and $p60_{217–225}$-specific $CD8^+$ T cells in the spleen after infection with the indicated dosages of $ActA^-$ LM (alive or heat killed). *C*, CB6F1 mice were treated as described above, except LM infection was with 1×10^8 CFU LLO^- LM. Individual mice are shown from four independent experiments. α-, Anti-. LOD, limit of detection. *, $p < 0.025$ vs hIg control $ActA^-$ LM.

host cell phagosome (28, 29). A lower number of $OVA_{257–264}$-specific $CD8^+$ T cells was measured in mice infected with 1×10^8 ($LD_{50} > 10^9$) LLO^- LM and treated with hIg compared with hIg-treated animals infected with $ActA^-$ LM (Fig. 4, compare *A* and *C*). Many MR-1 treated, LLO^- LM-infected animals had no $OVA_{257–264}$-specific response above the level of detection (7 of 12 animals). However, some animals did have measurable $OVA_{257–264}$-specific responses ranging from approximately 1×10^4 to 1×10^5 $CD8^+$ T cells/spleen. This indicates that infection with LLO^- LM can overcome CD40L blockade of cross-priming, but does so less efficiently than virulent LM or $ActA^-$ LM.

Neutralization of TNF or IFN-γ does not prevent a response to OVA-loaded splenocytes during infection

During LM infection, various proinflammatory cytokines, such as IFN-γ and TNF, are generated that could serve as activation signals for DCs. TNF in particular stimulates the maturation of DCs and promotes cross-presentation of model Ags in vivo in the absence of CD40L (30, 31). To determine whether TNF or IFN-γ is important for the ability of LM infection to overcome CD40L blockade of cross-priming, CB6F1 animals were treated with MR-1 and TNF- or IFN-γ-neutralizing Abs. Cytokine-neutralizing Abs were used at a dosage previously determined to be effective for neutralization in vivo (32). Animals were then infected with 1×10^6 $ActA^-$ LM and immunized with OVA-loaded splenocytes. Animals treated with TNF-neutralizing Abs had enlarged spleens ($3–4 \times 10^8$ splenocytes), and six of eight animals had organisms remaining in the liver 7 days after infection (data not shown). All other treatment groups had similar spleen sizes ($\sim 2 \times 10^8$ splenocytes) and had cleared the infection by day 7. The total number of $OVA_{257–264}$-specific $CD8^+$ T cell was not significantly different in αTNF-α- or αIFN-γ-treated animals compared with control rat Ig-treated animals (Fig. 5). However, the lack of statistical significance in TNF-depleted mice may be impacted by the increased level of infection in these animals. This experiment demonstrates that neutralization of these two cytokines under conditions of CD40L blockade and bacterial infection does not prevent naive $CD8^+$ T cell priming against OVA-loaded splenocytes.

Discussion

Here we examine the influence of CD40L-CD40 interactions on $CD8^+$ T cell priming against exogenous Ags during LM infection. Although it is accepted that the maturation of DCs is required for priming of $CD8^+$ T cells against exogenous Ags, the signals required for this maturation in vivo are unknown. LPS, bacterial DNA, CD40 stimulation, and TNF-α have all been shown to result in the maturation of DCs in vitro, and anti-CD40 Abs have been shown to promote $CD8^+$ T cell priming against exogenous model Ags in vivo in the absence of $CD4^+$ T cells (3, 7).

Although MR-1 treatment prevented $CD8^+$ T cell cross-priming against OVA-loaded splenocytes, it did not affect $CD8^+$ T cell priming against nonsecreted or secreted LM Ags. It has been shown that $CD4^+$ T cells or CD40L are not required for $CD8^+$ T cell priming under conditions of viral infection and that there is minimal impact on the splenic $CD8^+$ T cell response to secreted LM Ags in $CD40^{-/-}$ mice (8, 12). However, these studies did not address the issue of priming against Ags that do not have access to endogenous MHC class I processing pathways. It has additionally been demonstrated that memory $CD8^+$ T cell responses decline in the absence of CD40L. Although we saw a significant decline in the $CD8^+$ T cell memory response to $NP_{118–126}$, secondary responses to both $NP_{118–126}$ and $LLO_{91–99}$ were unaffected in MR-1-treated mice in our system. Since our MR-1 treatment was only

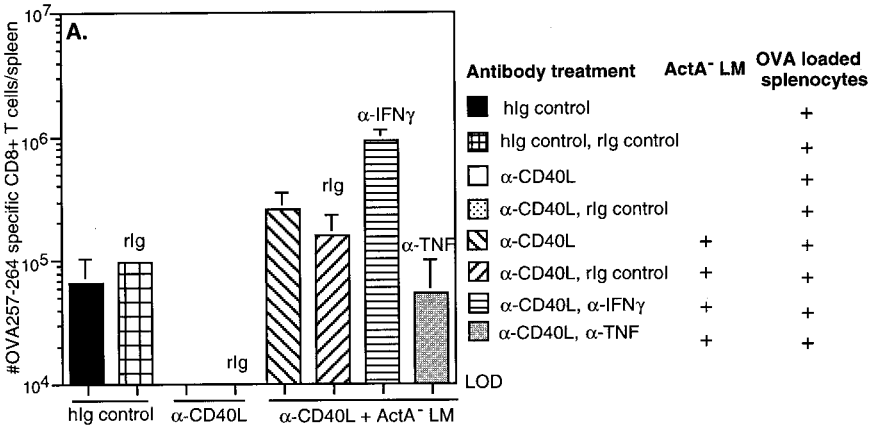

FIGURE 5. Neutralization of IFN-γ or TNF does not inhibit the ability of LM to overcome CD40L blockade of $CD8^+$ T cell cross-priming. CB6F1 animals were given i.p. injections of 0.25 mg hIg (control) or MR-1 on days -2, 0, 2, and 4. On day -1 mice were given i. p. injections of α-TNF-α (XT22 and XT3), α-IFN-γ (XMG1.2), or rat Ig control Abs. On day 0 mice were infected i. v. with 1×10^6 CFU $ActA^-$ LM. All mice were also immunized i.v. with $30–35 \times 10^6$ OVA-loaded BALB/c splenocytes. Intracellular staining for IFN-γ was performed on splenocytes on day 7. *A*, The total number of $OVA_{257–264}$-specific $CD8^+$ T cells in the spleen was calculated. Data are the mean $\pm$ SD of three independent experiments. α-, Anti-. LOD, limit of detection.

performed transiently during the initial exposure to Ag, it is unknown whether constant blockade of CD40L in these animals would affect primary or memory $CD8^+$ T cell responses after LM infection.

Consistent with the idea that immunization with model Ags may not reflect the host response to infection, we found that both virulent and attenuated LM could overcome CD40L blockade of $CD8^+$ T cell cross-priming. In addition, it was consistently seen that hIg-treated animals exhibited increased $CD8^+$ T cell responses in the presence of infection (Figs. 3–5). Cross-presentation requires bone marrow-derived DCs, which were recently reported to be of the $CD8\alpha^+$ or lymphoid lineage (33). An interesting hypothesis is that the increase in $CD8^+$ T cell priming against OVA in the presence of infection is due to an increase in the number of cells or types of cells (i.e., $CD8\alpha^-$) able to participate in cross-priming as effective APCs. Alternatively, when both CD40L and microbial signals are present, DCs show augmented IL-12 production in vivo, a situation that could result in increased T cell responses (34).

In contrast, LLO^- LM, which have a deletion in the *hly* gene, overcame CD40L blockade less efficiently in the majority of MR-1-treated animals. It is likely that the host response to LLO^- LM is different from the response to other strains of LM. Infection with LLO^- LM elicits lower IL-12 production compared with virulent LM, which may result in a cytokine environment that is not optimal for $CD8^+$ T cell cross-priming in the presence of MR-1 (35, 36). Previous experiments show that IL-12 is required in at least some models of exogenous Ag presentation, although the requirement for this cytokine in cross-presentation has not been examined (37). Recent evidence also demonstrated that CD40-mediated, $CD8^+$ T cell priming against exogenously processed heat-killed LM was dependent on endogenous IL-12 production (38).

The mechanism by which LM infection overcomes CD40L blockade of cross-priming is still unknown. However, there is a clear requirement for active infection, since the injection of large numbers of heat-killed bacteria (Fig. 4*B*) failed to stimulate measurable $CD8^+$ T cell priming against OVA-loaded splenocytes or LM Ags. This indicates that the presence of bacterial products such as the cell wall or bacterial DNA was not sufficient to overcome CD40L blockade at least at the levels tested. The ability of LM infection to overcome CD40L blockade was not inhibited by treatment with mAbs that efficiently neutralize IFN-γ or TNF, illustrating that multiple signals generated during bacterial infection may stimulate the maturation of DCs. The depletion of TNF or IFN-γ also does not prevent naive $CD8^+$ T cell priming against secreted or nonsecreted LM Ags in the presence or the absence of MR-1 (S. E. Hamilton and J. T. Harty, unpublished observations). Evidence has accumulated demonstrating the stimulatory properties of members of the TNF ligand superfamily (4-1BBL, OX40L, TNF-related apoptosis-inducing ligand, and TNF-related activation-induced cytokine) on DCs (39). 4-1BBL can influence $CD4^+$ T cell and $CD8^+$ T cell responses, particularly under suboptimal antigenic stimulation (40). Based on our results, it would be of interest to examine the effect of CD40L blockade on cross-priming in 4-$1BBL^{-/-}$ mice in the presence or the absence of LM infection and/or OVA-loaded splenocyte immunization. LM can also infect DCs themselves, suggesting that this could also be route to induce maturation during infection (41). This may be particularly relevant in experiments in which TNF was neutralized in mice resulting in increased levels of infection. Alternatively, another soluble molecule, such as IL-1, could be important for the maturation of dendritic cells during bacterial infection.

How cross-presentation results in either priming or tolerance of $CD8^+$ T cells is still under debate. Cross-presentation is a critical mechanism both for the induction of peripheral tolerance and for immune responses to Ags without obvious access to the cytosol of APCs (reviewed in Ref. 5). Our results support a model in which tolerance is the likely result when the APC environment is lacking in inflammatory stimuli. In this situation the APC would take up and present Ag, but would lack the costimulatory molecules required to effectively stimulate naive T cells. However, under conditions of infection or inflammation, changes in the APC environment, such as cytokine secretion or pathogen products, would promote maturation of DCs for effective stimulation of naive T cells. This poses a potential problem for the host, since the uptake of peripheral self Ags would presumably continue in a T cell stimulatory environment and increase the likelihood of autoimmunity. This is consistent with models of autoimmunity in which conditions often worsen during concurrent infections (42). How tolerance to self Ags can be maintained during infection requires further investigation.

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