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J Immunol 2001; 167:5603-5609; doi: 10.4049/jimmunol.167.10.5603
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Listeria monocytogenes Infection Overcomes the Requirement for CD40 Ligand in Exogenous Antigen Presentation to CD8+ T Cells

Sara E. Hamilton,* Amy R. Tvinneireim,† and John T. Harty2*†

In vivo priming of CD8+ T lymphocytes 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 (referred to as the endogenous or exogenous MHC class I presentation 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) 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
destruction of the organism (14). Consistent with this idea, infection of mice with recombinant LM strains expressing an H-2Ld-restricted epitope from the nucleoprotein of LCMV (NP118–126) as either a secreted or a nonsecreted fusion protein prime NP118–126-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-2b MHC) and CB6F1 (H-2b\# MHC) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Virulent LM strains used in this study were recombinant strain XFL304 (LM-NPns) expressing a nonsecreted fusion protein containing the LCMV NP118–126 epitope and strain XFL303 (LM-NPns) expressing the same fusion protein in secreted form (14). Attenuated LM strain DP-L1942 (Acta-/-LM), which carries an in-frame deletion in the acta gene (15), and attenuated LM strain DP-L2161 (listeriolysin O (LLO)-/-LM), which carries an in-frame deletion in the llo 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 × 10^5 LM-NPns or LM-NPns, 1 × 10^5 Acta-/-LM, and 1 × 10^5 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 µg/ml) in hypertonic medium (RPMI 1640, 0.5 M sucrose, 10% yolk-lysozyme 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% H2O) and incubated for 2 min at 37°C. Cells were washed twice in HBSS and resuspended in 60–70 × 10^4 splenocytes/ml. CB6F1 mice received 0.5 ml/mouse, i.v. for coinmunization with LM and OVA-loaded splenocytes, i.e. 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**

Hybrodias producing anti-CD40L mAb MR-1 (20) (a gift from Dr. T. Waldschmidt, University of Iowa, Iowa City, IA) and anti-TNF (XT32 and XT3) used in combination at a mass ratio of 1:1 (21) were grown in RPMI 1640 supplemented with 10% FCS, l-glutamine, and antibiotics. Hybrodias producing anti-IFN-γ (XMG1.2) (22) were grown in RPMI 1640 supplemented with 10% FCS, l-glutamine, and antibiotics (RPI0) (23). Ab was purined from plasma by affinity chromatography with protein G (Amerham Pharmacia, Piscataway, NJ). Each batch of Ab was concentrated to 2 mg/ml and assessed for endotoxin contamination by protein G (Amersham Pharmacia, Piscataway, NJ). 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 to yield MR-1 that could effectively block CD40L in vivo, MR-1- or hamster Ig (Ilg)-treated BALB/c mice were inoculated with SRBC. Immunization results in CD40L-dependent germinal center formation within 4 days in control IgG (Jackson ImmunoResearch, West Grove, PA)-treated animals or animals receiving no Ab treatment. In animals that received IgG 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 OVA257-264 in the context of H-2Kd, NP118–126 in the context of H-2Ld, LLO91–99, in the context of H-2Kd, or p60217-225 in the context of H-2Kd 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 µM 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 FcyIII/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 (10^5–10^7/well) were cultured with medium alone or with 100 nM synthetic peptide (LLO91–99 or NP118–126) 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 antisera (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-2b\#) animals were treated with MR-1 or hIg and injected i.v. with OVA-loaded BALB/c (H-2b) splenocytes. Priming of an H-2Kd-restricted CD8+ T cell response to the OVA257-264 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. OVA257-264-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. 1A. 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 OVA257-264 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 × 10^5/spleen in this experiment and is representative of 12 independent experiments (Fig. 1B). In contrast, CD8+ splenocytes from MR-1-treated, OVA-immunized animals failed to make a detectable IFN-γ response over background after
stimulation with synthetic OVA_{257-264} (Fig. 1, A and B). In total, 4 of 36 MR-1-treated mice and 33 of 36 hlg-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 hlg control Abs were infected with 2 × 10\(^3\) LM-NP\(_{118-126}\) (\(\sim\)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-\(\gamma\) intracellular staining 7 days after infection. Responses of approximately 3.3–3.9 × 10\(^5\) LLO\(_{91-99}\)-specific cells/spleen were detected from both groups of animals (Fig. 2A). Similarly, the total number of NP\(_{118-126}\) CD8\(^+\) splenocytes from both MR-1- and hlg-treated animals ranged from 6 to 8 × 10\(^4\) cells/spleen (Fig. 2B). Splenocytes from uninfected mice did not produce IFN-\(\gamma\) 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. Hartly, unpublished observations). MR-1- and hlg 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 (\(\sim\)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

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**FIGURE 1.** Cross-priming of OVA\(_{257-264}\)-specific CD8\(^+\) T cells is inhibited by CD40L-blocking Ab. CB6F1 (H-2\(^{bm}\)) mice were immunized i.v. with 30–35 × 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 hlg control Ab (■) or CD40L Ab MR-1 (□). On day 7 after OVA immunization intracellular staining for IFN-\(\gamma\) 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 hlg 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-.

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**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-NP\(_{118-126}\). On days −2, 0, 2, and 4 mice received i.p. injections of 0.25 mg hlg control Ab (■) or anti-CD40L Ab MR-1 (□). On day 7 after infection intracellular staining for IFN-\(\gamma\) 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-.

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**TABLE 1.** Cross-priming of CD8\(^+\) T cells against secreted and nonsecreted LM Ags under conditions of CD40L blockade. BALB/c mice were immunized i.v. with 2 × 10\(^3\) CFU LM-NP\(_{118-126}\). On days −2, 0, 2, and 4 mice received i.p. injections of 0.25 mg hlg control Ab (■) or anti-CD40L Ab MR-1 (□). On day 7 after infection intracellular staining for IFN-\(\gamma\) 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-.

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**TABLE 2.** Cross-priming of CD8\(^+\) T cells against secreted and nonsecreted LM Ags under conditions of CD40L blockade. BALB/c mice were immunized i.v. with 2 × 10\(^3\) CFU LM-NP\(_{118-126}\). On days −2, 0, 2, and 4 mice received i.p. injections of 0.25 mg hlg control Ab (■) or anti-CD40L Ab MR-1 (□). On day 7 after infection intracellular staining for IFN-\(\gamma\) 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-.
impact CD8⁺ T cell memory responses, mice treated with MR-1 or control hlg were infected with 2 × 10⁵ LM-NPns. Twenty-eight days after infection splenocytes were stimulated for 36 h with LLO₉₁₉₉₉₉ or NP₁₁₈₋₁₂₆ 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 NP₁₁₈₋₁₂₆ 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 NP₁₁₈₋₁₂₆-specific cells. However, since both LLO₉₁₉₉₉₉ and NP₁₁₈₋₁₂₆-specific cells were reduced in number, and a slightly larger spleen size was seen in the hlg control group, it is unlikely that this difference is biologically significant.

CD8⁺ T cell recall responses were also examined in animals that received transgenic CD40L blockade during primary infection with LM-NPns. For challenge, the mice were infected with LM-NPns, which is isogenic with LM-NPns except that LM-NPns secretes the NP₁₁₈₋₁₂₆ fusion protein and primes a 3- to 4-fold higher NP₁₁₈₋₁₂₆-specific response (14). On day 30 after primary infection, mice were challenged with 2 × 10⁵ LM-NPns (n=10 LD₅₀) for naive mice; Fig. 2, E and F). Both MR-1-treated and hlg-treated animals contained 3–5 × 10⁶ LLO₉₁₉₉₉₉-specific CD8⁺ T cells (Fig. 2E) and 2.4–2.7 × 10⁶ NP₁₁₈₋₁₂₆-specific CD8⁺ T cells (Fig. 2F) 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 hlg-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 NP₁₁₈₋₁₂₆ 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 OVA257–264-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 × 10⁵ LM-NPns that lacks the OVA257–264 epitope, and immunized with OVA-loaded splenocytes, made a substantial OVA257–264 response of 3.3 × 10⁹ CD8⁺ T cells/spleen (Fig. 3). This response was not significantly different from OVA-immunized, uninfected animals treated with hlg control Abs. There was a significant increase in the number of OVA257–264-specific CD8⁺ T cells (~6-fold) under conditions of infection and hlg 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 actA 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 × 10⁶ ActA⁺ LM (~0.1 LD₅₀). CB6F1 animals were treated with MR-1 or hlg as described, infected with ActA⁺ LM, and immunized with OVA-loaded splenocytes. Similar to the experiments with virulent LM, OVA257–264-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 10⁵ Ag specific cells/spleen and was equivalent to that seen in uninfected hlg-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 hlg-treated animals.

When infection with ActA⁻ LM was titrated, injection of as few as 1 × 10⁴ ActA⁻ LM (0.001 LD₅₀) overcame CD40L blockade of cross-priming (Fig. 4B). A lower dose of 1 × 10³ ActA⁺ LM resulted in measurable priming against an endogenous, secreted LM epitope (p60217–223) 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 OVA257–264-specific CD8⁺ T cell priming. Immunization with 1 × 10⁶ 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 hlg and immunized with OVA-loaded splenocytes (Fig. 4C). 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
host cell phagosome (28, 29). A lower number of OVA_{257-264}\textsuperscript{+} specific CD8\textsuperscript{+} T cells was measured in mice infected with 1 \times 10^8 (LD\textsubscript{50} > 10^6) LLO\textsuperscript{+} LM and treated with hlg compared with hlg-treated animals infected with ActA\textsuperscript{−} LM (Fig. 4, compare A and C). Many MR-1 treated, LLO\textsuperscript{−} LM-infected animals had no OVA_{257-264}\textsuperscript{+} specific response above the level of detection (7 of 12 animals). However, some animals did have measurable OVA_{257-264}\textsuperscript{+} specific responses ranging from approximately 1 \times 10^3 to 1 \times 10^5 CD8\textsuperscript{+} T cells/spleen. This indicates that infection with LLO\textsuperscript{−} LM can overcome CD40L blockade of cross-priming, but does so less efficiently than virulent LM or ActA\textsuperscript{−} LM.

Neutralization of TNF or IFN-\(\gamma\) does not prevent a response to OVA-loaded splenocytes during infection

During LM infection, various proinflammatory cytokines, such as IFN-\(\gamma\) 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-\(\gamma\) 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-\(\gamma\)-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 \times 10^6 ActA\textsuperscript{−} 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}\textsuperscript{+} specific CD8\textsuperscript{+} T cells was not significantly different in \(\alpha\)TNF-\(\alpha\) or \(\alpha\)IFN-\(\gamma\)-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\textsuperscript{+} T cell priming against OVA-loaded splenocytes.

Discussion

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

Although MR-1 treatment prevented CD8\textsuperscript{+} T cell cross-priming against OVA-loaded splenocytes, it did not affect CD8\textsuperscript{+} T cell priming against nonsecreted or secreted LM Ags. It has been shown that CD4\textsuperscript{+} T cells or CD40L are not required for CD8\textsuperscript{+} T cell priming under conditions of viral infection and that there is minimal impact on the splenic CD8\textsuperscript{+} T cell response to secreted LM Ags in CD40\textsuperscript{−/−} 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\textsuperscript{+} T cell responses decline in the absence of CD40L. Although we saw a significant decline in the CD8\textsuperscript{+} 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 4. Attenuated LM overcomes MR-1 blockade of CD8\textsuperscript{+} T cell cross-priming. A, CB6F1 mice were given i.p. injections of 0.25 mg hlg 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 1 \times 10^6 CFU ActA\textsuperscript{−} LM (■ and □). Mice were also immunized i.v. with 30–35 \times 10^6 OVA-loaded BALB/c splenocytes. Intracellular IFN-\(\gamma\)-staining was performed on splenocytes on day 7. The total number of OVA_{257-264}\textsuperscript{+} specific CD8\textsuperscript{+} T cells in the spleen was calculated. Data are the means \pm SD of three independent experiments, each with three mice per group. B, The total numbers of OVA_{257-264} and p60_{121-225} specific CD8\textsuperscript{+} T cells in the spleen after infection with the indicated dosages of ActA\textsuperscript{−} LM (alive or heat killed), C, CB6F1 mice were treated as described above, except LM infection was with 1 \times 10^8 CFU LLO\textsuperscript{−} LM. Individual mice are shown from four independent experiments. α-, Anti-. LOD, limit of detection. *p < 0.025 vs hlg control ActA\textsuperscript{−} LM.
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 hlg-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 CD8α⁻ 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., CD8α⁻) 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. 4B) 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.

Acknowledgments

We thank J. Cowdery, T. Waldschmidt, and R. Lynch for reagents. We also thank S. Perlman for critical reading of the manuscript.

References


