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Neutrophil Involvement in Cross-Priming CD8\(^+\) T Cell Responses to Bacterial Antigens\(^1\)

Amy R. Tvinnerem,\(^\ast\) Sara E. Hamilton,\(^\dagger\) and John T. Harty\(^2\*\dagger\)

Substantial CD8\(^+\) T cell responses are generated after infection of mice with recombinant *Listeria monocytogenes* strains expressing a model epitope (lymphocytic choriomeningitis virus NP\(_{118-126}\)) in secreted and nonsecreted forms. *L. monocytogenes* gains access to the cytosol of infected cells, where secreted Ags can be accessed by the endogenous MHC class I presentation pathway. However, the route of presentation of the nonsecreted Ag in vivo remains undefined. In this study we show that neutrophil-enriched peritoneal exudate cells from *L. monocytogenes*-infected mice can serve as substrates for in vitro cross-presentation of both nonsecreted and secreted Ag by dendritic cells as well as for in vivo cross-priming of CD8\(^+\) T cells. In addition, specific neutrophil depletion in vivo by low dose treatment with either of two Ly6G-specific mAb substantially decreased the relative CD8\(^+\) T cell response against the nonsecreted, but not the secreted, Ag compared with control Ab-treated mice. Thus, neutrophils not only provide rapid innate defense against infection, but also contribute to shaping the specificity and breadth of the CD8\(^+\) T cell response. In addition, cross-presentation of bacterial Ags from neutrophils may explain how CD8\(^+\) T cell responses are generated against Ags from extracellular bacterial pathogens. *The Journal of Immunology*, 2004, 173: 1994–2002.

Major histocompatibility complex class I-restricted CD8\(^+\) T cells respond to infections with viral, protozoan, and bacterial pathogens (1–3). Because microbial pathogens exhibit a substantial range of complexity, the immune system may be confronted with a few (10 or less for some viruses) or many (several thousand for bacteria and protozoa) potential target Ags, depending on the infection. This complexity is magnified in the case of bacterial and protozoan pathogens not only by the large number of potential Ags, but also because these microbes carry out protein synthesis independently of the infected host cell. Thus, bacteria and protozoa generate potential Ags that are sequestered from the host by the pathogen cell membrane. The idea that such Ag compartmentalization could be an important determinant of host immunity against bacterial and protozoan pathogens has received considerable experimental attention (4–8).

In the case of the intracellular bacterial pathogen *L. monocytogenes* (LM),\(^3\) CD8\(^+\) T cell responses have been documented against Ags that occupy secreted and nonsecreted bacterial compartments (9–12). However, only secreted proteins served as targets for effective CD8\(^+\) T cell immunity after challenge of immune mice (9, 10). This result suggested that secreted and nonsecreted Ags were displayed to the immune system in fundamentally different ways that promoted or prevented effective immunity. However, the finding of CD8\(^+\) T cell priming against both secreted and nonsecreted Ags also demonstrated that dendritic cells (DC), APC that uniquely stimulate clonal expansion of naive CD8\(^+\) T cells (13), were able to acquire Ags from diverse bacterial compartments. Because LM enters the cytosol of infected host cells, including DC, it was likely that secreted proteins were directly accessible to the endogenous MHC class I Ag processing and presentation pathway. In contrast, nonsecreted bacterial Ags in cytosolic LM would be sequestered from the MHC class I processing pathway by the bacterial membrane. Thus, destruction of LM, a process that takes place in membrane-bound phagocytic vesicles, would be required to expose nonsecreted Ags to the host immune system where they would be processed via an exogenous route of MHC class I presentation (14).

Destruction of bacteria by professional phagocytes, such as neutrophils, macrophages, and DC, occurs in membrane-bound structures to prevent host cell toxicity (15). DC appear to possess an efficient phagosome to cytosol transport system that allows exogenous Ags to be processed by the endogenous MHC class I presentation pathway (16). Regurgitation and recycling of phagosomes have also been suggested as pathways for direct presentation of exogenous bacterial Ags by infected cells (17, 18). In addition to these direct routes of Ag presentation, DCs can acquire and present Ags from apoptotic and necrotic cells via cross-presentation pathways (19–21). The contributions of direct and cross-presentation pathways to stimulation of CD8\(^+\) T cell responses to nonsecreted bacterial Ags in vivo remain to be determined.

Enhanced microbial activity of macrophages and DC would be expected to increase the CD8\(^+\) T cell response to nonsecreted bacterial Ags by increasing the amount of Ag available for presentation after direct infection. IFN-γ activates the microbial activities of macrophages; however, the CD8\(^+\) T cell response to a nonsecreted LM Ag was similar in wild-type and IFN-γ deficient mice (12). Thus, we considered the possibility that the CD8\(^+\) T cell response to nonsecreted LM Ags involved cross-presentation. Two requirements must be met for cross-presentation of nonsecreted LM Ags. First, death of the bacteria must occur to release...

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3 Abbreviations used in this paper: LM, *Listeria monocytogenes*; DC, bone marrow-derived dendritic cell; ICS, intracellular cytokine staining; LCMV, lymphocytic choriomeningitis virus; LLO, listeriolysin O; LM-NPns, LCMV NP\(_{118-126}\) epitope as nonsecreted fusion protein; LM-NPs, LCMV NP\(_{118-126}\) epitope as secreted fusion protein; MOI, multiplicity of infection; NP, nucleoprotein; PEC, peritoneal exudate cell.
the nonsecreted Ag and expose the protein to the host cell’s processing machinery. Second, the infected host cell must die to provide a substrate for uptake by DC and cross-priming of T cells. Neutrophils are ideal intermediate cells for cross-presentation of nonsecreted bacterial Ags because not only are they capable of killing LM, without the need for activation by IFN-γ (22), but they also undergo apoptosis following microbial ingestion after bacterial or viral infection (23–26). In this report we addressed the role of neutrophils in cross-priming CD8⁺ T cell responses against nonsecreted LM Ags. Our results suggest that neutrophils not only participate in innate defense against infection, but also provide nonsecreted bacterial Ags for cross-presentation to the adaptive immune system.

Materials and Methods

Mice

Female BALB/c (H-2d MHC), C57BL/6 (H-2b MHC), and CB6 F1 (H-2bxd MHC) mice were purchased from the National Cancer Institute (Bethesda, MD).

Bacteria

All bacterial strains used in this study are derived from LM strain 10403s and are described in Table I. Virulent and attenuated actA-deficient recombinant LM strains expressing the lymphocytic choriomeningitis virus (LCMV) NP₁₁₈–₁₂₆ epitope as a secreted (LM-NPs [XFL303]) and actA-deficient LM-NPs or nonsecreted (LM-NPs [XFL304]) fusion protein have been described (9, 11). Attenuated listeriolysin O (LLO)-deficient strains of LM were generated by in-frame deletion of the bltA gene from LM-NPs and LM-NPs using a construct provided by D. Portnoy (University of California, Berkeley, CA) as previously described (27). The growth and maintenance of all LM strains were described previously (28). The actual number of CFU injected was determined for each experiment by plate count.

Dendritic cells

CD11c⁺, B7.2⁺⁺, MHC class II⁺⁺ DC were generated from the bone marrow of BALB/c mice as previously described (29, 30). Bone marrow cells were cultured in medium supplemented with 1000 U/ml rGM-CSF (BD Pharmingen, San Diego, CA), and 25 U/ml rIL-4 (PeproTech, Rocky Hill, NJ) for DC cultures. On day 5 of culture, loosely adherent cells were harvested by pipetting. The resultant cell population was depleted of neutrophils by complement depletion with the Ly-6G-specific Ab RB6.8C5. After extensive washing, the DC were used for in vitro studies.

Neutrophil-enriched peritoneal exudate cells (PEC)

Neutrophil-enriched PEC were obtained from C57BL/6 or BALB/c mice by peritoneal lavage 5–6 h after i.p. infection with virulent LM, LM-NPs, or LM-NPs (all at 1×10⁶/i.v./injection), actA-deficient LM, actA-deficient NPs, actA-deficient LM-NPs (all at 3×10⁹/i.v.), or 16 h after infection with 1×10⁹ LLO-deficient LM (27). The 16 h point was used with LLO-deficient bacteria to reduce the number of viable bacteria in the PEC population for adoptive transfer studies. Sixty to 85% of the PEC were neutrophils as determined by Diff-Quik (Dade Behring, Deerfield, IL) staining of cytoplasm slides or by flow cytometric analysis using Ly-6G-specific Abs.

In vitro cross-presentation assay

Immune DC (3×10⁶) from day 5 of culture were mixed with 3×10⁶ or 1×10⁶ PEC obtained 1 h after infected mice. TNP peptide was added (Miltenyi Biotech, Auburn, CA) and a combination of PE-conjugated Abs. Briefly, RBC-depleted PEC were incubated for 30 min at 4°C in the presence of FcγRIIIA-specific Ab (2A3G2; 1/50) and PE-conjugated Abs specific for CD3 (BD Pharmingen), B220 (BD Pharmingen), CD11c (BD Pharmingen), class II (BD Pharmingen), and F4/80 (Caltag Laboratories, Burlingame, CA). The cells were washed and resuspended at 1×10⁹ cells/μl of RPMI 20. Twenty microliters of anti-PE microbeads/10⁶ cells were added to the cell suspension, followed by incubation at 6°C for 15–30 min. After incubation, the cells were washed and run over an LS selection column (Miltenyi Biotech) according to the manufacturer’s directions. The neutrophil-enriched PEC were reinfected with LLO-deficient LM, LLO-deficient LM-NPs, or LLO-deficient LM-NPs at an multiplicity of infection (MOI) of 1 for 1 h, followed by a 2-h treatment with 5 μg/ml gentamicin to kill extracellular bacteria. Reinjected neutrophil-enriched PEC (2×10⁶) were injected i.v. into CB6 F1 (H-2b/d) mice, and equivalent aliquots were lysed with 1% Triton X-100 and plated to determine the number of viable bacteria in the PEC populations. Seven days later, the number of NP₁₁₈–₁₂₆-specific CD8⁺ T cells (H-2L d restricted) was determined by flow cytometry using a cell sorter to detect IFN-γ. Between 1 and 2 h after injection, the cells were incubated overnight at 37°C. After overnight incubation, 3×10⁶ CFSE-labeled, Ag-specific CD8⁺ T cell lines were added to the cultures. These CD8⁺ T cell lines, specific for NP₁₁₈–₁₂₆, or LLO peptide, were generated and maintained as previously described (31). The cultures were incubated at 37°C for an additional 6 h in the presence of brefeldin A. The percentage of CFSE-labeled CD8⁺ T cells stimulated under each culture condition was determined by intracellular cytokine staining for IFN-γ.

Detection of Ag-specific CD8⁺ T cells by intracellular cytokine staining of splenocytes

Peptide-stimulated intracellular cytokine staining to detect epitope-specific CD8⁺ T cells was performed as previously described (12, 32). The percentage of IFN-γ⁺ CD8⁺ T cells in unstimulated samples from each mouse was subtracted from the peptide-stimulated value to determine the percentage of Ag-specific CD8⁺ T cells. The total number of epitope-specific CD8⁺ T cells per spleen was calculated from the percentage of IFN-γ⁺ CD8⁺ T cells, the percentage of CD8⁺ T cells in each sample, and the total number of cells per spleen.

Adoptive transfer of neutrophil-enriched PEC and mAb

PEC from C57BL/6 (H-2b⁺) mice infected with LLO-deficient LM (NP₁₁₈–₁₂₆ absent) for 16 h were negatively selected using anti-Ly-6G microbeads (Miltenyi Biotech, Auburn, CA) and a combination of PE-conjugated Abs. Briefly, RBC-depleted PEC were incubated for 30 min at 4°C in the presence of FcγRIIIA-specific Ab (2A3G2; 1/50) and PE-conjugated Abs specific for CD3 (BD Pharmingen), B220 (BD Pharmingen), CD11c (BD Pharmingen), class II (BD Pharmingen), and F4/80 (Caltag Laboratories, Burlingame, CA). The cells were washed and resuspended at 1×10⁹ cells/μl of RPMI 20. Twenty microliters of anti-PE microbeads/10⁶ cells were added to the cell suspension, followed by incubation at 6°C for 15–30 min. After incubation, the cells were washed and run over an LS selection column (Miltenyi Biotech) according to the manufacturer’s directions. The neutrophil-enriched PEC were reinfected with LLO-deficient LM, LLO-deficient LM-NPs, or LLO-deficient LM-NPs at an multiplicity of infection (MOI) of 1 for 1 h, followed by a 2-h treatment with 5 μg/ml gentamicin to kill extracellular bacteria. Reinjected neutrophil-enriched PEC (2×10⁶) were injected i.v. into CB6 F1 (H-2b/d) mice, and equivalent aliquots were lysed with 1% Triton X-100 and plated to determine the number of viable bacteria in the PEC populations. Seven days later, the number of NP₁₁₈–₁₂₆-specific CD8⁺ T cells (H-2L d restricted) was determined by flow cytometry using a cell sorter to detect IFN-γ. Between 1 and 2 h after injection, the cells were incubated overnight at 37°C. After overnight incubation, 3×10⁶ CFSE-labeled, Ag-specific CD8⁺ T cells lines were added to the cultures. These CD8⁺ T cell lines, specific for NP₁₁₈–₁₂₆, or LLO peptide, were generated and maintained as previously described (31). The cultures were incubated at 37°C for an additional 6 h in the presence of brefeldin A. The percentage of CFSE-labeled CD8⁺ T cells stimulated under each culture condition was determined by intracellular cytokine staining for IFN-γ.

Table I. Bacterial strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>~LD₅₀</th>
<th>NP₁₁₈–₁₂₆ Expression</th>
<th>Ref. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>10403s (LM)</td>
<td>10⁹</td>
<td>None</td>
<td>71</td>
</tr>
<tr>
<td>XFL-304 (LM-NPs)</td>
<td>10⁹</td>
<td>Non secreted</td>
<td>9</td>
</tr>
<tr>
<td>XFL-303 (LM-NPs)</td>
<td>10⁹</td>
<td>Secreted</td>
<td>9</td>
</tr>
<tr>
<td>DP-LI942 (actA-deficient LM)</td>
<td>10⁹</td>
<td>None</td>
<td>38</td>
</tr>
<tr>
<td>actA-deficient LM-NPs</td>
<td>10⁹</td>
<td>Secreted</td>
<td>11</td>
</tr>
<tr>
<td>actA-deficient LM-NPs</td>
<td>10⁹</td>
<td>Secreted</td>
<td>11</td>
</tr>
<tr>
<td>DP-LI216 (LLO-deficient LM)</td>
<td>&gt;10⁹</td>
<td>None</td>
<td>38</td>
</tr>
<tr>
<td>LLO-deficient LM-NPs</td>
<td>&gt;10⁹</td>
<td>Non secreted</td>
<td>This paper</td>
</tr>
<tr>
<td>LLO-deficient LM-NPs</td>
<td>&gt;10⁹</td>
<td>Secreted</td>
<td>This paper</td>
</tr>
</tbody>
</table>

Mice were treated with the indicated amount (200 or 25 μg) of RB6.8C5 (Ly-6G/Gr-1 specific) (33, 34), 25 μg of Nimp-R14 (Ly-6G/Gr-1 specific) (35), or rat Ig control Ab 1 day before infection with actA-deficient
LM-NPs or actA-deficient LM-NPns. One day after Ab treatment, the effectiveness of neutrophil depletion was determined by Diff-Quik (Dade Behring) staining of cytospin slides from peripheral blood or by flow cytometric analysis using RB6.8C5- and CD8-specific (e Bioscience, San Diego, CA) Abs. The low dose (25 µg) RB6.8C5 treatment of mice consistently depleted 85–90% of neutrophils from the peripheral blood without significantly impacting the frequency of CD8$^+$ cells. The impact of low dose RB6.8C5 treatment on plasmacytoid DC was also determined in spleen 1 day after mAb injection. Spleen cells were phenotyped for the expression of CD11c (N418, Cy5 conjugated), B220 (6B2, Texas Red conjugated), Ly6C (15.1, biotinylated), or Ly6G (RB6.8C5, biotinylated). FITC-conjugated Abs specific for CD3, Ter119, or CD19 were used to gate out T and B cells. Briefly, $1 \times 10^8$ splenocytes were incubated in an Ab mixture in the presence of 2.4G2 and rat serum for 30 min on ice. Cells were washed and incubated for an additional 30 min in the presence of PE-conjugated strepavidin. The cells were washed and fixed using 1% formaldehyde before analysis.

**Results**

**Presentation of secreted and nonsecreted LM Ags by neutrophil-enriched PEC from infected mice**

CD8$^+$ T cell responses against peptides from several secreted proteins are detected after LM infection of BALB/c mice (7). In addition, substantial CD8$^+$ T cell responses are observed against the LCMV NP$_{118-126}$ epitope after infection of BALB/c mice with rLM expressing this peptide as a secreted (strain LM-NPs) or nonsecreted (strain LM-NPns) fusion protein (9). Similarly, infection of C57BL/6 mice with rLM expressing a different LCMV epitope as a nonsecreted fusion protein also resulted in a substantial CD8$^+$ T cell response against the recombinant Ag (10). Thus, CD8$^+$ T cell responses can be evoked against both secreted and nonsecreted LM Ags. Although the cytosolic residence of LM should allow secreted proteins to be processed by the endogenous MHC class I presentation pathway, it is not known how the immune system accesses nonsecreted LM Ags for presentation to naive CD8$^+$ T cells.

Direct infection of macrophage-like cells with LM-NPs resulted in poor presentation of the nonsecreted Ag to previously activated CD8$^+$ T cells (9). In addition, IFN-γ is not required for CD8$^+$ T cell priming against the nonsecreted Ag in vivo (12) despite an essential function of this cytokine in activation of bacterial killing mechanisms in macrophages. Because the destruction of LM is required to expose the nonsecreted Ag to the host MHC class I processing machinery, these data suggested a role for cells other than macrophages in generating a CD8$^+$ T cell response to nonsecreted Ags in vivo. Based on their ability to kill bacteria in an IFN-γ-independent fashion, we considered a potential role for neutrophils in priming CD8$^+$ T cells against nonsecreted LM Ags.

Several studies have shown the ability of neutrophils to present Ags to previously activated CD8$^+$ T cells in vitro (36, 37). In addition, costimulatory molecule expression has been detected on neutrophils; thus, these cells could potentially serve as APC for activation of naive CD8$^+$ T cells. However, it is not known whether neutrophils present either secreted or nonsecreted LM Ags to CD8$^+$ T cells during in vivo infection. To begin to address this question, BALB/c mice were infected by i.p. injection with various attenuated (actA-deficient) rLM strains, derived from LM, LM-NPs, and LM-NPns strains. We used attenuated actA-deficient rLM (38) to deliver high initial doses ($3 \times 10^8$ CFU/animal) of bacteria in vivo (see Table I for the LM strains used in this study). Six hours after infection, the majority of PEC obtained by peritoneal lavage were neutrophils, as detected by Gr-1 expression (Fig. 1A). Presentation of secreted and nonsecreted Ags by the neutrophil-enriched PEC was determined by their ability to stimulate IFN-γ production after coculture with Ag-specific CD8$^+$ T cell lines. PEC obtained from actA-deficient LM infected mice failed to stimulate IFN-γ production above background ($\leq$5%) from NP$_{118-126}$-specific CD8$^+$ T cells (Fig. 1, B and F) unless synthetic NP$_{118-126}$ peptide was added to the coculture (Fig. 1C). However, PEC from mice infected with actA-deficient LM-NPs stimulated $\leq$30% of NP$_{118-126}$-specific CD8$^+$ T cells to produce IFN-γ (Fig. 1, D and F) demonstrating that these cells were capable of presenting the secreted epitope after in vivo infection. In contrast, PEC from actA-deficient LM-NPns-infected mice stimulated IFN-γ production by a fraction of NP$_{118-126}$-specific CD8$^+$ T cells that was only slightly above the background obtained by incubation of the T cells alone (Fig. 1, E and F). Thus, under these conditions, presentation of secreted LM Ags by neutrophils can occur.
conditions, neutrophil-enriched PEC from infected mice did not efficiently present a nonsecreted bacterial Ag to previously activated CD8⁺ T cells. This outcome was not due to gross alterations in the level of in vivo infection, because PEC populations elicited after infection with each of the rLM strains stimulated a similar frequency of IFN-γ production by CD8⁺ T cells against a secreted Ag (LLO₀₁₋₀₉₀) (39) common to all the rLM (Fig. 1F). Thus, it seems unlikely that LM-infected neutrophils in vivo directly activate naive CD8⁺ T cells specific for the nonsecreted Ag.

**Neutrophil-enriched PEC as a substrate for DC cross-presentation of secreted and nonsecreted bacterial Ags in vitro**

Over the last several years it has become clear that DC can obtain Ags by uptake of necrotic and apoptotic cells, a process called cross-presentation. Because ingestion of bacteria results in the rapid induction of a death program in neutrophils (23–26, 40), we developed an in vitro assay to determine whether LM-infected neutrophils could serve as a substrate for cross-presentation of both secreted and nonsecreted Ags. DC were obtained by culturing bone marrow cells from H-2d mice with IL-4 and GM-CSF. After 5 days, the cultures contained 60–80% CD11c⁺ cells (Fig. 2A), that were CD8⁻ (data not shown). The majority of CD11c⁺ cells were MHC class II⁺ (data not shown) and B7-2⁺ (Fig. 2B). These populations represent primarily immature DC, because further incubation with LPS generates a CD11c⁺ population that is uniformly MHC II and B7-2⁺ (data not shown) (30, 41, 42). DC cultures were mixed with neutrophil-enriched PEC obtained from H-2d MHC mice 6 h after infection with LM, LM-NPns, or LM-NPs (all virulent strains). PEC from H-2d mice are unable to directly present the NP₁₁₈₋₁₂₆ (H-2L⁻ restricted) or LLO₀₁₋₀₉₀ (H-2K² restricted) epitopes to CD8⁺ T cells; thus, stimulation of Ag-specific CD8⁺ T cells in this system would require cross-presentation by H-2d MHC DC. After overnight incubation, H-2d-restricted CD8⁺ T cell lines specific for LLO₀₁₋₀₉₀ or NP₁₁₈₋₁₂₆ were added, and stimulation of these cells by cross-presentation was detected by intracellular cytokine staining for IFN-γ. As shown in Fig. 2, C–E, stimulation of IFN-γ production by NP₁₁₈₋₁₂₆-specific CD8⁺ T cells was Ag specific, as DC cocultured with PEC elicited by LM infected stimulation only background IFN-γ production (4%; Fig. 2C). In contrast, DC cocultured with PEC elicited with LM-NPns (Fig. 2D) or LM-NPs (Fig. 2E) were able to stimulate IFN-γ production from a substantial and similar fraction (30%) of NP₁₁₈₋₁₂₆-specific CD8⁺ T cells. These data suggest that neutrophil-enriched PEC can serve as substrate cells for in vitro cross-presentation of both secreted and nonsecreted bacterial Ags.

One potential complication to this experimental design was that viable LM in the PEC (determined by plating PEC lysates) could directly infect the DC and contribute to the level of T cell stimulation. To control for this, we determined the level of CD8⁺ T cell stimulation after direct infection of DC by varying doses of LM or LM-NPns that cover the range of viable bacteria found in the neutrophil-enriched PEC. The fraction of LLO₀₁₋₀₉₀-specific CD8⁺ T cells stimulated to produce IFN-γ after incubation with LM- or LM-NPns-infected DC was infection dose dependent and required an MOI >0.1 for detectable stimulation of IFN-γ production (Fig. 2, F and G, lines). However, the fraction of IFN-γ⁺ LLO₀₁₋₀₉₀-specific CD8⁺ T cells stimulated to produce IFN-γ in the cross-presentation assay (Fig. 2, F and G, bars) was substantially higher than that obtained by direct infection with the same number of viable bacteria found in the PEC lysates. This result was obtained at two input doses of infected PEC, where the number of viable bacteria in the PEC populations differed by 10-fold. Thus, we conclude that neutrophil-enriched PEC from LM-infected mice can serve as substrates for in vitro cross-presentation of secreted LM Ags.

Similarly, neutrophil-enriched PEC obtained from LM-NPns-infected mice served as substrates for cross-presentation of the nonsecreted NP₁₁₈₋₁₂₆ epitope as detected by the fraction of

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**FIGURE 2.** DC cross-present secreted and nonsecreted bacterial Ags from neutrophil-enriched PEC in vitro. Day 5 DC cultures derived from BALB/c mice (H-2d MHC) were stained for the expression of CD11c (A) or B7.2 (B; dark lines; thin lines represent isotype control staining). C–E, Representative intracellular IFN-γ staining of NP₁₁₈₋₁₂₆-specific CD8⁺ T cells after incubation with DC and neutrophil-enriched PEC elicited from C57BL/6 (H-2b MHC) mice by LM (C), LM-NPns (D), or LM-NPs (E) infection. F–I, Histograms represent the percentage of LLO₀₁₋₀₉₀-specific (F and G) or NP₁₁₈₋₁₂₆-specific (H and I) CD8⁺ T cells stimulated to produce IFN-γ after incubation with H-2d DC and two input numbers (3 × 10⁵ or 3 × 10⁶) of H-2d PEC elicited by infection with LM (F and H) or LM-NPns (G and I). The number of viable LM in each PEC population was determined after detergent lysis and plating on selective media; the histograms representing the degree of stimulation in the cross-presentation assay are plotted at the MOI that would be possible for direct infection by the number of bacteria in the PEC population. In all cases, the number of viable bacteria in the highest input number of PEC resulted in a MOI <1. To control for CD8⁺ T cell stimulation after direct infection of DC by the small number of viable bacterial in the PEC population, DC were directly infected with MOI of 1, 0.1, and 0.01 LM (F and H) or LM-NPns (G and I), and the percentage of IFN-γ-producing T cells was determined (F–I, lines). This format allows direct comparison of Ag-specific CD8⁺ T cell activation by DC incubated with bacteria alone or after cross-presentation of PEC that contain a small number of bacteria. The average ± SD of three replicates is shown for a representative experiment of seven.
Adoptive transfer of neutrophil-enriched PEC from infected mice to naive mice results in cross-priming of naive CD8+ T cells

Although the preceding experiments demonstrate cross-presentation of the LM Ag in neutrophil-enriched PEC in vitro, we next determined whether neutrophil-enriched PEC could also act as substrate cells for cross-priming CD8+ T cell responses in vivo. Preliminary studies were performed by adoptively transferring neutrophil-enriched PEC from actA-deficient, LM-NPns-infected H-2b mice to H-2b/splmice and determining the number of NP118,126-specific CD8+ T cells in the spleen 7 days later. Although recipient mice generated NP118,126 CD8+ T cells, the number of Ag-specific CD8+ T cells was only slightly greater than the number of CD8+ T cells generated by direct infection with the number of viable bacteria present in the adoptively transferred PEC (data not shown). To circumvent this problem, we used LLO-deficient LM strains, which are much weaker at stimulating CD8+ T cell responses in vivo due to their inability to enter the host cytosol and replicate in infected hosts (43). Neutrophil-enriched PEC used for adoptive transfers were obtained from H-2b mice infected with LLO-deficient LM (NP118,126 absent; Fig. 3A). Groups of H-2bsplmice received direct infection with graded doses of LLO-deficient LM-NPns to determine the CD8+ T cell response against the nonsecreted Ag that could be attributed to various levels of direct infection with the attenuated LM-NPns (Fig. 3B). Before adoptive transfer, the PEC were reinfected in vitro with LLO-deficient LM, LLO-deficient LM-NPns, or LLO-deficient LM-NPns to synchronize exposure of the neutrophils to the recombinant Ags and ensure that all neutrophils had the chance to ingest bacteria. After gentamicin treatment to kill extracellular bacteria, the reinfeeted, neutrophil-enriched PEC were adoptively transferred to H-2bsplmice. The number of viable bacteria in the PEC populations was also determined by plating of PEC lysates. Expansion of NP118,126-specific CD8+ T cells in the spleens of the infected or adoptive transfer recipient mice was determined by intracellular cytokine staining for IFN-γ 7 days after transfer or infection. The adoptively transferred PEC contained <5 × 10^7 LLO-deficient LM-NPns, a dose of bacteria that was insufficient to stimulate a detectable NP118,126-specific CD8+ T cell response after direct infection (Fig. 3B). Mice that received neutrophil-enriched PEC infected with LLO-deficient LM-NPns (Fig. 3C) or LLO-deficient LM-NPns (not shown) generated a detectable NP118,126-specific CD8+ T cell response in the spleen on day 7 postinfection. In contrast, mice that received PEC infected with LLO-deficient LM (no NP118,126 epitope) did not generate a detectable NP118,126-specific CD8+ T cell response (Fig. 3C). Together, these results are consistent with the idea that neutrophil-enriched PEC, generated by a combination of in vivo and in vitro infection, are a substrate for in vivo cross-priming of CD8+ T cells against secreted and nonsecreted bacterial Ags by DC.

Treatment of mice with low doses of Ly-6G-specific Ab RB6.8C5 depletes neutrophils, but not the plasmacytoid-like DC subset, in spleen

Next, we determined whether neutrophils play a role in cross-presentation of nonsecreted bacterial Ags during infection. To address this question, we depleted mice of neutrophils using an Ly-6G-specific Ab RB6.8C5 (33). However, this Ab has been reported to deplete lymphocytes at doses >50 μg (34). In addition, an IFN-α-producing, murine DC subset with plasmacytoid morphology has recently been described (44, 45) that is depleted in vivo by low doses (500 μg) of RB6.8C5 Ab. Although it is presently unclear whether the plasmacytoid DC contributes to stimulation of naive T cells, it is a potent producer of type 1 IFN and could indirectly participate in CD8+ T cell priming. In particular, there is evidence that type I IFN-γ is important in cross-priming CD8+ T cells after viral infection (46). Therefore, we first determined whether treatment of mice with a low dose of RB6.8C5 resulted in specific neutrophil depletion. Neutrophil depletion in the peripheral blood was equally effective in mice treated with 25 μg of RB6.8C5 (Fig. 4B) and 200 μg of RB6.8C5 (Fig. 4C), as determined by flow cytometry or differential count (data not shown). However, treatment with 200 μg of RB6.8C5 resulted in depletion of CD8+ -expressing cells, whereas treatment with 25 μg of RB6.8C5 resulted in little or no depletion of this cell population (data not shown). Furthermore, a population of B220high and Ly6Chigh plasmacytoid DC can be detected in the spleen of control
Ig-treated mice (Fig. 4D), whereas injection of 200 μg of RB6.8CS resulted in almost complete elimination of these cells (Fig. 4E). In contrast, the majority of plasmacytoid DC remained at day 1 after treatment of mice with 25 μg of RB6.8CS (Fig. 4E). Thus, low dose treatment of mice with RB6.8CS resulted in substantial and relatively specific depletion of neutrophils.

**Neutrophil depletion before infection reduces the number of CD8^+ T cells specific for nonsecreted bacterial Ags**

To address the in vivo role of neutrophils in priming CD8^+ T cells against the nonsecreted LM Ag, BALB/c mice were treated with 25 μg of either of two distinct neutrophil-specific depleting Abs (RB6.8CS and Nimp-R14) (35). This treatment regimen resulted in 85–90% depletion of neutrophils from the peripheral blood as determined by flow cytometry (Fig. 5, A–C) and differential counts (data not shown). The neutrophil-depleted mice were infected with actA-deficient LM-NPs or actA-deficient LM-NPns, and the bacterial load was determined on day 1 postinfection. Neutrophil depletion increased the bacterial load in the livers (10- to 100-fold) and spleens (∼10-fold) at day 1 postinfection compared with that in control mice (data not shown). The magnitude of the Ag-specific CD8^+ T cell response was determined 7 days after infection. Neutrophil depletion with low dose of RB6.8CS before actA-deficient LM-NPs infection resulted in a modest increase in the CD8^+ T cell responses to LLO_91–99 and NP_118–126 probably as a result of the increased bacterial load (data not shown). However, RB6.8CS treatment did not alter the ratio of LLO_91–99 to NP_118–126^specific CD8^+ T cells after LM-NPs infection (both epitopes secreted) compared with that in mice treated with control Ab (Fig. 5D). In contrast, although neutrophil depletion before actA-deficient LM-NPns infection caused a similar modest increase in the LLO_91–99 response compared with control Ab treatment, it resulted in substantially reduced priming against the nonsecreted NP_118–126 epitope. This caused an increase in the ratio of LLO_91–99 to NP_118–126^specific CD8^+ T cells after LM-NPns infection from an average of ∼3 in rlg-treated mice to >11 in RB6.8CS-treated mice (Fig. 5E). The ratio of LLO_91–99^specific CD8^+ T cell to NP_118–126^specific Cd8^+ T cells in each mouse is the most accurate measurement because it controls for differences in the magnitude of the response between individual mice. Similar results were obtained after depletion of neutrophils with the NimpR14 mAb. Again, neutrophil depletion did not change the ratio of LLO_91–99^specific CD8^+ T cells to NP_118–126^specific CD8^+ T cells compared with that in Ig-treated mice after actA-deficient LM-NPns infection (Fig. 5F), but caused an increase from ∼2 to >8 in this ratio after actA-deficient LM-NPns infection (Fig. 5G).

**Discussion**

Efficient stimulation of CD8^+ T cell responses during primary infection is thought to require Ag presentation by mature DC (47–51). Consistent with this notion, primary CD8^+ T cell responses to secreted LM Ags, which are accessible to the endogenous MHC class I Ag presentation pathway (52), were ablated in conditional DC knockout mice (53). However, it is also clear that DC can present nonsecreted Ags from LM, which are not readily accessible to the endogenous MHC class I pathway, to prime CD8^+ T cell responses after infection (9, 10). DC could acquire secreted and nonsecreted Ags by phagocytosis or by direct membrane contact with Ag-loaded DCs.
nonsecreted LM Ags after infection or through cross-presentation of Ags from cells that are able to destroy LM, but still die as a result of infection or activation. Our results are consistent with a role for neutrophils in cross-priming CD8+ T cells against nonsecreted LM Ags in vivo.

Our previous results showed similar CD8+ T cell responses to nonsecreted LM Ags in wild-type and IFN-γ-deficient mice (12). Because IFN-γ is a potent cytokine in activating the microbial activities of macrophages and DC, it seemed unlikely that these cells were directly killing LM to expose the nonsecreted Ag and activate CD8+ T cells after infection. Therefore, we focused our attention on a cross-priming mechanism to evoke CD8+ T cells against nonsecreted LM Ags. We hypothesized that the substrate cell for cross-presentation would need to elaborate antimicrobial activity to destroy LM and expose the nonsecreted Ag for processing. Neutrophils were attractive substrates for cross-presentation because they are recruited to sites of infection very early and are responsible for decreased bacterial numbers in the liver that occurs within 6 h of i.v. infection with LM (54–57). Once the neutrophils have become activated or ingest bacteria, they die within a few hours. Thus, neutrophils that ingest LM could provide substrate for DC-mediated phagocytosis and cross-presentation (20, 21, 58). Alternatively, neutrophils that ingest and kill LM could directly activate naive CD8+ T cells. In contrast to this idea, neutrophil-enriched PEC from LM-NPns-infected mice were unable to efficiently present the nonsecreted Ag and stimulate even previously activated CD8+ T cells. Although neutrophils can express some costimulatory molecules (59, 60), we believe it unlikely that they serve as direct stimulators of naive CD8+ T cells specific for nonsecreted Ags.

However, neutrophil-enriched PEC from infected mice can serve as substrate cells for in vitro cross-presentation of both secreted and nonsecreted LM Ags by DC. Although these in vitro results are consistent with a role for neutrophils as substrates for cross-presentation in vivo, many cell types appear to function as substrates for cross-presentation using similar in vitro assays (20, 21, 58, 61). Interestingly, under the conditions we used for our experiments, in vitro cross-presentation of the secreted and nonsecreted Ag by DC was similar in magnitude, as judged by the percentage of NP118–126-specific CD8+ T cells stimulated to produce IFN-γ in the cultures. This result may be due to the fact that neutrophils destroy LM and initially process Ags in vacuoles. In this case, the absolute level of Ag may be more critical to the degree of cross-presentation than the compartmentalization of the Ag. Previous analyses showed similar steady state levels of the secreted and nonsecreted Ags in LM-NPs and LM-NPns (9), which could account for the similar stimulation of NP118–126-specific CD8+ T cells in the in vitro cross-presentation assay. Alternatively, it is possible that the in vitro experimental conditions resulted in saturation of the cross-presentation capacity of the system, leading to similar levels of stimulation with neutrophil-enriched PEC from LM-NPns- and LM-NPns-infected mice.

To extend the relevance of the in vitro results, we also showed that adoptive transfer of LM-NPs-infected (not shown) or LM-NPns-infected neutrophil-enriched PEC resulted in cross-presentation of CD8+ T cells against both secreted and nonsecreted LM Ags in vivo. Our ability to measure cross-presentation after adoptive transfer required that we control for the level of CD8+ T cell priming due to direct infection because it is unavoidable that some viable bacteria (generally in the range of several hundred CFU per mouse) are transferred in the PEC population. To this end, we used LLO-deficient LM strains to elicit the PEC, which were reinfected in vitro with LLO-deficient LM, LLO-deficient LM-NPns, or LLO-deficient LM-NPs. These LLO-deficient LM strains are highly attenuated (LD50 > 108) (43) and in our hands only stimulated detectable CD8+ T cell responses at direct infection doses of > 104 CFU/mouse, numbers of organisms that are orders of magnitude higher than the number of viable bacteria in the adoptively transferred PEC populations. Thus, the CD8+ T cell responses we observed after adoptive transfer of infected PEC could only have occurred by a cross-priming mechanism. Similar to our in vitro results, CD8+ T cell responses were cross-primed in vivo against both secreted (not shown) and nonsecreted LM Ags after adoptive transfer of infected, neutrophil-enriched PEC. These data suggest that LM-infected neutrophils are potential substrates for in vivo cross-priming of CD8+ T cells.

Finally, we show that in vivo depletion of neutrophils with a low dose of two distinct Ly-6G-specific mAb specifically reduced CD8+ T cell priming against the nonsecreted Ag without interfering with the CD8+ T cell response against the recombinant or control secreted epitopes. High dose treatment (200–500 μg) with Ly-6G-specific mAb effectively depleted neutrophils, but also results in depletion of other cell types, including CD8+ T cells and plasmacytoid DC (34, 44, 66). This nonspecific depletion is thought to occur due to cross-reactivity with Ly-6C, an activation marker expressed by many cell types (67). We used a low dose (25 μg) treatment regimen with the Ly-6G-specific mAb, which depleted 80–90% of circulating neutrophils, but had minimal impact on survival of CD8+ T cells or plasmacytoid DC. Thus, we concluded that inhibition of priming against the nonsecreted Ag is a consequence of neutrophil depletion, rather than depletion of some other cell type.

With regard to the results with in vivo neutrophil depletion there are two issues worthy of note. First, the level of in vivo neutrophil depletion we achieved reduced, but did not eliminate, CD8+ T cell priming against the nonsecreted Ag. Thus, other cell types may serve as substrates for cross-presentation of the nonsecreted Ag during LM infection. Alternatively, neutrophils that escaped depletion could be sufficient to generate the reduced level of CD8+ T cell response. It is also possible that DC may be able to destroy LM and present the nonsecreted Ag at some level after direct infection. Consistent with this idea, direct in vitro infection of DC with LM-NPns resulted in stimulation of NPs118–126-specific CD8+ T cells, albeit at a lower level than stimulation of LLC09–099-specific CD8+ T cell directed at a secreted Ag (see Fig. 2). Resolution of this issue will require generation of an experimental system where neutrophils can be quantitatively depleted.

Secondly, in vitro studies and adoptive transfer studies showed that neutrophils can be substrates for cross-presentation of both secreted and nonsecreted Ags, whereas in vivo neutrophil depletion specifically inhibited CD8+ T cell priming against the nonsecreted Ag. One explanation for this result would suggest that presentation of the secreted Ag can occur by either direct infection of DC or cross-presentation, but the direct route is sufficient to activate a maximal CD8+ T cell response during infection. Alternatively, LM infection of any cell type should result in initial processing of the secreted Ag by the endogenous MHC class I pathway, although only cells that can kill LM should be able to begin to process the nonsecreted Ag. Although it is unclear whether processing during cross-presentation occurs in the initially infected cell, the DC, or both, exposure of the secreted Ag in any infected cell may increase the number of cells that can serve as substrates for cross-presentation, such that depletion of neutrophils does not limit CD8+ T cell priming against the secreted Ag. In contrast, the smaller number of cells that can kill LM to expose the nonsecreted Ag for initial processing may limit the number of substrate cells for cross-presentation such that neutrophil depletion significantly decreases the CD8+ T cell response.
Together, these data reveal a previously unappreciated role for neutrophils as substrates for cross-priming of CD8+ T cell responses against bacterial Ags that are not directly accessible to the endogenous MHC class I presentation pathway. These results may also explain how CD8+ T cells respond to Ags expressed by bacteria that occupy extracellular environments (68–70), although the relevance of CD8+ T cell responses against extracellular bacteria for immune regulation or protective immunity is currently unclear. In conclusion, our results serve to expand and strengthen the idea that the innate immune system, in this case neutrophils, is critical in shaping the specificity and breadth of adaptive (CD8+ T cell) immune responses.

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References


