Neutrophils Process Exogenous Bacteria Via an Alternate Class I MHC Processing Pathway for Presentation of Peptides to T Lymphocytes

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Neutrophils Process Exogenous Bacteria Via an Alternate Class I MHC Processing Pathway for Presentation of Peptides to T Lymphocytes

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Peptides that are presented by class I MHC (MHC-I) molecules derive from cytosolic Ags processed via the conventional MHC-I pathway or exogenous Ags processed via alternate MHC-I processing mechanisms. Alternate MHC-I processing by macrophages and dendritic cells allows presentation of peptides from particulate Ags, including bacteria. Despite the established phagocytic activity of neutrophils, MHC-I processing and presentation of phagocytosed Ags by neutrophils has not been investigated. Murine neutrophils from peritoneal exudates were shown to express MHC-I molecules and tested for the ability to process HB101.Crl-OVA, Escherichia coli transfected to express a fusion protein containing the 257–264 epitope of OVA. Neutrophils were found to process HB101.Crl-OVA and present OVA257–264-Kb complexes to CD8+ T hybridoma cells via a pathway that was resistant to brefeldin A, an inhibitor of anterograde endoplasmic reticulum-Golgi transport, and lactacystin, a proteasome inhibitor. These results suggest that neutrophils process phagocytosed bacteria via a vacuolar alternate MHC-I pathway that does not involve cytosolic processing. In addition, neutrophils were found to secrete or “regurgitate” processed peptide that was subsequently presented by neighboring prefixed macrophages or dendritic cells. Thus, neutrophils may influence T cell responses to bacteria, either by directly presenting peptide-MHC-I complexes or by delivering peptides to other APCs for presentation. Hypothetically, neutrophils may directly present peptide to effector T cells in vivo at sites of inflammation, inducing cytokine production, whereas dendritic cells in receipt of neutrophil-derived antigenic peptides may migrate to lymphoid organs to initiate T cell responses. The Journal of Immunology, 2001, 167: 2538–2546.

Neutrophils are the cellular hallmark of acute inflammation and rapidly accumulate in large numbers at sites of infection. During their short life span (hours to days) neutrophils perform many functions of host defense, including phagocytosis and killing of bacteria with reactive oxygen intermediates and other mechanisms. Although neutrophils have been viewed primarily in terms of their contributions to innate immunity, they also have the ability to influence adaptive immunity (1). Unstimulated circulating neutrophils express class I MHC (MHC-I) molecules (1–3). Stimulation of neutrophils with GM-CSF increases the expression of these molecules and induces the expression of many other molecules including cytokines and, under some circumstances, class II MHC (MHC-II) molecules (particularly when IFN-γ is also present) (4–6). Constitutive or regulated expression of these and other proteins allows neutrophils to influence both innate and adaptive immunity (1).

Although neutrophils generally have not been appreciated to have significant function as APCs, they have been shown to present peptide-MHC-I complexes; exogenous peptide added to neutrophils in vitro can bind cell surface MHC-I and stimulate memory CD8+ T cells (7). In addition, neutrophils stimulated with GM-CSF and IFN-γ were found to express MHC-I-II and mediate superantigen-induced T cell activation, but were not found to process protein Ag for MHC-II-restricted presentation to T cells (4, 5). Thus, neutrophils have been shown to possess certain Ag-presenting functions, but their ability to actively process exogenous Ag has not been established.

The conventional MHC-I Ag-processing pathway generally involves the processing of cytosol-derived Ag. Exogenous Ag can be processed via alternate MHC-I Ag-processing mechanisms, which may involve delivery of exogenous Ag from vacuolar compartments to the cytosol for cytosolic processing (8–10) or processing wholly within vacuolar compartments without access to the cytosol (i.e., vacuolar alternate MHC-I Ag processing) (11–17). Particulate Ags, including bacteria, are efficiently processed by macrophages and dendritic cells for MHC-I presentation.

Although neutrophils phagocytose bacteria, express MHC-I, and can present exogenous synthetic peptides, previous studies have not tested whether neutrophils can process Ag expressed by bacteria for MHC-I presentation. The studies presented here establish that neutrophils process phagocytosed bacteria via an alternate MHC-I Ag-processing pathway that allows MHC-I presentation of peptides derived from the bacteria. Although this processing was inhibited by cytochalasin D, which blocks phagocytosis, it was not affected by lactacystin, a proteasome inhibitor, or brefeldin A, which blocks anterograde transport from the endoplasmic reticulum (ER) through the Golgi apparatus. These results indicate that neutrophils process phagocytosed bacteria via a vacuolar alternate MHC-I pathway. In addition, we observed that neutrophils release or “regurgitate” processed peptide into the extracellular space, and this peptide can bind MHC-I on neighboring cells (e.g., macrophages or dendritic cells) for presentation to CD8+ T cells, although it remains to be determined whether the predominant mechanism for neutrophil generation of peptide-MHC-I complexes involves

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3 Abbreviations used in this paper: MHC-I, class I MHC; MHC-II, class II MHC; ER, endoplasmic reticulum; MFV, mean fluorescence value.
intravascular binding or regurgitation and surface binding. Our model suggests that neutrophils may directly present bacterial Ags to stimulate local effector T cell responses, including the production of cytokines, particularly when effector T cells and neutrophils are colocalized at sites of inflammation. Additionally, neutrophils may regurgitate peptides to load MHC-I molecules on professional APCs, e.g., dendritic cells, which could either elicit local effector T cell responses or migrate to lymph nodes to stimulate primary T cell responses. These are the first reported studies of the ability of neutrophils to process phagocytosed bacteria for presentation to T cells.

Materials and Methods

Cell preparation and culture

Cells were cultured at 37°C in a 5% CO2 atmosphere in standard medium composed of DMEM (Life Technologies, Grand Island, NY) with 10% FCS (HyClone Laboratories, Logan, UT), 5 x 10^{-5} M 2-ME, 116 mg/L l-arginine hydrochloride, 36 mg/L l-asparagine, 2 g/L NaHCO3, 1 mM sodium pyruvate, and antibiotics. Neutrophils were elicited from B6D2F1/J (H-2b) or CBA/J (H-2d) mice (The Jackson Laboratory, Bar Harbor, ME) by i.p. injection of 1.5 ml sterile thioglycolate (29 mg/ml) without resazurin (Difco, Detroit, MI). Four hours later, peritoneal exudate cells were harvested by i.p. injection of Con A (100 μg/10 ml of cell suspension) before to use. To produce heat-killed bacteria, bacteria were resuspended in PBS, incubated at 65°C for 60 min, and resuspended in standard medium.

In the basic Ag-processing protocol, neutrophils (0.5-2 x 10^6/ml) or control macrophages were incubated overnight at 37°C in U-bottom 96 well plates (Falcon 3077; BD Biosciences, Franklin Lakes, NJ) with heat-killed E. coli (10^5 to 3 x 10^6 cells/well) and CD8OVA1.3 T hybridoma cells (10^4/well), which detect OVA257-264-Kb complexes (11). Plates were centrifuged, and supernatants (0.1 ml) were harvested to determine IL-2 content as a measure of T cell response (see CTL-2 bioassay for IL-2 below).

A second protocol allowed the use of viable bacteria and/or pharmacologic manipulation of the Ag-processing incubation before fixation of neutrophils and subsequent incubation with CD8OVA1.3 cells. Neutrophils (2 x 10^6/well in 96-well plates or 10^5 cells in 2 ml polypropylene tubes) were incubated for 15–20 min in standard medium (without antibiotics for experiments with live bacteria) with or without brefeldin A (0.5–1 μg/ml; Sigma-Aldrich, St. Louis, MO), lactacystin (10–40 μM; purchased from E. J. Corey, Harvard University, Cambridge, MA), or cytochalasin D (10 μg/ml; Sigma-Aldrich). Bacteria were added for 90–120 min in the control incubation or absence of inhibitor. Neutrophils were fixed for 20 min in 1% paraformaldehyde in PBS, washed, incubated for 20 min with 0.2 M lysine in standard medium, and washed three times with standard medium. CD8OVA1.3 T hybridoma cells (10^4/well) were added, the plates were incubated 20–24 h, and supernatants were harvested for an IL-2 assay (see CTL-2 bioassay for IL-2). A third protocol was designed to test for peptide regurgitation. Dendritic cells (10^5/well) or Con A-elicted macrophages (CD8OVA1.3 T hybridoma cells (10^4/well)) were fixed with paraformaldehyde, washed, and incubated overnight with 10^9 viable neutrophils from CBA/J mice, various doses of heat-killed HB101.Crt-OVA, and 10^5 CD8OVA1.3 T hybridoma cells. Supernatants were harvested for an IL-2 assay.

Peritoneal macrophages were used in control experiments to confirm the activity of inhibitors for blockade of the conventional MHC-I Ag-processing pathway, because these cells were more amenable to electroporation than neutrophils. Cells were resuspended at 8 x 10^6/ml in DMEM with or without brefeldin A (0.5–1 μg/ml) or lactacystin (10–40 μg/ml), incubated for 10 min at 37°C in polypropylene tubes with mixing, and cooled to 4°C. OVA protein (Sigma-Aldrich, catalog no. A-5503) was added (0.6 mg/ml per 1 ml plate concentration), and macrophages were electroporated at 4°C with 200V, 800 μF, and low resistance settings using a Cell-Porator and 4-mm gap cuvettes (Life Technologies) (21). Macrophages were washed, plated (10^5/well), incubated for 2 h at 37°C in standard medium with the continued presence or absence of inhibitor, fixed, and washed. CD8OVA1.3 T hybridoma cells were added for 24 h, and supernatants were harvested as described above.

CTLL-2 bioassay for IL-2

Supernatants from Ag-processing experiments were frozen, thawed, and assayed for IL-2 using a colorimetric CTLL-2 bioassay (18, 22). IL-2-dependent CTLL-2 cells were washed three times and incubated (5 x 10^4/well) in the supernatants for 20–24 h at 37°C. Alamar blue (Accumed, Chicago, IL) was added (15 μl/well) to assess metabolic activity. The plates were incubated for 20–24 h, and the extent of Alamar blue reduction was determined by the difference in optical density at 550 and 595 nm using a Bio-Rad (Hercules, CA) model 550 microplate spectrophotometer (18).

Flow cytometry

To assess neutrophil purity, 5 x 10^5 cells were stained for 30 min at 4°C in FACS buffer (PBS with 1% BSA) containing 10 μg/ml RB6-8C5 anti-Gr-1 (BD Pharmingen) or rat IgG2b isotype control (Caltag Laboratories), washed three times with FACS buffer, incubated with FITC-conjugated goat F(ab')2 anti-rat IgG H and L (Caltag Laboratories) for 1 h, washed three times, and analyzed with a flow cytometer (FACScan, BD Biosciences; or Epics elite ESP, Beckman Coulter, Miami, FL). To assess MHC-I expression, neutrophils were stained with biotinylated 28-8-6 anti-H-2Kb (BD Pharmingen) or biotinylated mouse IgG2a isotype control Ab (BD Pharmingen) and streptavidin-APC or streptavidin-APC-Cy-Chrome (BD Pharmingen). Fluorescein-labeled bacteria were used to assess phagocytosis. Heat-killed HB101. Crt-OVA (1 ml at 10^8 bacteria/ml in PBS (pH 9.1)) were depleted by
Results

Purification of neutrophils for analysis of MHC-I expression and Ag-processing function

Neutrophils phagocytose and kill bacteria but have not been demonstrated to process bacterial Ags for presentation to T cells. Because neutrophils, with some exceptions (4–6), do not generally express MHC-II molecules, their potential ability to function as APCs for CD4 T cell responses appears limited. However, neutrophils do express MHC-I (1–3) and have been shown to present MHC-I-restricted peptides (7). Thus, we propose that alternate MHC-I Ag-processing mechanisms could allow the processing of bacteria that are phagocytosed by neutrophils.

To establish a system for analysis of MHC-I expression and Ag processing, murine neutrophils were isolated from thioglycolate-induced acute peritoneal exudates. Exudate cells consisted primarily of neutrophils with small numbers of macrophages and lymphocytes (66% of exudate cells were neutrophils by microscopic analysis of stained cells, and 64% had high expression of Gr-1, a neutrophil marker, by flow cytometry; Fig. 1A). Purification of neutrophils on Percoll density gradients resulted in neutrophil purity of 93–99% (range in different experiments) by morphology and a virtually identical percentage of cells with high expression of Gr-1 by flow cytometry (Fig. 1A). In contrast, resident peritoneal macrophages from naive mice or bone marrow-derived macrophages had no significant expression of Gr-1, and macrophages from acute thioglycolate-elicited peritoneal exudates had at most only low expression of Gr-1 (data not shown, corresponding to gate M2 in Fig. 1A, below the high expression gate containing neutrophils). In some experiments, neutrophils isolated on Percoll gradients were further purified by treatment with F4/80 and complement to increase neutrophil purity by an additional 1–3% (as assessed by morphology). Flow cytometry also revealed that 99% of neutrophils expressed MHC-I (Fig. 1B). These approaches provided highly purified neutrophil preparations for analysis of Ag-processing function.

Phagocytosis of bacteria by neutrophils

To assess bacterial uptake, neutrophils were incubated for 60 min with varying numbers of fluorescent-labeled heat-killed HB101.Crl-OVA (E. coli bearing a fusion protein that contains the OVA257–264 epitope) and evaluated by flow cytometry (Fig. 1C). To calculate the number of bacteria per neutrophil, events were first selected by gating to include fluorescent-positive events (gate M1, Fig. 1C) with scatter properties of intact neutrophils. The mean fluorochrome value (MFV) of these events was then divided by the MFV of isolated fluorescent-labeled HB101.Crl-OVA to obtain mean bacterial uptake per neutrophil (Table I). Of neutrophils that internalized bacteria (i.e., fluorescent-positive neutrophils), the mean bacterial uptake per neutrophil ranged from 0.9 to 5.9 at different bacterium:neutrophil ratios (Table I). Theoretically, these numbers may slightly underestimate bacterial uptake, because bacterial degradation may decrease cell-associated fluorescence. However, at lower limiting bacterial dilutions (bacterium:neutrophil ratio from 1:1 to 10:1), this approach indicated that fluorescein-positive neutrophils (M1 gate of Fig. 1C) contained an average of close to 1 bacterium per neutrophil (Table I). Most of the variation in fluorescence of these cells was explained by variation in the level of labeling of individual bacteria (Fig. 1C). If bacterial degradation or other artifacts substantially affected this calculation, the number of bacteria per fluorescent-positive neutrophil would not approach 1 (e.g., degradation of bacteria would reduce the MFV of neutrophils at limiting bacterial dilutions). Therefore, bacterial uptake can be approximated by dividing the MFV of neutrophils by the MFV of isolated bacteria.

Subsequent experiments showed that detectable Ag presentation was produced with 10⁵ neutrophils and 1×10⁶ bacteria/well (i.e., with a bacterium:neutrophil ratio from 10:1 to 30:1). Under these conditions, the proportion of neutrophils that contained bacteria ranged from 25.7 to 44.0%, and neutrophils that contained bacteria had a mean bacterial uptake of 1.1–1.8 (Fig. 1C and Table I). In conclusion, conditions that produced detectable Ag presentation (see Alternate MHC-I processing of bacterial Ag by neutrophils) involved phagocytosis of as little as 1–2 bacteria/neutrophil.

The calculations of total bacterial uptake do not reveal whether neutrophil-associated bacteria were intracellular or on the cell surface. To address this question, neutrophils were incubated with
fluorescein-labeled bacteria for 60 min, transferred to 4°C without fixation, and directly analyzed by flow cytometry with and without addition of ethidium bromide (100 μg/ml) to quench the fluorescence of extracellular or surface-bound bacteria as previously described (24–26). The fluorescence of isolated bacteria (without exposure to neutrophils) was decreased by 88% when quenched by 100 μg/ml ethidium bromide (data not shown). When neutrophils were exposed to bacteria at 4°C, the fluorescence of neutrophil-associated bacteria was decreased by 92% with ethidium bromide (Fig. 2), indicating that essentially all of these bacteria were surface bound. Uptake of bacteria was greater at 37°C than at 4°C, and bacterial fluorescence was decreased by only 44% with ethidium bromide (Fig. 2). These results indicate that ~56% of neutrophil-associated bacteria were intracellular after a 60-min incubation at 37°C (the presence of some bacteria at the cell surface is consistent with the lack of a chase period).

Alternate MHC-I processing of bacterial Ag by neutrophils

To examine processing of phagocytosed bacteria, B6D2F1/J neutrophils were purified on Percoll gradients and incubated overnight with heat-killed HB101.Crl-OVA and CD80VA1.3 T cell hybridoma cells (which recognize OVA257-264-Kb complexes). T cell stimulation was determined by IL-2 secretion as assessed with a colorimetric CTLL-2 bioassay. Ag expressed by bacteria was processed and presented by neutrophils to CD8OVA1.3 T hybridoma cells (Fig. 3A). Ag presentation was detected with as few as 1–3 × 10^6 bacteria/well (bacterium:neutrophil ratio from 10:1 to 30:1), corresponding to phagocytosis of only 1–2 bacteria by 25.7–44% of neutrophils (see above and Table I). No significant presentation was observed when T hybridoma cells alone were incubated with bacteria (i.e., in the absence of neutrophils; data not shown) or when T hybridoma cells were incubated with bacteria and MHC-disparate (H-2^k) neutrophils (data not shown and section on peptide regurgitation). Significant Ag presentation was observed with as few as 2 × 10^4 neutrophils/well (97–98% pure neutrophils; data not shown). Heat-killed bacteria were used to avoid bacterial contamination in the previous experiments (which used a simple, non-fixation protocol), but similar results were obtained in experiments with live bacteria and a fixation protocol (Fig. 3B). In addition, alternate MHC-I processing of HB101.Crl-OVA was inhibited by cytochalasin D, which inhibits actin-dependent functions such as phagocytosis (Fig. 4), indicating that phagocytic internalization of bacteria by neutrophils was necessary for alternate MHC-I processing and presentation of bacterial Ag.

To ensure that Ag-processing activity was mediated by neutrophils, neutrophil purity was enhanced by combining Percoll gradient purification with depletion of contaminating macrophages with F4/80 Ab and complement. Neutrophils purified by this approach retained Ag processing and presentation activity, indicating that this activity was truly attributable to neutrophils (Fig. 5). In addition, macrophages were isolated from acute, thioglycolate-induced peritoneal exudates using Percoll gradients. The Ag processing and presentation activity of neutrophils was then compared with that of macrophages isolated from the same exudate (Fig. 6). Neutrophils and macrophages were both capable of alternate MHC-I Ag processing and presentation, but neutrophils were more

### Table I. Uptake of fluorescent bacteria by neutrophils assessed by flow cytometry

<table>
<thead>
<tr>
<th>Bacteria:Neutrophil Ratio</th>
<th>Neutrophils with Bacteria (%)</th>
<th>MFV of Neutrophils in Positive Gate</th>
<th>MFV Less Neutrophil Background</th>
<th>Bacteria/Neutrophil (Mean)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrophils only</td>
<td>0</td>
<td>1.9d</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Bacteria only</td>
<td>NA</td>
<td>39.9f</td>
<td>39.9f</td>
<td>NA</td>
</tr>
<tr>
<td>1:1</td>
<td>6.2</td>
<td>52.4</td>
<td>50.5</td>
<td>1.3</td>
</tr>
<tr>
<td>3:1</td>
<td>16.1</td>
<td>38.6</td>
<td>36.7</td>
<td>0.9</td>
</tr>
<tr>
<td>10:1</td>
<td>25.7</td>
<td>47.1</td>
<td>45.2</td>
<td>1.1</td>
</tr>
<tr>
<td>30:1</td>
<td>44.0</td>
<td>75.4</td>
<td>73.5</td>
<td>1.8</td>
</tr>
<tr>
<td>100:1</td>
<td>66.1</td>
<td>113.6</td>
<td>111.7</td>
<td>2.8</td>
</tr>
<tr>
<td>300:1</td>
<td>90.8</td>
<td>236.4</td>
<td>234.5</td>
<td>5.9</td>
</tr>
</tbody>
</table>

* Percentage of neutrophils in positive gate (M1 gate, Fig. 1C).
* MFV of neutrophils within positive gate.
* Bacteria per neutrophil within positive gate (neutrophils with bacteria). This value was computed by dividing MFV less neutrophil background by 39.9 (the MFV of isolated bacteria).
* MFV of all neutrophils without bacteria (“neutrophil background”).
* NA, Not applicable.
* MFV of bacteria.

**FIGURE 2.** Internalization of bacteria by phagocytosis at 37°C. Neutrophils (5 × 10^5/ml; 93% pure by morphology) were incubated with rocking in 1 ml standard medium with fluorescein-labeled heat-killed HB101.Crl-OVA (1.5 × 10^7 bacteria/ml) for 60 min, transferred to 4°C without fixation, and directly analyzed by flow cytometry with (+EB) or without (No EB) addition of ethidium bromide (100 μg/ml) to quench the fluorescence of extracellular or surface-bound bacteria.
efficient than macrophages at the same titer (10^5 cells/well). Furthermore, numbers of macrophages that could potentially contaminate 93–99% pure neutrophil preparations (1–7% of the population, i.e., 1–7/10^5 cells in wells containing 10^5 cells) generated small or undetectable levels of presentation, significantly (p < 0.01, Fig. 6) below the level produced by 10^5 neutrophils. These observations indicate that alternate MHC-I Ag-processing activity of purified neutrophil preparations was truly attributable to neutrophils.

**Cytosolic vs vacuolar alternate MHC-I Ag processing by neutrophils**

Alternate MHC-I Ag processing may involve cytosolic Ag processing, following escape of Ag from the phagosome into the cytosol, or vacuolar Ag processing within the phagosome, followed by peptide binding to MHC-I molecules within the vacuole. A third possibility is that vacuolar processing is followed by secretion or regurgitation of peptide and binding of peptide to MHC-I at the cell surface. More than one of these mechanisms may contribute to alternate MHC-I Ag processing, and their relative contributions may vary under different conditions.

To determine the mechanism(s) of neutrophil Ag processing, we used inhibitors with differential effects on cytosolic and vacuolar processing mechanisms. Proteasome inhibitors and brefeldin A (an inhibitor of anterograde ER-Golgi transport) inhibit cytosolic processing and the conventional MHC-I pathway (21, 27–30) but do not block vacuolar alternate MHC-I Ag processing (11–16, 21). Neutrophils were incubated with brefeldin A, lactacystin (a proteasome inhibitor), or control medium for 15 min and then incubated for 90 min with HB101.Crl-OVA in the continued presence or absence of the inhibitors. The neutrophils were then fixed, washed, and incubated overnight with CD8OVA1.3 T hybridoma cells to determine the extent of Ag processing. Alternate MHC-I Ag-processing activity of neutrophils was completely resistant to both lactacystin and brefeldin A (Fig. 7A). To provide a positive control for the pharmacologic activities of lactacystin and brefeldin A, we confirmed that both inhibitors were able to block the processing of OVA protein that was electroporated into the cytosol of macrophages (Fig. 7B; neutrophils were not used because extensive cell death occurred with these cells upon electroporation). The continued processing of bacterial Ag by neutrophils under conditions that block cytosolic processing indicates that alternate MHC-I Ag processing by neutrophils occurs by vacuolar mechanisms.

**FIGURE 5.** Processing of HB101.Crl-OVA by purified neutrophils. Neutrophils were purified by Percoll gradient isolation followed by treatment with F4/80 and complement to deplete residual macrophages, resulting in a 94% pure neutrophil preparation. Processing of HB101.Crl-OVA was assessed as in Fig. 3A.
Role of peptide regurgitation in alternate MHC-I Ag processing by neutrophils and capacity of neutrophils to deliver antigenic peptides to other APCs

Peptides generated by vacuolar alternate MHC-I Ag processing may bind to MHC-I molecules within vacuolar compartments or may be regurgitated into the surrounding milieu and bind to MHC-I molecules on the surface of the same cell or a neighboring cell (11, 31). To evaluate peptide regurgitation, we incubated viable MHC-mismatched (CBA/J) neutrophils with HB101.Crl-OVA and fixed B6D2F1/J macrophages. Viable CBA/J neutrophils were capable of phagocytic processing of bacteria but lacked expression of Kb to present OVA peptide to CD8OVA1.3 T cells, whereas the fixed B6D2F1/J macrophages were unable to phagocytose or process bacteria but were capable of presenting exogenous peptide to CD8OVA1.3 T cells. Processing and presentation of bacterial Ag occurred under these conditions, although neither viable CBA/J neutrophils nor fixed B6D2F1/J macrophages alone elicited a response after overnight incubation with HB101.Crl-OVA and CD8OVA1.3 cells (Fig. 8A). Thus, neutrophils can process bacterial Ags and regurgitate peptides that bind to MHC-I molecules on macrophages for subsequent presentation to T cells. Fixed dendritic cells were also shown to bind and present peptides regurgitated by CBA/J neutrophils (Fig. 8B). In addition, chloroquine and ammonium chloride inhibited the ability of neutrophils to process HB101.Crl-OVA for regurgitation of peptides onto fixed macrophages for presentation to T cells (Fig. 9). This observation indicates that processing for regurgitation was dependent on intracellular vacuolar processing mechanisms that are inhibited by these lysosomotropic amines (which disrupt vacuolar pH gradients). We conclude that neutrophils phagocytose and process bacterial Ags and that presentation to T cells is mediated both directly by neutrophils and indirectly by professional APCs, e.g., macrophages and dendritic cells, after regurgitation of peptide from neutrophils.

Discussion

This report provides the first demonstration that neutrophils can process bacteria for MHC-I presentation to T cells. Macrophages are also capable of alternate MHC-I Ag processing, but neutrophil preparations used in these experiments did not contain sufficient numbers of contaminating macrophages to explain the processing activity. Small numbers (20,000/well) of highly purified neutrophils produced detectable Ag presentation (with a maximum of 200-1400 contaminating macrophages/well, too few to produce detectable presentation). Thus, neutrophils actively process bacteria via alternate MHC-I processing mechanisms and directly present the resulting peptide-MHC-I complexes to T cells. Neutrophils are most likely limited to the presentation of MHC-I-restricted Ag, because they generally lack MHC-II molecules.
and CD8OVA1.3 T hybridoma cells (10^5/well). Secretion of IL-2 was measured with a colorimetric CTLL-2 bioassay as described in Fig. 3. Macrophages or dendritic cells (Mac at 10^5/well or DC at 10^4/well), incubated overnight with heat-killed HB101.Crl-OVA, previously fixed B6D2F1/J APCs (macrophages at 10^5/well or dendritic cells at 10^4/well), and CD8OVA1.3 T hybridoma cells (10^5/well), secreted IL-2 was measured with a colorimetric CTLL-2 bioassay as described in Fig. 3. A. Regurgitation of peptides by neutrophils for presentation by macrophages. B. Regurgitation of peptides by neutrophils for presentation by dendritic cells.

FIGURE 8. Neutrophils process Ag and regurgitate peptides that can be presented by MHC-I molecules on neighboring fixed macrophages or dendritic cells. To assess peptide regurgitation and delivery of neutrophil-derived peptides to other APCs, viable CBA/J neutrophils (10^7/well) were incubated overnight with heat-killed HB101.Crl-OVA, previously fixed B6D2F1/J APCs (macrophages at 10^5/well or dendritic cells at 10^4/well), and CD8OVA1.3 T hybridoma cells (10^5/well). Secretion of IL-2 was measured with a colorimetric CTLL-2 bioassay as described in Fig. 3. A. Regurgitation of peptides by neutrophils for presentation by macrophages. B. Regurgitation of peptides by neutrophils for presentation by dendritic cells.

(with some specific exceptions; Ref. 4–6). Previous studies have suggested that vacuolar alternate MHC-I Ag processing may involve binding of peptides to MHC-I molecules either within vacuolar compartments (e.g., phagosomes) or on the cell surface (following peptide regurgitation), and it is possible that both of these mechanisms occur. We observed that peptides were regurgitated by MHC-mismatched neutrophils and bound to MHC-I molecules on fixed macrophages or dendritic cells for presentation to T cells. This observation suggests two interesting conclusions. First, peptide regurgitation may contribute, at least in part, to generation of peptide-MHC-I complexes on neutrophils and direct presentation of Ag by neutrophils to T cells (“auto-regurgitation”). This does not exclude additional generation of peptide-MHC-I complexes within intracellular vacuolar compartments. Second, peptide regurgitation may allow neutrophils to deliver peptides to other APCs, e.g., macrophages and dendritic cells (“trans-regurgitation”). In this case, neutrophils would indirectly contribute to Ag presentation by performing the initial proteolytic steps of Ag processing, but the final steps of direct presentation to T cells would be mediated by professional APCs. Professional APCs have properties (life span, costimulator expression, and the ability to migrate to lymph nodes) that promote their ability to induce T cell responses. Thus, neutrophils may provide both direct and indirect contributions to presentation of Ag to T cells.

The indirect and direct contributions of neutrophils to Ag presentation may affect different stages of T cell responses. Neutrophils primarily encounter and phagocytose bacteria at peripheral (nonlymphoid) sites of infection and inflammation, and this tissue localization provides potential interaction with effector or memory T cells but little chance of interaction with naive T cells, which are primarily localized within lymphoid tissues. Furthermore, in agreement with other studies, we found that costimulatory molecules (CD80 or CD86) were not expressed or expressed at very low levels by neutrophils (data not shown), which limits their potential to activate naive T cells effectively. Therefore, direct Ag...
presentation by neutrophils is unlikely to impact on priming of T cell responses. Effector T cells may respond to direct Ag presentation by neutrophils to activate various effector mechanisms, including cytokine production, which may influence immune responses and host defense. Direct presentation of Ag by neutrophils may also contribute to the amplification of CD8 responses or the longevity of CD8 T cell memory.

In contrast, neutrophils may contribute indirectly to presentation of Ags for priming of T cell responses. Professional APCs such as macrophages and dendritic cells are often resident in tissues and may also migrate to sites of inflammation. Thus, neutrophils present at a site of acute infection or inflammation are likely to encounter both bacterial Ag and professional APCs. Peptide presentation by neutrophils, which are highly phagocytic and may be present in large numbers, may contribute substantially to formation of peptide-MHC-I complexes on nearby professional APCs. Dendritic cells in receipt of neutrophil-derived peptides may migrate to lymph nodes and present Ag to activate naive T cells, providing a mechanism for neutrophils to indirectly contribute to priming of T cell responses.

Neutrophils directly present bacterial Ags on MHC-I molecules but not MHC-II molecules, but one can consider the possibility that neutrophils contribute indirectly to MHC-II-restricted Ag presentation via regurgitation of peptides onto professional APCs. Stern and colleagues (32, 33) have recently suggested that peptide-MHC-II complexes may be formed on the surface of dendritic cells, although their system did not involve any contribution of neutrophils. Although previous studies have demonstrated that at least some MHC-I Ag presentation can result from peptide regurgitation (11, 31), peptide regurgitation has not been demonstrated at least some MHC-I Ag presentation can result from peptide regurgitation by neutrophils, which are highly phagocytic and may be present in large numbers, may contribute substantially to formation of peptide-MHC-I complexes on nearby professional APCs. Dendritic cells in receipt of neutrophil-derived peptides may migrate to lymph nodes and present Ag to activate naive T cells, providing a mechanism for neutrophils to indirectly contribute to priming of T cell responses.

Neutrophils may make unique contributions to processing of bacterial Ags. The high phagocytic capacity of neutrophils makes them a repository of bacterial Ags for processing. Direct or indirect contributions by neutrophils could allow enhanced presentation and generation of T cell responses to these Ags, which would otherwise be lost to the immune system. In addition, the extensive capacities of neutrophils for phagocytosis, microbicidal functions, and catabolism of phagocytosed material may make them uniquely capable of processing certain bacteria, especially pathogenic bacteria that are resistant to host defense mechanisms. These bacteria may be more efficiently killed and processed by neutrophils than by other cells (e.g., dendritic cells), which may have less developed microbicidal functions.

In summary, the results presented here provide the first demonstration that neutrophils can mediate alternate MHC-I Ag processing of bacterial Ags and their direct presentation to T cells. Neutrophils can also contribute to the formation of peptide-MHC-I complexes on other APCs, providing an indirect contribution to presentation of bacterial Ags. Although neutrophils were once thought to provide only microbialicidal function to innate immune defense, they are now appreciated to influence adaptive immunity, including T cell responses, via a number of mechanisms, including cytokine secretion, synthesis and expression of MHC molecules, and formation of peptide-MHC complexes.

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References