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Lipopolysaccharide Induces Rapid Production of IL-10 by Monocytes in the Presence of Apoptotic Neutrophils

Aideen Byrne* and Denis J. Reen†

There is growing evidence that apoptotic neutrophils have an active role to play in the regulation and resolution of inflammation following phagocytosis by macrophages and dendritic cells. However, their influence on activated blood monocytes, freshly recruited to sites of inflammation, has not been defined. In this work, we examined the effect of apoptotic neutrophils on cytokine production by LPS-activated monocytes. Monocytes stimulated with LPS in the presence of apoptotic neutrophils for 18 h elicited an immunosuppressive cytokine response, with enhanced IL-10 and TGF-β production and only minimal TNF-α and IL-1β cytokine production. Time-kinetic studies demonstrated that IL-10 production was markedly accelerated in the presence of apoptotic neutrophils, whereas there was a sustained reduction in the production of TNF-α and IL-1β. This suppression of proinflammatory production was not reversible by depletion of IL-10 or TGF-β or by addition of exogenous IFN-γ. It was demonstrated, using Transwell experiments, that monocyte-apoptotic cell contact was required for induction of the immunosuppressive monocyte response. The response of monocytes contrasted with that of human monocyte-derived macrophages in which there was a reduction in IL-10 production. We conclude from these data that interaction between activated monocytes and apoptotic neutrophils creates a unique response, which changes an activated monocyte from being a promoter of the inflammatory cascade into a cell primed to deactivate itself and other cells. 

Monocytes are recognized as key promoters of acute and chronic inflammatory responses (1–3). They are activated and attracted to sites of inflammation to augment and prolong the response to microbial stimulation initiated by tissue macrophages (2). Their initial response begins with the rapid production of TNF-α (3). This proinflammatory cytokine is the principal mediator of the inflammatory response. TNF-α induces the release of other cytokines (IL-1β, IL-6), eicosanoids, reactive oxygen species, and the activation of the complement and coagulation cascades (4). The second phase of the monocyte response involves the production of IL-10, a potent immunosuppressive cytokine that is detectable within 8 h of exposure to LPS (5). IL-10 down-regulates the production of the proinflammatory cytokines (6) and plays a role in the development of LPS tolerance (7) whereby, after initial stimulation with LPS, monocytes become tolerant to further LPS challenges (8). TGF-β is also released by activated monocytes. It is a pleiotropic cytokine with both pro- and anti-inflammatory properties (9). Its actions on activated cells of the monocyte-macrophage lineage are generally suppressive, including the down-regulation of TNF-α, IFN-γ, and monocyte chemoattractant protein-1 (MCP-1) (10). The balance between the production of pro- and anti-inflammatory cytokines by the monocyte determines the effectiveness of the inflammatory response (11). The cytokine profile of monocytes is recognized as a critical indicator of survival in septic patients, where a high IL-10/TNF-α ratio in febrile patients is associated with increased mortality (12).

Monocytes are also central to the pathogenesis of chronic inflammation (3, 13). Rheumatoid arthritis and inflammatory bowel disease are characterized by heightened expression, at the site of inflammation, of MCP-1 and MCP-3 (14, 15). This results in enhanced recruitment of monocytes from the bloodstream to the site of inflammation that generate a sustained inflammatory cascade, giving rise to tissue destruction (16). Inhibitors of monocyte-derived cytokines such as TNF-α, using blocking Abs, has been effective in the treatment of these conditions (17, 18).

Neutrophil apoptosis has been shown to impact on the inflammatory response in a number of ways (19). It regulates inflammation by reducing the number of cells capable of fuelling an inflammatory reaction. Apoptotic cells maintain their membrane integrity and are rapidly ingested by macrophages, thus preventing the release of toxic granules (20). Their uptake by phagocytes does not provoke the release of proinflammatory cytokines (21). In fact, uptake of apoptotic neutrophils by LPS-stimulated human monocyte-derived macrophages (HMDM) inhibits the production of TNF-α, IL-1β, and IL-10 through the induction of TGF-β (22). Although the effect of apoptotic neutrophils on HMDM has been explored extensively (19, 20, 23), their impact on cytokine production by activated blood monocytes has not as yet been defined. However, apoptotic neutrophils have been shown to produce a monocyte-specific chemotactic factor, thereby contributing to the recruitment of these proinflammatory cells to sites of inflammation (24).

Monocytes differ from mature macrophages in that they are unable to phagocytose apoptotic cells (25). Apoptotic lymphocytes have been shown to alter cytokine production in LPS-activated monocytes, including an augmentation of IL-10 (26). However, as apoptotic neutrophils and monocytes are considered to play such critical and yet opposing roles in determining the outcome of the inflammatory response, it was essential that the relationship between these two cell types be defined.
In this study we report that, through cell-cell contact, apoptotic neutrophils interact with monocytes, altering their primary response to LPS. This altered response is immunosuppressive in nature, with an accelerated and exaggerated release of IL-10, enhanced production of TGF-β, and only minimal production of the proinflammatory cytokines TNF-α and IL-1-β.

Materials and Methods

Reagents

IMDM and HBSS were purchased from BioWhittaker (Wokingham, U.K.). Human AB serum was purchased from Quest Biomedical (West Midlands, U.K.).

Human IFN-γ, human IL-10, anti-human IL-10 Ab, and anti-human TGF-β Ab were all purchased from R&D Systems (Oxon, U.K.). LPS (Escherichia coli serotype 055:B5) was obtained from Sigma-Aldrich (Dublin, Ireland). Anti-human CD16(FITC) and CD14(FITC) CD45(PE) Abs, and rabbit anti-human IgG (clone 2D1H30) IgG1 Ab were purchased from BD Biosciences (Oxford, U.K.). Rabbit anti-mouse IgG was purchased from Dako (Cambridgeshire, U.K.).

Every effort was made to ensure minimal contamination with LPS in our system. All steps in the neutrophil and monocyte purification protocols and culturing were conducted using certified nonpyrogenic plastics (Corning/Costar, Bucks, U.K.) and media, certified to have an endotoxin level of 0.005 endotoxin units (EU)/ml (BioWhittaker). All solutions were made up with endotoxin-free water (Nanopure, Barnstead, U.K.).

Monocyte and macrophage isolation

Blood was obtained from healthy adult volunteers and placed in sterile tubes containing PBS/0.1 M EDTA (4 ml/20 ml blood). It was diluted 1/1 with PBS. PBMC were separated by density centrifugation through Ficoll-Hypaque (Lymphoprep, Nycosmed, Oslo, Sweden), washed twice in cold IMDM, and resuspended in IMDM/10% human AB serum (3 × 10^6/ml). The mononuclear cell suspension was plated (1 ml/well) in 24-well plates (Corning/Costar, Cornning, NY). After incubation for 45 min at 37°C, the nonadherent cells were removed by washing with warm HBSS containing Ca^2+ and Mg^2+ (BioWhittaker). After a 15-min incubation at 4°C, monocytes were retrieved by vigorous washing of the plates with cold PBS. They were washed and resuspended in IMDM without serum (0.5 × 10^6/ml). Cell viability, by ethidium bromide and acridine orange staining, was >90%. Monocyte purity, determined using two-color flow cytometric analysis (CD14/CD45), was >90%. Human monocyte-derived macrophages were generated from adherence-purified monocytes (1 × 10^6/ml) cultured for 7 days in IMEM, 10% human AB serum, and 2 mM glutamine in 24-well culture plates. The medium was changed on day 3.

Induction of apoptosis in purified human neutrophils

Neutrophils were purified from whole blood by dextran sedimentation and centrifugation on a Percoll gradient as previously described (27). The purity of the neutrophil population, as assessed by single-color flow cytometric analysis of CD15 expression, was >90%. In some experiments fresh neutrophils were suspended in IMDM (5 × 10^6/ml) in the absence of serum directly after isolation and added to monocyte cultures (10/1).

For the purpose of inducing apoptosis, the neutrophils (2 × 10^6/ml) were suspended in IMDM (BioWhittaker) with 10% autologous serum and incubated for 24 h at 37°C in 5% CO2 to induce apoptosis. They were then harvested and washed twice with HBSS without Ca^2+ and Mg^2+ (Bio-Whittaker) and resuspended in IMDM (5 × 10^6/ml) in the absence of serum. Apoptosis at this time was >80% as determined by Annexin V positivity and loss of cell surface expression of CD16 (28) Cell viability, under UV illumination using ethidium bromide and acridine orange staining, was >90%. To opsonize apoptotic neutrophils, the apoptotic neutrophils were rotated end-to-end at 4°C with mouse anti-human CD45 Ab for 30 min. After three washes, the secondary Ab, rabbit anti-mouse IgG, was added for an additional 30 min. The cells were washed again and resuspended in IMDM (5 × 10^6/ml) without serum (22).

Coculture of monocytes and macrophages with apoptotic neutrophils

Purified monocytes in IMDM (0.25 × 10^6/ml) or HDM were stimulated with LPS (10 µg/ml) in the presence or the absence of apoptotic neutrophils or opsonized apoptotic neutrophils (2.5 × 10^6/ml) for 18 h in 24-well plates (Costar, Bucks, U.K.). Apoptotic cells were added at the same time as the stimulus. Supernatants were then recovered and centrifuged at 500 × g for 10 min at 4°C to remove particulate debris and were stored in aliquots at −70°C. In time-course experiments, supernatants were harvested at 2, 4, 6, 8, 10, and 18 h. In experiments comparing the effects of freshly isolated and apoptotic neutrophils, supernatants were harvested at 8 h. In some experiments, monocytes (0.25 × 10^6 in 1 ml) were separated from apoptotic neutrophils (2.5 × 10^6 in 0.5 ml) using a 0.4-µm pore size Transwell filter (Costar). To investigate the role played by IL-10 and TGF-β in this assay, anti-human IL-10 Ab (10 µg/ml) and anti-human TGF-β Ab (100 µg/ml) were added to cultures. In other experiments, IFN-γ was added (1–500 ng/ml) to the cultures.

Collection of supernatants from apoptotic neutrophil cultures

After 24 h in culture at 37°C, aged apoptotic neutrophils (3 × 10^6/ml) were incubated in serum-free IMEM with LPS (10 µg/ml) for 18 h at 37°C. The supernatants were then recovered, centrifuged at 500 × g for 10 min at 4°C, and then filtered through a 0.2-µm pore size filter to remove all particulate matter. They were then used to resuspend isolated monocytes.

These fresh monocytes (2 × 10^6/ml) were, in turn, incubated for 18 h at 37°C in the presence or the absence of LPS (10 µg/ml). Supernatants were harvested and stored as before.

Analysis of cytokines

Cytokine concentrations in the culture supernatants were determined by ELISA, using TNF-α-matched Ab pairs (R&D Systems), human TGF-β, and IL-1β (Quantikine kits; R&D Systems), and OptEIA human IL-10 sets (BD PharMingen, Oxford, U.K.). Assays were performed according to the instructions provided. Color development was assessed using a VERSAmax microplate reader (Biosciences, Dublin, Ireland).

Statistical analysis

The Mann-Whitney nonparametric test was performed using the software SPSS for Windows.

Results

Apoptotic neutrophils regulate pro- and anti-inflammatory cytokine production by LPS-activated monocytes

There is growing evidence that apoptotic neutrophils have an active role to play in the regulation and resolution of inflammation following phagocytosis by macrophages and dendritic cells (19, 29). However, their influence on activated blood monocytes, freshly recruited to sites of inflammation, has not been defined. Freshly isolated human monocytes (0.25 × 10^6/ml) were incubated with LPS (10 µg/ml) for 18 h in the presence or the absence of aged neutrophils (10/1), and the effect on cytokine production was measured. In the presence of apoptotic neutrophils, monocyte production of TNF-α and IL-1-β was reduced by 88 ± 7 and 86 ± 11%, respectively (Fig. 1, A and B). This inhibitory effect was not dependent on the concentration of LPS used (Fig. 1E). The production of IL-10 was increased 4-fold (Fig. 1C), and TGF-β production was increased 2-fold (Fig. 1D). It has been previously reported that apoptotic lymphocytes enhance IL-10 production in monocytes (26) and produce IL-10 themselves in response to LPS (30). In our system, neither IL-10 nor TGF-β was produced by the apoptotic neutrophils in either the presence or the absence of LPS (10 µg/ml), as measured by ELISA. In the absence of LPS stimulation, monocytes cultured in the presence of apoptotic neutrophils produced none of the four cytokines measured (Fig. 1, A–D).

Time course of cytokine production by monocytes in the presence of apoptotic neutrophils

The kinetics of cytokine production by LPS-stimulated monocytes in the presence of apoptotic neutrophils over 18 h were studied. In the presence of LPS (10 µg/ml), TNF-α and IL-1-β were detectable (78 ± 26 and 479 ± 99 pg/ml) by ELISA 4 h poststimulation (Fig. 2). These levels increased to 1585 ± 163 and 2854 ± 720 pg/ml, respectively, by 18 h. In the presence of apoptotic neutrophils, TNF-α was also detectable (39 ± 17 pg/ml) at 4 h poststimulation (Fig. 2A). However, at each time point after 6 h there was significantly less TNF-α produced than in cells incubated with LPS.
alone, with a maximum level of only 316 ± 100 pg/ml present at 10 h poststimulation. In the presence of apoptotic neutrophils there was a significant reduction in IL-1β levels at each time point (4–18 h inclusive), with a maximum level of 1070 ± 287 pg/ml recorded at 18 h poststimulation (Fig. 2B).

LPS stimulation induced IL-10 (10 ± 3 pg/ml) release by 8 h, which increased to 140 ± 16 pg/ml by 18 h. In contrast, IL-10 release was markedly enhanced in the presence of apoptotic neutrophils (20 ± 13 pg/ml) at 4 h. Actual production was significantly greater than with LPS stimulation alone at each time point to a maximum of 306 ± 39 pg/ml at 18 h (Fig. 2C).

The effect on cytokine production is specific to apoptotic neutrophils

To confirm that the pattern of cytokine change seen was the result of exposure to apoptotic neutrophils and not neutrophils, the effects of fresh and opsonized neutrophils on LPS-stimulated monocytes were examined. Monocytes (0.25 × 10⁶/ml) were stimulated with LPS (10 μg/ml) and incubated with freshly isolated or apoptotic neutrophils (2.5 × 10⁶/ml) for 8 h. This time point was chosen to minimize neutrophil death in the fresh neutrophil cultures. At 8 h the fresh neutrophil population was <15% Annexin positive. It had been established that the effects of apoptotic
neutrophils on cytokine production are apparent at this time point (Fig. 2). In the presence of apoptotic neutrophils, monocytes were activated with LPS in the presence of apoptotic neutrophils. Supernatants were harvested at 2, 4, 6, 8, 10, and 18 h. The data shown are the mean of five experiments ± SEM. *, There is a significant decrease in cytokine production vs that during culture with LPS alone (p < 0.005). **, There is a significant increase in cytokine production vs that during culture with LPS alone (p < 0.005).

Monocytes (0.25 × 10^6/ml) were stimulated with LPS (10 μg/ml) and incubated with apoptotic or opsonized apoptotic neutrophils (2.5 × 10^6/ml) for 18 h. TNF-α and IL-1β production were unaffected by the presence of opsonized apoptotic neutrophils. IL-10 production was increased from 342 ± 10 to 696 ± 203 pg/ml in the presence of apoptotic neutrophils and further increased to 1101 ± 203 pg/ml in the presence of opsonized neutrophils (Table II). This up-regulation of IL-10 by opsonized cells is consistent with published data (22, 31). These data collectively demonstrate that the induction of reduced proinflammatory cytokine production by monocytes coupled with the promotion of augmented IL-10 production are specific to apoptotic neutrophils.

The immunosuppressive effect of apoptotic neutrophils on monocytes is contact-dependent

The effect of apoptotic neutrophils on macrophage cytokine production occurs in association with phagocytosis by the macrophages. However, the part played by surface contact and actual phagocytosis has not been fully elucidated. Monocytes do not phagocyte apoptotic neutrophils (25), but do express a number of receptors capable of recognizing and binding them, including CD36 (32) and CD14 (33). We hypothesized that cell-cell contact was necessary and sufficient to generate the cytokine changes inducible in monocytes by apoptotic neutrophils. After overnight aging, apoptotic neutrophils (3 × 10^6/ml) were cultured in the
The inhibition of proinflammatory cytokine production is not mediated by either TGF-β or IL-10

IL-10 is a major inhibitor of TNF-α and IL-1β production (5). However, it has been previously shown in other models that down-regulation of TNF-α can be independent of a simultaneous increase in IL-10 production (34, 35). To explore whether the augmented monocyte-derived IL-10 output was responsible for the down-regulation of their proinflammatory cytokine production, monocytes were incubated in the presence or the absence of apoptotic neutrophils with anti-IL-10 (10 μg/ml).

In response to LPS stimulation, IL-1β production was 1132 ± 165 pg/ml (Fig. 4A). TNF-α production was 263 ± 55 pg/ml (Fig. 4B). In the presence of apoptotic neutrophils, IL-1β production was reduced to 162 ± 100 pg/ml (Fig. 4A). TNF-α production was reduced to 16 ± 3 pg/ml (Fig. 4B). These figures were only minimally increased with the addition of anti-IL-10 to 328 ± 101 and 61 ± 40 pg/ml. It has previously been shown that inhibition of proinflammatory cytokine production in the macrophage can be reversed using anti-TGF-β Ab (22). We explored whether this could be responsible for the reduction in proinflammatory cytokine output in the monocyte system. Monocytes were incubated in the presence or the absence of apoptotic neutrophils with anti-TGF-β Ab (100 μg/ml). In the presence of Ab, LPS induced production of TNF-α and IL-1β and increased from 310 ± 60 and 1444 ± 100 pg/ml to 527 ± 60 and 1998 ± 120 pg/ml, respectively. In the presence of apoptotic neutrophils, IL-1β production was minimally increased by the addition of anti-TGF-β Ab from 250 ± 20 to 541 ± 30 pg/ml. TNF-α production in the presence of apoptotic neutrophils was 58 ± 18 pg/ml, and this was unchanged by the addition of anti-TGF-β Ab (58.5 ± 22 pg/ml). These results indicate that the down-regulation of proinflammatory cytokines enforced by apoptotic neutrophils is independent of the induction of IL-10 or TGF-β.

The inhibitory effect of apoptotic neutrophils on the production of proinflammatory cytokines cannot be reversed by IFN-γ

Exposure to endotoxin leads to the secondary down-regulation of a number of LPS-driven responses, including the production of TNF-α. This phenomenon is called LPS tolerance. This repression of proinflammatory cytokine production can be reversed by the addition of IFN-γ (36). To examine the effect of IFN-γ on the down-regulation of TNF-α production induced by apoptotic neutrophils, monocytes were incubated with IFN-γ (1–500 ng/ml) alone or in combination with LPS (10 μg/ml) in the presence or the absence of apoptotic neutrophils. In the absence of apoptotic neutrophils, IFN-γ enhanced TNF-α production in a dose-dependent fashion, both alone (Fig. 5B) and in combination with LPS (Fig. 5A) to maximum levels of 1961 ± 180 and 2184 ± 388 pg/ml, respectively. This was in accordance with previous reports. However, IFN-γ was unable to reverse the suppression of LPS-driven TNF-α production induced by apoptotic neutrophils (Fig. 5A). Monocytes activated by IFN-γ alone also down-regulate their TNF-α production in the presence of apoptotic neutrophils (Fig. 5B). There was a small, dose-dependent increase in output when IFN-γ was added in combination with LPS (10 μg/ml), but only to a maximum of 376 ± 65 pg/ml. This was consistent with the small, dose-dependent increase in TNF-α production stimulated by

| Table I. Fresh neutrophils do not alter cytokine production in LPS-stimulated monocytes

<table>
<thead>
<tr>
<th></th>
<th>TNF-α (pg/ml)</th>
<th>IL-1β (pg/ml)</th>
<th>IL-10 (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>16 ± 2</td>
<td>10 ± 3</td>
<td>12 ± 2</td>
</tr>
<tr>
<td>LPS</td>
<td>711 ± 27</td>
<td>1870 ± 107</td>
<td>64 ± 37</td>
</tr>
<tr>
<td>LPS + Apop. Neutrophils</td>
<td>208 ± 100</td>
<td>1108 ± 229b</td>
<td>164 ± 16*b</td>
</tr>
<tr>
<td>LPS + Fresh Neutrophils</td>
<td>814 ± 210</td>
<td>1816 ± 150</td>
<td>77 ± 27</td>
</tr>
</tbody>
</table>

* LPS-stimulated monocytes were incubated in the presence of apoptotic or fresh neutrophils for 8 hr. TNF-α and IL-1β production was significantly decreased in the presence of apoptotic neutrophils, but remained unchanged in the presence of fresh neutrophils. IL-10 production was increased only in the presence of apoptotic neutrophils. Data represents the mean of four experiments ± SEM.

| Table II. Opsonized apoptotic neutrophils and apoptotic neutrophils exert different effects on cytokine production by LPS-stimulated monocytes

<table>
<thead>
<tr>
<th></th>
<th>TNF-α (pg/ml)</th>
<th>IL-1β (pg/ml)</th>
<th>IL-10 (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>15 ± 1</td>
<td>13.9 ± 7</td>
<td>7.8 ± 1</td>
</tr>
<tr>
<td>LPS</td>
<td>1559 ± 250</td>
<td>5555 ± 541</td>
<td>342 ± 10</td>
</tr>
<tr>
<td>LPS + Apop. Neutrophils</td>
<td>193 ± 12a</td>
<td>1102 ± 330ab</td>
<td>696 ± 79b</td>
</tr>
<tr>
<td>LPS + Opsonized Apop.</td>
<td>1110 ± 300</td>
<td>5264 ± 300</td>
<td>1101 ± 203d</td>
</tr>
</tbody>
</table>

* LPS-stimulated monocytes were incubated in the presence of apoptotic or opsonized apoptotic neutrophils for 18 hr. TNF-α and IL-1β production was significantly decreased in the presence of apoptotic neutrophils, but remained unchanged in the presence of opsonized apoptotic neutrophils. IL-10 production was increased by apoptotic neutrophils and significantly more by opsonized apoptotic neutrophils. Data represents the mean of four experiments ± SEM.

* There is a significant decrease in cytokine production vs stimulation with LPS alone (p < 0.05).

* There is a significant decrease in cytokine production vs stimulation with LPS alone (p < 0.05).

* There is a significant increase in IL-10 production than with apoptotic neutrophils (p < 0.05).
dution in IL-10 production (69 ± 18%) by the macrophages, as had been reported previously (Fig. 6B) (22).

**Discussion**

Balance between pro- and anti-inflammatory mediators is critical in preventing the innate immune response from becoming destructive to the host and in promoting resolution and repair (39). Here we describe a previously unrecognized immunosuppressive mechanism that can inhibit the proinflammatory drive and maintain homeostasis. We show that monocytes activated by LPS, while simultaneously contacting with apoptotic neutrophils, are early releasers of IL-10. This increased production of IL-10 is sustained over an 18-h period along with minimal release of TNF-α and IL-1β throughout that time.

The induction kinetics of proinflammatory genes differ from those of the IL-10 gene due to fundamental differences in molecular regulation (40, 41). As a result, LPS stimulation results in rapid release of TNF-α and IL-1β, whereas release of IL-10 is delayed (5). In the past, LPS-activated monocytes have been manipulated through the use of β-agonists (42), antioxidants (43), and adenosine receptor agonists (44, 45), resulting in reduced production of proinflammatory cytokines and augmented IL-10 production. We have shown that contact with apoptotic neutrophils has a similar quantitative effect (Fig. 1). However, unlike previous models of monocyte modulation (5, 42, 44), apoptotic neutrophils also induce an altered kinetic response to LPS stimulation. After stimulation with LPS in our model, monocytes produce IL-10, detectable by ELISA, after 8–10 h. However, in the presence of apoptotic neutrophils, IL-10 is detectable as early as 4 h after stimulation (Fig. 2C). Monocytes are traditionally seen as promoters of the inflammatory cascade (46, 47). We show that by becoming primarily IL-10-producing cells, through contact with apoptotic neutrophils, they also have a role as suppressors of inflammation.

We have also shown that in this model activated monocytes augment their release of TGF-β. This extends the immunosuppressive phenotype that they achieve through interaction with apoptotic neutrophils. TGF-β not only inhibits release of proinflammatory cytokines, but also promotes tissue repair through recruitment of fibroblasts and the promotion of matrix deposition, including fibronectin and collagen (10, 48).

It might be argued that these monocytes could be described as endotoxin tolerant due to their suppressed proinflammatory response to LPS stimulation. Endotoxin-tolerant cells produce only minimal levels of proinflammatory cytokines upon LPS stimulation and normal or increased amounts of IL-10 (36, 49). However, tolerance is defined as a failure to respond to secondary challenge after primary stimulation with LPS (50). In our model apoptotic cells and monocytes are added to the culture plates simultaneously and then stimulated with LPS. Therefore, this work is not a model of LPS tolerance, but instead demonstrates that in the presence of apoptotic neutrophils, monocytes on exposure to LPS become IL-10-producing, immunosuppressive cells.

LPS-tolerant cells can be triggered to produce TNF-α when IFN-γ is added to the culture (36, 51). Our work shows that, in contrast, the suppressive effect of apoptotic neutrophils on TNF-α production cannot be reversed by IFN-γ (Fig. 5A). IFN-γ induction of TNF-α was also inhibited by the presence of apoptotic neutrophils, demonstrating that the inhibitory mechanism is not specific to LPS stimulation alone (Fig. 5B).

IL-10 is recognized as a major deactivating cytokine, capable of inhibiting the production of TNF-α and IL-1β (6). Here we report that the inhibition of TNF-α and IL-1β is not reversed by the depletion of IL-10 (Fig. 4). This pattern of down-regulation of

**FIGURE 3.** The inhibitory effect of apoptotic neutrophils on TNF-α production and its enhancing effect on IL-10 production are cell contact dependent. A, Apoptotic neutrophils (3 × 10^9/ml) were cultured in the presence of LPS (10 μg/ml) for 18 h. Supernatants were harvested from these cultures and added to monocytes (0.25 × 10^9/ml) for an additional 18 h in the presence of LPS (10 μg/ml). The data shown are the mean of three experiments ± SEM (p < 0.05). B, LPS-activated monocytes were separated from apoptotic neutrophils using 0.4-μm pore size Transwell filters. The data shown are the mean of five experiments ± SEM (p < 0.005). *, There is a significant decrease in cytokine production vs that during culture with LPS alone. **, There is a significant increase in cytokine production vs that during culture with LPS alone.

**Apoptotic neutrophils have a contrasting effect on LPS-activated monocytes and macrophages**

It has been suggested that contrasting reports of cytokine profiles in the presence of apoptotic neutrophils were due to differences in the models used (37). Following the same model system as before, apoptotic neutrophils (2.5 × 10^9/ml) were incubated with 7-day-old monocyte-derived macrophages (0.25 × 10^9/ml) and stimulated with LPS (10 μg/ml) for 18 h. Phagocytosis of the apoptotic cells by macrophages was confirmed by staining for myeloperoxidase activity (38). As with the monocyte model, we observed a reduction in TNF-α (71 ± 13%; Fig. 6A) and a 2-fold increase in TGF-β release (Fig. 6C) in the presence of apoptotic neutrophils. However, in contrast to the monocyte model, we observed a re-
TNF-α, independent of IL-10, has been demonstrated before using acetylcholine to alter cytokine production (52). Similarly, the inhibitory effect of cAMP-elevating drugs on TNF-α production is independent of their elevating effect on IL-10 levels (53).

TGF-β is a pleiotropic cytokine that has a contrasting effect on resting and activated cells of the innate immune system (48, 54). We have shown that LPS-induced TNF-α and IL-1β production can be augmented in the presence of anti-TGF-β Ab, but that, in contrast, the inhibitory effect of apoptotic neutrophils on these cytokines is not reversed (Fig. 4, C and D). The dampening of TNF-α production is mediated through numerous, mechanically distinct, mechanisms involving diverse, intracellular signaling pathways (55–57). The particular mechanism in our model remains to be elucidated.

We applied our model to HMDM and showed that, in contrast to monocytes, these cells down-regulate their production not only of TNF-α but also of IL-10, while increasing their production of TGF-β (Fig. 6). This is consistent with previous work by Fadok et al. (22). Therefore, using the same experimental conditions we have shown that monocytes and macrophages differentially alter their cytokine profile in the presence of apoptotic neutrophils. This demonstrates that monocytes are not just precursor macrophages, but that they have an independent role in inflammation. The differential cytokine output from monocytes and macrophages in the presence of apoptotic neutrophils may be due to an alternative signaling mechanism. We hypothesize that signaling occurs downstream of a receptor ligand mechanism. We have shown that contact between monocytes and apoptotic neutrophils is necessary for a change in cytokine profile to occur. In the macrophage, ligation of the phosphatidyserine receptor using mab217 creates a similar cytokine response to apoptotic neutrophils (58). Monocytes do not express the phosphatidyserine receptor (58).

There are, however, a multitude of other receptors involved in the tethering of apoptotic neutrophils by macrophages (CD36 (32), CD14 (33), vitronectin (38), SR-BI, scavenger receptor A (59), CD91 (60), and CD68 (19)) with an undefined role in cytokine regulation in the presence of apoptotic neutrophils. A number of these are expressed on the monocyte, including CD36, CD14, CD68, and CD91. Interestingly, both ourselves and Voll (26) have shown that a reduction in TNF-α and a simultaneous increase in IL-10 can be achieved through the ligation of the CD36 receptor (data not shown), while Fadok (37) reports that this is not the case in the macrophage. Monocytes do not phagocytose apoptotic cells, unlike macrophages (25, 61). However, an actual role for phagocytosis of apoptotic neutrophils, independent of surface ligation, has not been defined. In the macrophage model it has been shown that the critical structure required from an apoptotic neutrophil to generate an anti-inflammatory signal is phosphatidyserine-containing membrane (62).

We have shown that in the presence of fresh neutrophils, which do not express phosphatidyserine on their surface, there is no inhibition of proinflammatory cytokine production. Similarly, in the presence of opsonized apoptotic neutrophils, which would trigger the CD16 receptor, we show that LPS stimulation drives a proinflammatory response. This is a previously reported phenomenon of CD16 ligation (22, 31).

This is the first report to define the interaction between apoptotic neutrophils and blood monocytes. Voll et al. (26) showed that in the presence of apoptotic lymphocytes, monocytes also enhanced production of IL-10 at 18 h coupled with reduced proinflammatory cytokine production. Interestingly, it has been shown that in sepsis, apoptosis of lymphocytes is greatly enhanced (63, 64), whereas apoptosis of neutrophils is delayed (65, 66). This may reflect a mechanism for maintaining a balance between the two cell types as

![FIGURE 4](http://www.jimmunol.org/). The inhibition of proinflammatory cytokine production by apoptotic neutrophils after 24-h incubation is independent of the increase in IL-10 production or TGF-β production. The inhibition of IL-1β and TNF-α is not abolished in the presence of anti-IL-10 (10 μg/ml; A and B) or in the presence of anti-TGF-β Ab (100 μg/ml; C and D). The data shown are the mean of three experiments ± SEM. * There is a significant decrease in cytokine production vs that during stimulation with LPS alone (p < 0.05). ** There is a significant increase in cytokine production vs that during stimulation with LPS alone (p < 0.05).
a way of regulating cytokine production (67). It has been suggested that inhibition of lymphocyte apoptosis might be an effective therapeutic target in sepsis (68, 69). However, it would now appear that a fuller understanding is needed of the specific role played by each of these two apoptotic cell types in the regulation of IL-10 and proinflammatory cytokine production before targeting one alone (70).

Sepsis occurs when there is an overwhelming inflammatory response to microbial invasion (4). Sepsis is now known to have two stages (11). The first stage is dominated by a proinflammatory drive. The second phase is associated with high levels of IL-10. This is the time when patients are particularly vulnerable to opportunistic infection (71). IL-10 levels have been demonstrated as sensitive indicators of high mortality risk (39).

To date, apoptotic cells have been described as playing a role in the resolution of inflammation (22). Our work suggests that their role is more complex and that it is possible that in sepsis, through their effects on monocytes, they may contribute to the high levels of IL-10 described, which often lead to life-threatening immunosuppression.

The modulation of activated monocytes into IL-10-producing cells by apoptotic neutrophils may also have significance to the treatment of chronic inflammatory conditions such as rheumatoid
In conclusion, we have shown that after contact with apoptotic neutrophils, monocytes no longer act as promoters of the inflammatory cascade. Instead, they generate a unique anti-inflammatory response to LPS stimulation characterized by accelerated release of IL-10 and enhanced production of TGF-β. It is likely that this is evidence of a previously unrecognized mechanism through which inflammation is regulated that has implications for the treatment of both acute and chronic inflammatory conditions.

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References


