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Apoptotic Cells and Innate Immune Stimuli Combine to Regulate Macrophage Cytokine Secretion

Mark Lucas, Lynda M. Stuart, John Savill, and Adam Lacy-Hulbert

Macrophage interactions with apoptotic cells can suppress inflammatory responses, but cell death by apoptosis may also trigger inflammation. We now report that murine macrophages exposed to the combination of apoptotic cells and archetypal ligands for Toll-like receptors (TLRs) 2, 4, and 9 mount cytokine responses that differ importantly from those elicited by either class of stimulus alone. TLR ligands induced early and sustained secretion of TNF-α, macrophage-inflammation protein (MIP) 1α and MIP-2 with later secretion of IL-10, IL-12, and TGF-β1; apoptotic cells alone stimulated late TGF-β1 secretion only. The combination of apoptotic cells and TLR ligands enhanced early secretion of TNF-α, MIP-1α, and MIP-2 and increased late TGF-β1 secretion, while suppressing late TNF-α, IL-10, and IL-12 by mechanisms which could nevertheless be overridden by IFN-γ. We propose that this combinational macrophage cytokine response to apoptotic cells and TLR ligands may contribute to recruitment and activation of innate immune defense when cell death occurs at infected inflamed sites while promoting later resolution with diminished engagement of adaptive immunity. The Journal of Immunology, 2003, 171: 2610–2615.

Inflammation and tissue remodeling are highly dynamic processes, the kinetics of which are poorly understood. Essential to both is activation of local cells and, later, the recruitment of specialized effector cells such as macrophages and other cells of the innate immune system (1). How pathogens are able to orchestrate these processes is fairly well defined; via pathogen-associated molecular pattern recognition receptors, infectious agents stimulate both proinflammatory cytokine production and chemokine release (2, 3). However, little is known of how anti-inflammatory signals from endogenous products found in inflamed sites are integrated with stimulators of the innate immune system (1). How pathogens are able to orchestrate these processes is fairly well defined; via pathogen-associated molecular pattern recognition receptors, infectious agents stimulate both proinflammatory cytokine production and chemokine release (2, 3). However, little is known of how anti-inflammatory signals from endogenous products found in inflamed sites are integrated with stimulators of the innate immune system during inflammation.

Apoptosis, or programmed cell death, occurs at high rates in infected, inflamed, or remodeling tissue. Free apoptotic cells (ac) are rarely found in tissue, even during inflammation, as they are rapidly removed by either local nonprofessional phagocytes or specialized phagocytes such as macrophages, and this clearance is believed to have significant regulatory effects upon inflammatory and immune responses (4). It has recently been shown that ac death may play an important role in the resolution phase of inflammation, switching off production of proinflammatory mediators and stimulating production of anti-inflammatory cytokines such as TGF-β from recruited macrophages that have internalized ac (5, 6). The involvement of receptors of the innate immune system (CD14, integrin αmβ2, SRA, CD36) in clearance of ac suggests there could be subtle interactions between the latter and engagement of innate immune responses. Such subtleties could explain apparently contradictory data on deliberate administration of ac in vivo, where both suppression and stimulation of the acute inflammatory response have been reported (6, 7).

In this study, we demonstrate that signals generated by ac are integrated with signals from LPS to promote macrophage responses that are qualitatively different from responses to stimuli given individually. Apoptotic cells possessed the capacity to activate macrophages for rapid and augmented, but temporally limited, secretion of proinflammatory mediators when coadministered with a number of innate immune stimuli; in the case of LPS this required Toll-like receptor (TLR) 4 function. Apoptotic cellderived macrophages later down-regulated proinflammatory responses, as has been demonstrated previously. Apoptotic cell death may therefore play an important role in early events in inflammation triggered by innate immune stimuli derived from invading bacteria or tissue damage. We postulate that this early proinflammatory response contributes to the swift recruitment and arming of innate immune cells when cell death occurs at an inflamed site. Furthermore, our data demonstrate how clearance of ac may add “value” to innate immune responses, broadening the repertoire of macrophage cytokine secretion triggered by constituents of microorganisms.

Materials and Methods

Mice

BALB/c and C3H/HeJ mice were purchased from B & K Universal (Hull, U.K.) and maintained in specific pathogen-free conditions at the University of Edinburgh. Housing and animal procedures were approved by the U.K. Government Home Office. Bone marrow-derived macrophages were prepared from 8- to 12-wk-old mice.

Macrophage culture

Macrophages were cultured as described previously (8–10). Briefly, for mouse bone marrow-derived macrophages, femurs from BALB/c or C3H/HeJ were removed, cleaned, and bone marrow flushed through with DMEM supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10% heat-inactivated FBS, and 10% conditioned supernatant from L929 cells, which was changed on day 2 and every 3 days subsequently. Macrophages were used between days 7 and 10 of culture.

For human monocyte-derived macrophages, mononuclear cells were isolated from the blood of normal donors and plated at 4 × 10⁶ cells/well in 24-well tissue culture plates. Lymphocytes were removed by washing after culture for 1 h. Monocytes were cultured for a further 5 days in DMEM supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% autologous platelet-rich plasma-derived serum.

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Generation of ac

Human neutrophils were extracted from peripheral blood of healthy volunteers as described previously (11). Briefly, blood was separated using dextran sedimentation and a Percoll gradient. This yielded highly pure human neutrophils (>95%), which were allowed to undergo constitutive apoptosis by aging overnight in Iscove’s modified DMEM with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% autologous platelet-rich plasma-derived serum. After this period, the cells were routinely 60–80% apoptotic, assessed by cytopathic morphology. No significant necrosis was detected, as assessed by trypan blue exclusion (<1% positive). Aged thymocytes were used as an alternative source of ac. Briefly, whole mouse thymus was removed and disaggregated in RPMI 1640 supplemented with 1% FBS to yield a single-cell suspension. Thymocytes were aged overnight in Iscove’s basal medium (90% v/v) and supernatants were harvested for cytokine analysis by ELISA (using duoset kits; R&D Systems, Minneapolis, MN). Alternatively, macrophages were washed as above, cultured with ac for 2 h, then re-washed to remove noningested cells before stimulation with LPS, SAC, or CpG DNA (1 μg/ml; gift from Prof. D. Gray, CAPB, University of Edinburgh, Edinburgh, U.K.). All conditions were set up in triplicate and repeated on at least three separate occasions.

Results

Apopotic cells and LPS in combination stimulate a pattern of cytokine response distinct from individual stimuli

Recent studies have suggested that the induction of apoptosis may be associated with inflammatory responses in vivo (7). To investigate this phenomenon, we followed the response of macrophages to ac and LPS in vitro. We prepared mouse bone marrow-derived macrophages, stimulated them with human apoptotic neutrophils in the presence or absence of 0.5 μg/ml LPS, harvested cell supernatants over a 24-h period, and determined cytokine levels by ELISA. As previously described in this well-established model (4, 12), human apoptotic neutrophils did not stimulate proinflammatory responses (no increase in TNF-α, IL-10, IL-12, macrophage-inflammatory protein (MIP) 1α, or MIP-2 over control unstimulated cells was seen; data not shown). However, when macrophages were also stimulated with LPS, we saw substantial release of these cytokines and chemokines (Figs. 1 and 2).

The pattern of cytokine response was significantly different from that produced by macrophages stimulated with LPS alone. Doubly stimulated macrophages (i.e., exposed to ac and LPS) released significantly more of the proinflammatory cytokine TNF-α over the first 2–4 h following LPS stimulation (Fig. 1A). However, after 24 h, no TNF-α could be detected in doubly stimulated macrophage culture supernatants, whereas sustained TNF-α production occurred in cultures of macrophages treated with LPS alone. We therefore examined the kinetics of LPS-induced TNF-α release. Supernatants from macrophages stimulated with ac and LPS or LPS alone were harvested after discrete time intervals and replaced with fresh medium (Fig. 1B). LPS stimulation alone caused a steady release of TNF-α over 24 h. However, macrophages that were stimulated with LPS following ingestion of ac showed an initial release of TNF-α over the first 4 h, which had fallen to undetectable levels by 8–24 h. Therefore, ac and LPS in combination selectively engaged an early inflammatory response in macrophages, limiting TNF-α production to a 4- to 6-h period following LPS stimulation.

We confirmed that LPS was stimulating the macrophages rather than the ac preparation to produce cytokines because apoptotic human neutrophils produced no detectable TNF-α when treated with LPS for 24 h (data not shown). Furthermore, the same pattern of TNF-α production was seen when macrophages were incubated with human apoptotic neutrophils for 2 h and extensively washed before stimulation with LPS, conditions under which ac were not exposed to LPS (Fig. 1C).

To determine whether this pattern of cytokine production was shared with other LPS-induced factors, we measured levels of chemokines MIP-1α and MIP-2 produced by macrophages stimulated with ac and LPS or with either stimulus alone. Macrophages treated with ac alone produced no detectable MIP-1α or MIP-2 (data not shown). However, treatment with LPS stimulated rapid release of both chemokines (Fig. 2) and coadministration of ac with LPS increased early (2–4 h) release of MIP-1α in keeping with the results observed for TNF-α.
with effects on TNF-α secretion but had no effect on MIP-2. Furthermore, unlike TNF-α secretion, later (8–24 h) release of MIP-1α and MIP-2 stimulated by LPS was unaffected by coadministration of ac. LPS stimulated the production of the immunoregulatory cytokines IL-10 and IL-12 by macrophages (Fig. 2B). Apoptotic cells alone did not stimulate production of either cytokine by macrophages (data not shown), and when LPS and ac were coadministered, macrophages produced no detectable IL-10 or IL-12p40 (Fig. 2B), demonstrating that ac were also able to block completely the production of these cytokines in response to LPS, in keeping with late effects on TNF-α secretion.

The altered cytokine profiles (augmented early TNF-α production, inhibited IL-12 production, and unchanged MIP-1α production) were independent of the source or species of ac or macrophages, as the same effects were seen with LPS treatment of mouse bone marrow-derived macrophages incubated with apoptotic mouse thymocytes (Fig. 3) or human monocyte-derived macrophages incubated with apoptotic human neutrophils (data not shown).

In summary, ac and LPS in combination selectively engaged an early inflammatory response in macrophages, producing chemokines, limiting increased TNF-α production to a 4- to 6-h period following LPS stimulation, and preventing production of IL-10 or IL-12.

Apoptotic cell-induced macrophage TGF-β1 release does not block early LPS-driven TNF-α secretion

The only cytokine among those examined that we could detect following stimulation of macrophages with ac alone was the anti-inflammatory cytokine TGF-β1 (Fig. 4). Low levels of TGF-β1 were produced soon after interaction with ac, but high levels were seen after 8–24 h. TGF-β1 was also produced after treatment of macrophages with LPS, although at lower levels than stimulation with ac and LPS.

It has been reported that the inhibition of proinflammatory cytokine production by human macrophages following ac ingestion is due to paracrine suppressive actions of TGF-β1, released after stimulation of the phosphatidylserine receptor by ac (5, 6). In earlier work, we used soluble TGF-β receptor to confirm the predominant role for ac-directed release of TGF-β1 in late suppression of TNF-α secretion triggered by LPS stimulation of murine bone marrow-derived macrophages (13). Interestingly, in our current studies, the timing of TNF-α down-regulation (after 6 h) correlated with the induction of secretion of TGF-β1. Therefore, it appeared possible that the inhibition of TNF-α secretion following interaction with ac required a “threshold” concentration of TGF-β1 to be
potent inflammatory stimulus. We found that macrophages treated with IFN-γ and ac retained early enhancement of TNF-α secretion when exposed to ac, but failed to exhibit late suppression (Fig. 5). This demonstrated that the apparently robust macrophage anti-inflammatory phenotype induced 24 h after exposure to ac combined with LPS could nevertheless be modulated by a powerful inflammatory stimulus such as IFN-γ.

The combinatorial cytokine response to LPS and ac requires functional TLR4 but can be elicited by other TLR ligands

The identification of the LPS receptor CD14 as a potential receptor for ac (14) suggested to us that the combinatorial effects of LPS and ac might not be shared with other innate immune-activating agents. We therefore stimulated macrophages with other proinflammatory stimuli that do not require CD14 as a coreceptor, after treatment with ac. SAC and CpG DNA, major ligands for TLR2 and TLR9, respectively, produced broadly similar results to LPS, with early TNF-α release enhanced by coadministration of ac.
which nevertheless suppressed TNF-α release at 24 h (Fig. 6). These data indicated that modulation of interaction between CD14 and TLR4 was not required for these effects. However, when we incubated macrophages from C3H/HeJ mice, which are deficient in TLR4 signaling, with ac and then LPS, we observed no significant release of TNF-α (Table I), confirming that functional macrophage TLR4 was essential for the combinatorial response to LPS and ac. Importantly, incubation of C3H/HeJ macrophages with ac and a TLR2 ligand (SAC) demonstrated that early stimulation and late suppression of TNF-α secretion could be elicited in such macrophages (Table I). These experiments also confirmed that the combined effects of LPS and ac on macrophages were not due to factors released by ac in response to LPS, but were due to a combination of LPS-derived and ac-derived signals integrated within macrophages. Furthermore, the data also excluded the unlikely possibilities that increases in early TNF-α secretion were due to artifactual stimulation of macrophages by undetected necrotic cells through TLR2 (15), or that the events detected were selectively driven via stimulation of TLR4 by heat shock proteins released from dying cells rather than by LPS (16).

**Discussion**

Macrophages remove ac in healthy tissues, but this role is greatly increased in inflammatory and immune responses. The responses of macrophages to this process are tightly regulated. Internalization and/or binding of ac has been shown to down-regulate the response of macrophages (5, 17), monocytes (18, 19), and dendritic cells (13) to microbial stimuli. By providing an attractive mechanism for control of the innate and adaptive immune systems, this interesting phenomenon has become a recent focus of investigation. In this study, we demonstrate that following ac interaction, macrophages engaged an altered inflammatory program when stimulated with LPS. Specifically, macrophages incubated with ac produced increased amounts of TNF-α and the chemokine MIP-1α immediately following LPS stimulation compared with macrophages stimulated with LPS alone. After 24 h, such doubly stimulated macrophages continued to produce MIP-1α and MIP-2, but no TNF-α was produced and increased levels of TGF-β1 could be detected. LPS-induced production of IL-10 and IL-12 was completely blocked by incubation of macrophages with ac. Similar results were seen with other innate stimuli (CpG DNA or SAC) but not when macrophages were stimulated with IFN-γ. Thus, combined stimulation of macrophages with ac and TLR ligands yielded a phenotype distinct from that induced by either ligand alone; ac elicited secretion of TGF-β1 only, whereas LPS alone triggered prolonged production of TNF-α, but together they stimulated a third pattern of response, apparently capable of enhancing early acute inflammatory responses but nevertheless promoting later resolution.

These data provide important new insights into the significance of ac-macrophage interactions during inflammation. The dynamics of inflammation are complex and temporal changes within the inflammatory environment cannot be ignored. The proposed role of ac as anti-inflammatory agents promoting resolution (6) is not inconsistent with early stimulation by ac of an inflammatory response. This may be of importance in infections (e.g., vaccinia, *Listeria, Trypanosoma cruzi*; (20–23)) where apoptosis is a prominent accompaniment of inflammatory tissue damage. Furthermore, experiments by Lorrimore et al. (24) demonstrated that soon after whole body exposure of mice to ionizing radiation, both thymus and spleen showed increased macrophage activation and neutrophil infiltration. Importantly, these effects were due to stimulation by ac rather than direct, radiation-induced activation of macrophages (24, 25). Similarly, inhibition of apoptosis in a renal ischemia/reperfusion model reduced inflammation and neutrophil infiltration, again suggesting that apoptosis might augment the early inflammatory response (26). Importantly, our in vitro observations demonstrated that macrophages stimulated with both LPS and ac exhibited increased secretion of TNF-α coupled with retained secretion of chemokines MIP-1α and MIP-2. Other workers have also implicated these chemokines in neutrophil recruitment by ac and have further shown the critical dependence upon macrophages (7). Our data suggest, however, that ac alone are not capable of eliciting an inflammatory response, and that it is the combination of ac and innate immune stimuli that promotes proinflammatory cytokine production in vitro.

However, ac do not merely amplify the proinflammatory effects of LPS. Macrophages stimulated with ac and LPS adopted a more complex phenotype that represents more than simple “addition” of responses to the two stimuli. We observed that the combination of TLR ligands and ac completely blocked production of IL-12 and IL-10, which would normally engage and regulate the adaptive immune system, suggesting that this combinatorial stimulus selectively primes an innate rather than an adaptive immune response. This may be beneficial, since adaptive immune responses to ac-derived self-Ags are likely to induce autoimmunity (4). Nevertheless, proinflammatory responses to the endogenous “danger signal” IFN-γ were not suppressed by exposure to ac, providing a potential mechanism for allowing adaptive immune responses to microorganisms despite the presence of ac.

This article does not address the intracellular mechanisms by which macrophages integrate signals derived from ligation of TLRs and exposure to ac. However, the data obtained with C3H/HeJ mouse macrophages clearly indicated that, in the case of LPS, such integration required functional TLR4. Further studies will be required to elucidate the mechanisms by which ac down-regulate TNF-α, IL-10, and IL-12 secretion. Our preliminary data (not shown) suggest that this occurs not at the transcriptional level, but

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**Table I. C3H/HeJ macrophages do not produce significant levels of TNF-α in response to LPS or apoptotic cells, but do respond to SAC**

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>1</th>
<th>2</th>
<th>4</th>
<th>8</th>
<th>24</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPS</td>
<td>16.6 ± 1.1</td>
<td>17.9 ± 2.0</td>
<td>17.9 ± 2.4</td>
<td>22.9 ± 1.7</td>
<td>24.4 ± 1.8</td>
</tr>
<tr>
<td>LPS + ac</td>
<td>25.9 ± 0.8</td>
<td>25.4 ± 1.3</td>
<td>28.7 ± 0.6</td>
<td>34.1 ± 1.2</td>
<td>35.5 ± 10.4</td>
</tr>
<tr>
<td>ac</td>
<td>20.3 ± 1.4</td>
<td>15.5 ± 0.2</td>
<td>17.9 ± 0.8</td>
<td>15.5 ± 0.2</td>
<td>14.3 ± 0.4</td>
</tr>
<tr>
<td>SAC</td>
<td>181.3 ± 26.1</td>
<td>869.2 ± 8.1</td>
<td>1347.5 ± 95.5</td>
<td>1733.5 ± 54.5</td>
<td>2279 ± 145.6</td>
</tr>
<tr>
<td>SAC + ac</td>
<td>337.8 ± 0.9</td>
<td>1298.5 ± 122.3</td>
<td>1742.0 ± 261.6</td>
<td>1549 ± 434.2</td>
<td>24.9 ± 3.2</td>
</tr>
</tbody>
</table>

* Bone marrow-derived macrophages from C3H/HeJ macrophages were incubated with LPS or SAC, apoptotic human neutrophils, or LPS/SAC with ac for the indicated times. Culture supernatants were harvested and TNF-α was measured by ELISA. Data are presented as mean values (picograms per milliliter) ± SD from three independent experiments.
posttranslationally or by modulating release from preformed stores. TGF-β1 has been proposed as a major regulator of ac-induced reprogramming of macrophages and in earlier work we confirmed that TGF-β1 was indeed essential for the “late” effects of ac upon murine macrophages (24-h down-regulation of TNF-α) (13). However, increases in TGF-β1 cannot be detected until 4–8 h after ac uptake, suggesting that there are early signals that act directly on the macrophage, priming the cell for early proinflammatory cytokine release and later secretion of TGF-β1. Ongoing work in the laboratory is addressing the possible role of ligation of macrophage receptors for ac in such early events.

In summary, it has been suggested that macrophage clearance of dying cells is important in limiting the attack against “self,” aiding, rather than abetting, the resolution of inflammation. However, interaction with ac primes macrophages not to be simply anti-inflammatory as previously suggested, but to respond to “danger signals” in a more complex way, producing a restricted, self-limiting inflammatory response. Furthermore, through coligation of phagocytic receptors ac expand the repertoire of responses to TLR ligands. Our observations of early “enhancement” and late “switch off” of macrophage proinflammatory responses not only add to previous work but also reconcile many paradoxical observations between studies which demonstrate either pro- or anti-inflammatory effects of ac. Thus, ac death may be central to the resolution of inflammation, first recruiting and arming effector cells and then rapidly deactivating them after they have served their functional purpose.

Acknowledgments
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References