Phagocyte Recognition of Dead and Dying Cells

Regulation of macrophage cytokine production by phagocytosis of apoptotic and post-apoptotic cells

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Recognition and removal is the final common event in the lives of most apoptotic cells in vivo. Macrophages are particularly talented at this task and therefore play a central role in the resolution of inflammation. Because macrophages remove apoptotic cells prior to their lysis, the release of potentially toxic and/or pro-inflammatory intracellular contents is prevented. Uptake of apoptotic cells has more complex benefits than simple removal of noxious substances, however. It was shown by Meagher et al. and then by Stern et al. that uptake of apoptotic neutrophils or eosinophils by human monocyte-derived macrophages did not induce secretion of granulocyte-macrophage colony stimulating factor (GM-CSF) or thromboxane B2 [1,2]. This was in marked contrast with necrotic or opsonized cells, which induced both. We subsequently found that apoptotic cells actually inhibited the production of many pro-inflammatory cytokines by macrophages stimulated with either lipopolysaccharide (LPS) or zymosan through an autocrine/paracrine mechanism involving transforming growth factor (TGF)β [3]. Apoptotic cell uptake was found to suppress production of GM-CSF, interleukin (IL)-1β, IL-8, IL-10, and tumour necrosis factor (TNF)α. In contrast with the other cytokines, TGFβ levels were increased. By co-incubating LPS, macrophages, and apoptotic cells in the presence of anti-TGFβ antibodies, we were able to reverse the suppression of the other cytokines, suggesting that the autocrine production of TGFβ was responsible, in part, for the cytokine inhibition we observed. In these studies, we used macrophages which utilized primarily α,β3 and CD36 by which to recognize apoptotic cells; uptake of apoptotic cells by these macrophages was not inhibited by phosphatidylinerine (PS) liposomes or by water-soluble PS analogues. We therefore went on to examine the effects of apoptotic cells on PS-recognizing macrophages. We induced the ability to recognize PS in human macrophages using β-glucan [4]. Phagocytosis by these macrophages was no longer inhibited by Arg-Gly-Asp-Ser or anti-α,β3 antibodies; rather, PS liposomes inhibited uptake. The effects on cytokine production were the same, suggesting that macrophages using mechanisms other than α,β3/thrombospondin/CD36 for uptake were capable of exhibiting the suppressive effects [4].

Other investigators have also addressed the effects of apoptotic cell uptake on macrophage cytokine production. Voll et al. examined the effects of apoptotic human peripheral lymphocytes on human peripheral blood mononuclear cells stimulated with LPS [5]. They observed a decrease in IL-1β, IL-2, and TNFα levels; however, in contrast with our findings, they observed an increase in IL-10. Increased IL-10 was also observed using an antibody against CD36, one of the important receptors for apoptotic cells on human macrophages. They have suggested that the increase in IL-10 could result in immunosuppression in conditions associated with increased apoptosis, including cancer, exposure to radiation, pregnancy, and viral infections such as those with human immunodeficiency virus.

Uchimura et al. have studied the effects of apoptotic lymphocyte uptake on mouse thioglycollate-elicited peritoneal exudate macrophages [6]. As a source of apoptotic cells, they used the CTLL-2 T lymphocyte line deprived of IL-2 for 28 h. They examined mRNA levels in the macrophages using reverse transcription-PCR. They found that macrophages co-cultured with CTLL-2 cells had increased levels of mRNA for ILK-1α, IL-1α, IL-1β, IL-10, IL-6, and MIP2, although in the case of IL-10, normal CTLL-2 cells induced the same effect. The levels for TGFβ were slightly lower for those macrophages cultured with CTLL-2 cells than for those cultured alone. Although they did not measure cytokine protein levels in the cell culture supernatants, they did show that these supernatants induced a neutrophilic infiltrate when injected into the peritoneal cavity of mice.

Abbreviations used: GM-CSF, granulocyte-macrophage colony stimulating factor; LPS, lipopolysaccharide; TGF, transforming growth factor; IL, interleukin; TNF, tumour necrosis factor; PS, phosphatidylinerine.

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How can one account for the differences in the findings in these studies? There are several possibilities. First, is it possible that different types of apoptotic cells could have different effects? Voll and Uchimura used apoptotic lymphocytes, whereas we used primarily neutrophils, although we found that apoptotic Jurkat T cells had the same effect as neutrophils [3]. Could the differences relate to the way in which apoptosis was induced? This seems unlikely, as both we and Voll et al. used U.V. irradiation to induce apoptosis in neutrophils or lymphocytes [3,5]. Could some of the differences relate to differences between mice and men? Lastly, could the presence of necrotic cells have affected the results, as Uchimura reported that 50% of their apoptotic populations was Trypan Blue-positive?

We set out to answer these questions by studying the effects of apoptotic cells in mouse and human macrophages using either neutrophils or Jurkat T cells as targets. More importantly we wished to begin to determine the mechanisms by which apoptotic cells induced their suppressive effects on macrophage cytokine production and to understand why necrotic cells were pro-inflammatory. The mouse macrophages we used included bone marrow-derived macrophages, thioglycollate-elicited peritoneal macrophages, and the macrophage cell line J774, all of which have been shown to phagocytose apoptotic cells [7–9]. For all apoptotic populations, Trypan Blue staining did not exceed 2–3%; for all necrotic populations, Trypan Blue staining exceeded 95%. In preliminary experiments we found that apoptotic cells failed to stimulate cytokines except for TGFβ in mouse bone marrow-derived macrophages, thioglycollate-elicited macrophages, or J774 cells. In contrast, necrotic cells appeared to stimulate production of MIP2, TNFα, and IL-10. Necrotic cells also failed to stimulate TGFβ to the same extent as apoptotic cells. In zymosan-stimulated bone marrow macrophages or LPS-stimulated J774 cells, apoptotic cells suppressed MIP2, TNFα, and IL-10, but stimulated TGFβ. Necrotic cells did not appear to inhibit MIP2 production, but markedly inhibited the production of TNFα and IL-10 although not to the same extent as apoptotic cells. Similar results were seen when Jurkat T cells were used as phagocytic targets, or when human macrophages were used as phagocytes. These results suggest that necrotic cells induce low levels of inflammatory cytokines and IL-10 in unstimulated macrophages, but may suppress at least some cytokines in stimulated macrophages.

One of the potential differences between apoptotic cells and necrotic cells is the permeability of the latter with the release of intracellular proteases. We wondered whether proteases derived from the necrotic cells could play a role in the more pro-inflammatory effects of the necrotic cell. We therefore performed the assays in the presence or absence of leupeptin, aprotinin, or PMSF. Preliminary results suggested that leupeptin and aprotinin induced no changes in the effects of apoptotic or necrotic cells on macrophage cytokine production. In contrast, the presence of PMSF appeared to alter the responses to necrotic cells such that they behaved more like apoptotic cells. PMSF had very little effect on zymosan stimulation or the ability of either apoptotic neutrophils or apoptotic Jurkat cells to inhibit MIP2 production. In contrast, PMSF was able to abrogate the pro-inflammatory effects of necrotic cells; this was particularly marked for neutrophils compared with Jurkat T cells; neutrophils would be expected to carry a larger arsenal of proteases than lymphocytes. Similar results are seen for TNFα and IL-10. These data suggest that serine proteases contribute to the inflammatory effects of necrotic cells; however, it seems clear that necrotic cells must also have anti-inflammatory components as well. In our studies, necrotic cells did not stimulate cytokine production to the same extent as the inflammatory stimuli LPS or zymosan, and they had some inhibitory effects on cytokine production stimulated by these two substances.

The mechanisms by which apoptotic cells signal the macrophage to become anti-inflammatory are not known at this time, but are an active area of research. We have examined the effects of apoptotic cells or TGFβ on NFκB and AP-1, and have seen no changes in the activity of these transcription factors, suggesting that the inhibition may operate downstream of transcription.

Another potential explanation for the anti-inflammatory effects of apoptotic cells is the hypothesis that the receptor which recognizes the cell determines the final outcome. Meagher and Stern showed that if apoptotic neutrophils or eosinophils were opsonized with antibody, they were able to induce GM-CSF and thromboxane production, whereas their unopsonized counterparts did not. In addition, Manfredi et al. [10] have shown that apoptotic lymphocytes can be
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opsonized with sera from patients with antiphospholipid syndrome; many of the antiphospholipid antibodies are believed to recognize epitopes composed of β2GP1 bound to exposed PS on the surface of the apoptotic cell. Uptake of these opsonized apoptotic cells was associated with TNFα secretion by human macrophages [10]. These data suggest that uptake by the Fc receptor was pro-inflammatory, whereas uptake by the α,β3/thrombospondin/CD36 mechanism was not [1,2]. We also found that opsonized apoptotic cells were pro-inflammatory and that they failed to induced production of TGFβ [3,4]. Uptake of unopsonized apoptotic cells by macrophages which use either the α,β3/thrombospondin/CD36 or PS recognition mechanisms are inhibited from producing pro-inflammatory cytokines; interestingly, CD36 appears to be common to both uptake mechanisms [4]. Voll et al. found that an anti-CD36 antibody was able to inhibit production of TNFα and IL-12 by LPS-stimulated peripheral blood mononuclear cells [5]. We have used anti-α,β3 and anti-CD36 antibodies, alone and with cross-linking, and found that they actually stimulate cytokine production, so the picture is far from clear. Perhaps another as yet unidentified receptor mediates the anti-inflammatory effect.

In summary, the uptake of apoptotic cells not only fails to stimulate cytokine production, but induces the macrophage to become anti-inflammatory (Figure 1). Our work suggests that macrophage-secreted TGFβ plays a major role in the induction of the anti-inflammatory phenotype; however, IL-10 and other factors may also play a role. The mechanisms by which apoptotic cells induce this response are not known. Necrotic cells tend to be more pro-inflammatory and this appears to be related, in part, to the activity of serine proteases. Clearly, these findings need to be confirmed in vivo; however, we can hypothesize that the removal of apoptotic cells while still intact promotes the resolution of inflammation by preventing the release of potentially toxic materials and by inducing an anti-inflammatory phenotype in the macrophage.

**Figure 1**

A model for inhibitory effects of apoptotic cells on macrophage cytokine production

Apoptotic cells induce the secretion of TGFβ, which acts in an autocrine/paracrine fashion to inhibit the production of inflammatory cytokines and chemokines. Necrotic cells also have some anti-inflammatory activity; however, the anti-inflammatory signals are partially abrogated by the action of the serine proteases that are released.

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