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Recognition of apoptotic cells by human peripheral blood monocytes does not alter their ability to phagocytize and kill *Staphylococcus aureus*

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Summary

Introduction:

During acute inflammation, leukocyte infiltration is mostly neutrophilic, but later monocytes prevail. The majority of inflammatory cells, particularly neutrophilic polymorphonuclear leukocytes (PMNs), become apoptotic at later stages of inflammation and are phagocytosed by neighboring cells, mostly by macrophages. Recently, it has been found that human peripheral blood monocytes also recognize apoptotic cells, which primes them to increased production of interleukin (IL)-10 – a cytokine known to reduce phagocytes' ability to engulf and kill pathogens. Based on the above, we studied monocytes' ability to phagocytose and kill *Staphylococcus aureus* while in contact with apoptotic cells.

Materials and Methods:

Monocytes isolated by elutriation were co-cultured with apoptotic PMNs or Jurkat cells and exposed to viable, human serum-opsonized *S.aureus*. To induce apoptosis PMNs were cultured overnight while Jurkat cells were UV-treated. Apoptosis, phagocytosis of bacteria and intracellular superoxide production were measured by flow cytometry. Production of reactive oxygen species was also followed by measurement of chemiluminescence. The bactericidal effect was determined by standard colony forming units method.

Results:

Data presented show that contact of monocytes with apoptotic neutrophils and Jurkat cells had no influence on monocyte phagocytosis of *S. aureus*, the generation of reactive oxygen species, or the killing of bacteria.

Conclusion:

The data obtained suggest that monocytes attracted to the inflammatory site are not deficient in their ability to cope with pathogens after contact with apoptotic cells despite increased production of IL-10.

Key words:

apoptotic cell recognition • monocytes • *Staphylococcus aureus*

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INTRODUCTION

Recognition and phagocytosis of pathogens by resident macrophages, activation of mast cells and triggering of the kinin system all provide mediators responsible for the development of a tightly orchestrated inflammatory response^{1, 8, 23}. The accumulation of neutrophils (PMNs), monocytes and, finally lymphocytes, all attracted to the inflammation site, contribute to the native and, later, adaptive immune responses responsible for the elimination of invading pathogens. The majority of inflammatory cells, particularly PMNs, become apoptotic at later stages of inflammation and are phagocytosed by neighboring cells, in particular by macrophages. Apoptosis of PMNs and their phagocytosis by macrophages is crucial for the resolution of inflammation as it protects the tissues from the leakage of noxious contents from dying cells^{9, 13, 18, 20}. Some pro-inflammatory cytokines, including granulocyte-macrophage colony-stimulating factor, interleukin (IL)-1, and tumor necrosis factor, were even shown to upregulate macrophage capacity to phagocytize apoptotic PMNs¹⁶. In short, if the rate of cell death by apoptosis exceeds macrophage clearance capacity, or when apoptotic cells cannot be recognized by macrophages, the resolution of inflammation is delayed or becomes chronic^{15, 25}.

Recently it has become apparent that the uptake of apoptotic cells not only prevents the tissues from exposure to noxious contents released by inflammatory cells, but also that it has a direct anti-inflammatory effect. Fadok et al.⁷, and McDonald et al.¹² reported that phagocytosis of apoptotic cells by monocyte-derived macrophages resulted in the suppression of the lipopolysaccharide (LPS)-induced synthesis of proinflammatory cytokines, mostly due to increased production of transforming growth factor β .

Studies by Newman et al.¹⁴ indicated that monocytes did not phagocytize apoptotic cells, but acquired that ability only after differentiation into macrophages. However, recent findings indicate that peripheral blood monocytes recognize apoptotic cells^{4, 5}. It has been found that while in contact with apoptotic cells, LPS- or bacteria-stimulated monocytes produce much more anti-inflammatory cytokine IL-10, while pro-inflammatory cytokines are rather suppressed^{4, 5}.

IL-10 has a potent immunosuppressive activity, including impairment of antibacterial host defense²². It has been shown to inhibit the production of reactive oxygen intermediates (ROI) by PMNs and monocyte-derived macrophages and to reduce the antimicrobial activity of phagocytes^{11, 24}. Against this background, we ask whether the presence of apoptotic cells interferes with the uptake and killing of *Staphylococcus aureus* by peripheral blood monocytes.

MATERIALS AND METHODS

Monocytes

Peripheral blood mononuclear cells (PBMCs) were isolated by standard Ficoll/Paque (Pharmacia, Uppsala, Sweden) gradient centrifugation from EDTA-treated blood from healthy donors and then subjected to countercurrent centrifugal elutriation (Beckman JE-6B elutriation system equipped with a 5-ml Sanderson separation chamber) to obtain monocytes as previously described^{3, 5}. Monocytes were washed once with cold RPMI 1640 and kept on ice in RPMI 1640 culture medium supplemented with L-glutamine and 10% fetal calf serum, without antibiotics (all reagents from Gibco, Grand Island, NY, USA) until use.

Polymorphonuclear neutrophils

PMNs were isolated from erythrodeposits (left after PBMC isolation). These were suspended in a 1% solution of polyvinylalcohol in PBS (Merck, Hohenbrunn, Germany) and sedimented for 20 min at room temperature. PMN were collected from the upper part. The contaminating erythrocytes were lysed for 20 s with water, which resulted in about 90% pure population of PMNs as confirmed by Pappenheim staining. The PMNs were used immediately after harvest (fresh, non-apoptotic) or after 18–24 h of incubation at 37°C in humidified atmosphere containing 5% CO₂ in culture medium (aged, apoptotic). The proportion of annexin V-positive cells (see below) in aged PMNs ranged 53–71% whereas in freshly isolated never exceeded 9%.

Jurkat cells

Clone E6-1 (ATCC, TIB-152) was propagated in the above-defined culture medium with addition of 10 mM HEPES. Cells were used immediately after harvest and washing (fresh, nonapoptotic) or 18 h after 2-hour exposure to UV light (apoptotic). The proportion of annexin V-positive cells in UV-treated cells ranged 45–65 % whereas in non-treated did not exceed 12%.

Flow cytometry determination of apoptosis and cell viability

To determine apoptosis of PMNs or Jurkat cells, annexin V-binding assay was performed. Cells were suspended in staining buffer (HEPES buffer containing 150 mM NaCl, 5 mM KCl, 1 mM MgCl₂, and 1.8 mM CaCl₂, pH 7.4) and labeled with annexin V-PE (Bender MedSystems, Vienna, Austria) for 15 min on ice to detect phosphatidylserine expression on the outer cell

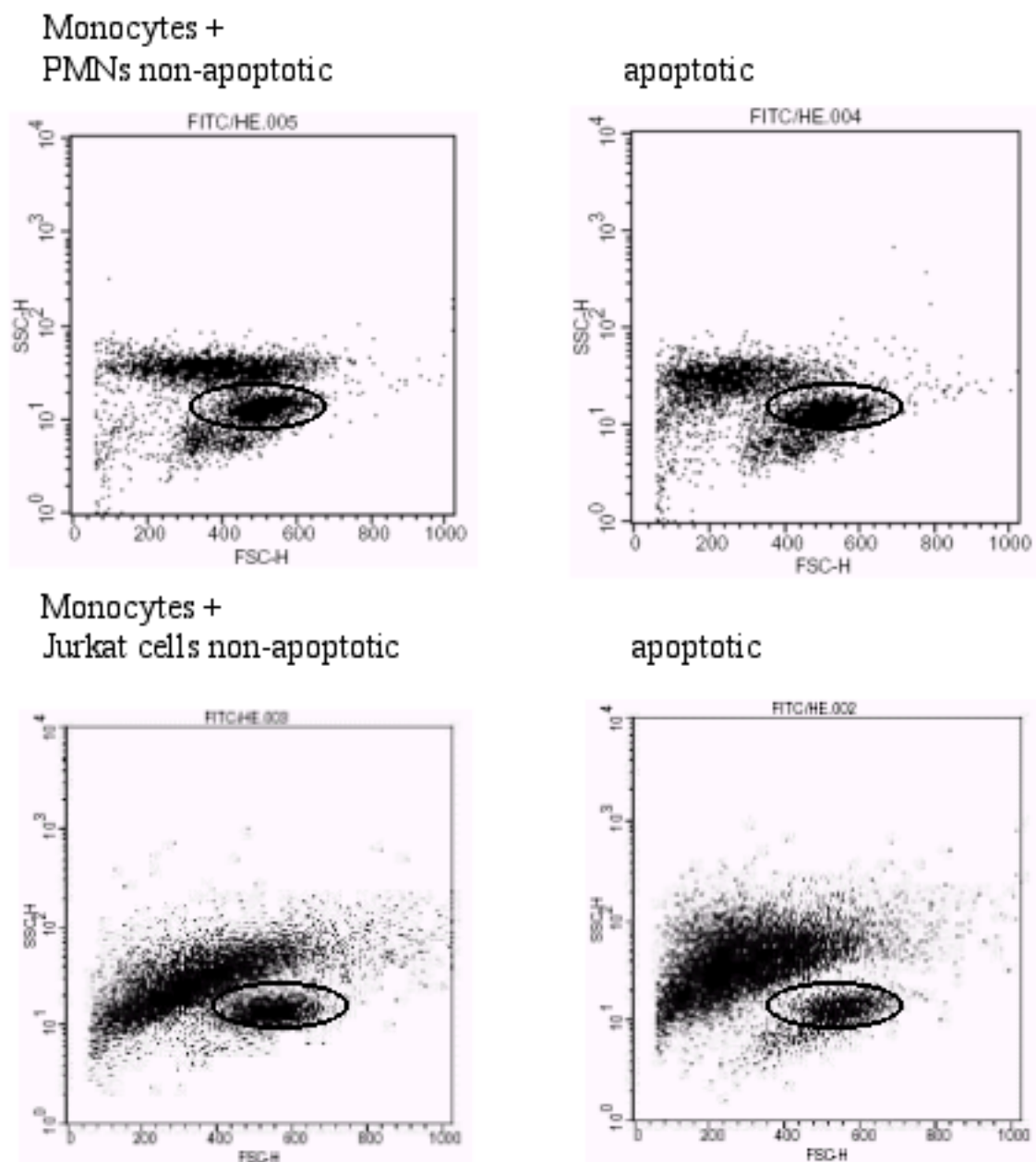


Figure 1. Scatter patterns of co-cultures of monocytes and PMNs or Jurkat cells. Dot-plot analysis of forward vs side scatter. Elliptic monocyte gate is indicated. Please note that monocytes can be easily distinguished from other cell populations.

membrane layer. After washing with staining buffer cells were analyzed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA). The cells' viability was determined by standard propidium iodide (PI) uptake. The percentage of annexin V-positive and PI-negative cells in populations of fresh and aged PMNs and untreated and UV-light-treated Jurkat cells ranged 1–10 and 50–83, respectively. The proportion of PI-positive cells did not exceed 10%.

Bacteria

S. aureus (ATCC 25923) was grown for 18 h in brain-heart infusion broth (Oxoid), washed twice with saline and opsonized (30 min, 37°C) in the presence of 10% normal human serum (pooled fresh human serum stored in aliquots at –70°C). Opsonized bacteria were additionally washed once with saline and their number was counted in a Thoma chamber. In some experiments,

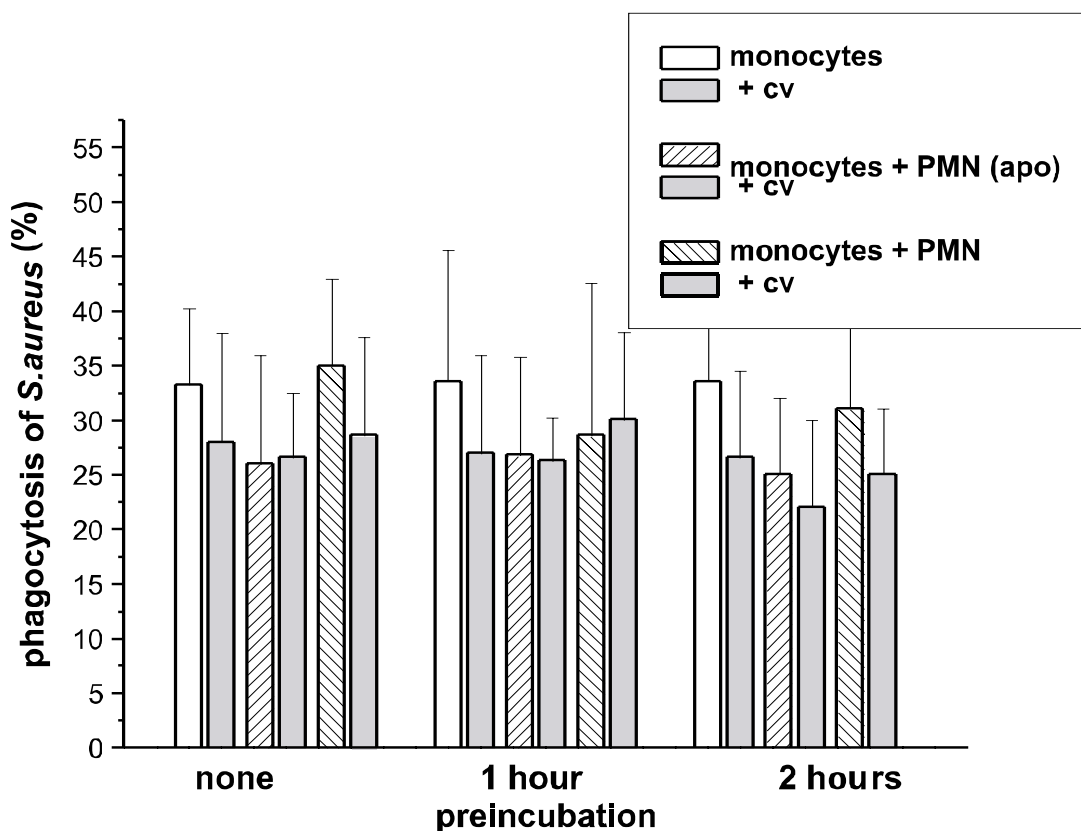


Figure 2. Effect of non-apoptotic and apoptotic PMNs on monocyte phagocytic activity. Monocytes were cultured alone or co-cultured with PMNs (either freshly isolated or apoptotic). FITC-labeled serum-opsonized bacteria were added to cultures either at the time of culture setting or 1–2 h later as indicated. The monocytes:bacteria ratio was 1:20. Monocytes were gated according to FSC vs SSC signals and each sample was evaluated twice: before and after addition of crystal violet (cv) to quench extracellular fluorescence. The data are mean $\pm$ SD of 4 independent experiments.

before opsonization bacteria were incubated for 2 h at 37°C in PBS containing 0.1% fluorescein isothiocyanate (FITC; BHD Chemicals Ltd., Poole, England) and subsequently washed free of unbound dye.

Latex particles

Latex particles (diameter, 0.8–1.0 μ m) were obtained from the Institute of Catalysis and Surface Chemistry, Polish Academy of Sciences (Cracow, Poland). They were washed twice with 70% ethanol and kept suspended in PBS at a concentration of 1.5×10^9 /ml.

Phagocytosis of bacteria

Monocytes (1×10^6) alone or mixed with PMNs or Jurkat cells were incubated (37°C) in Falcon 2054 tubes (Becton Dickinson Labware Europe, Le Pont De Croix, France) with suspensions of FITC-labeled opsonized bacteria in a total volume of 1 ml of RPMI 1640 without antibiotics. Unless stated otherwise, bacteria were added 1 h after cultures of monocytes or monocytes and other cells were. The phagocyte/bacterium ratio was 1:20. After 30 min incubation with

bacteria tubes were transferred to an ice bath and analyzed by flow cytometry. The analysis was performed using the CellQuest program to determinate the number of green cells (due to FITC-labeled bacteria) within the monocyte gate defined by FSC/SSC signals³. In some experiments, crystal violet (final concentration 0.25 μ g/ml) was added to quench fluorescence of bound but not internalized bacteria¹⁰.

ROI production by phagocytes

Intracellular superoxide production by monocytes was analyzed by using hydroethidine (HE; Sigma, St. Louis, MO, USA) as described elsewhere^{3,17}. Briefly, HE (final concentration 10 μ M) was added to cell samples 15 min after addition of FITC-labeled bacteria. The cells were incubated for another 15 min in 37°C and then were analyzed by flow cytometry.

Alternatively, the luminol-dependent chemiluminescence (CL) of phagocytes was measured after exposure to latex particles or serum-opsonized bacteria. Monocytes (0.1×10^6) alone or mixed with Jurkat cells were incubated with opsonized bacteria or latex beads (in

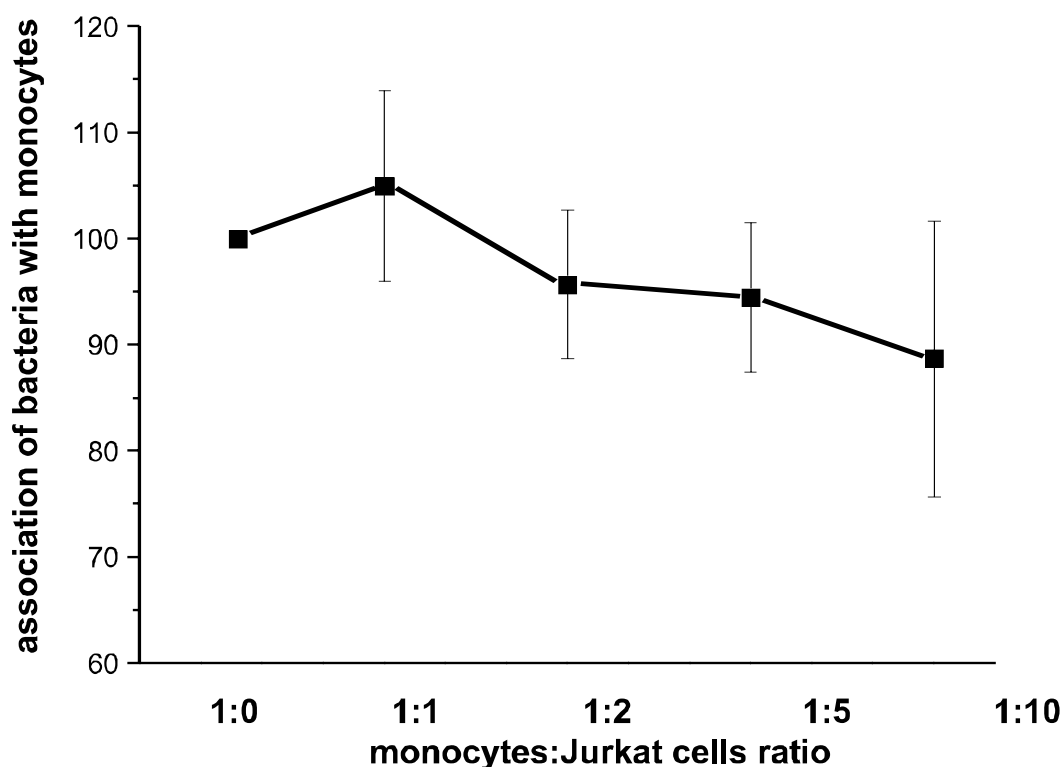


Figure 3. Effect of apoptotic Jurkat cells on phagocytosis of *S. aureus* by monocytes. Monocytes cultured for 1 h alone or as co-cultures with different numbers of apoptotic Jurkat cells were incubated with FITC-labeled fresh human serum-opsonized *S. aureus*. The ratio of phagocytes/bacteria was 1:20. After 30 min of incubation, samples were analyzed by flow cytometry. Monocytes were gated according to FSC vs SSC signals. The results are expressed as percentages of the results obtained in the group of monocytes cultured alone, considered in each experiment as 100%. The data are mean $\pm$ SD of 3 independent experiments.

a phagocyte/particle ratio of 1:100) in 0.5 ml of CL medium (see below). The incubation (10 or 30 min with latex beads and bacteria, respectively) took place in a 6-channel luminometer (Multi-Biolumat LB9505C; Berthold, Vienna, Austria) which allowed continuous monitoring of chemiluminescence. The CL medium consisted of Krebs-Ringer phosphate buffer containing glucose (11 mM) and bivalent cations (0.54 mM Ca^{2+} and 1.12 mM Mg^{2+}) plus 2 mM luminol (5-amino-2,3-dihydro-1,4-phthalazinedione; Sigma, St. Louis, MO, USA).

Bacterial killing

Bactericidal capacity of phagocytes was measured by a standard colony forming units (c.f.u.) method. Monocytes (3×10^6) alone or mixed with other cells were incubated (37°C) in siliconized glass tubes with opsonized bacteria (in a monocyte/bacterium ratio of 1:20) in a total volume of 1 ml. After 30 min of incubation, 1 ml of cold RPMI 1640 medium was added to the tubes and the cells were centrifuged (110 $\times$ g, 5 min) to separate phagocytic cells from free bacteria. Then the cells were resuspended in 1 ml of culture medium (with-

out antibiotics) and split into two 0.5-ml samples. One of these was immediately lysed with 2.5 ml distilled water. The second was additionally incubated for 1 h and lysed the same way. Serial dilutions of lysates in saline were plated onto sheep-blood agar plates. Colonies were counted after incubation of the plates for 24 h at 37°C, and the percentage of killed microorganisms was calculated, considering as 100% the value determined in the sample lysed immediately after phagocytosis.

Statistical analysis

Differences of means were compared using the Student's *t*-test and considered significant when $p < 0.05$.

RESULTS

The presence of apoptotic cells has no influence on monocytes' ability to phagocytose S. aureus

The phagocytic activity of monocytes was determined by flow cytometry. The differences in the scatter patterns of monocytes, PMNs and lymphocytes enabled a satisfacto-

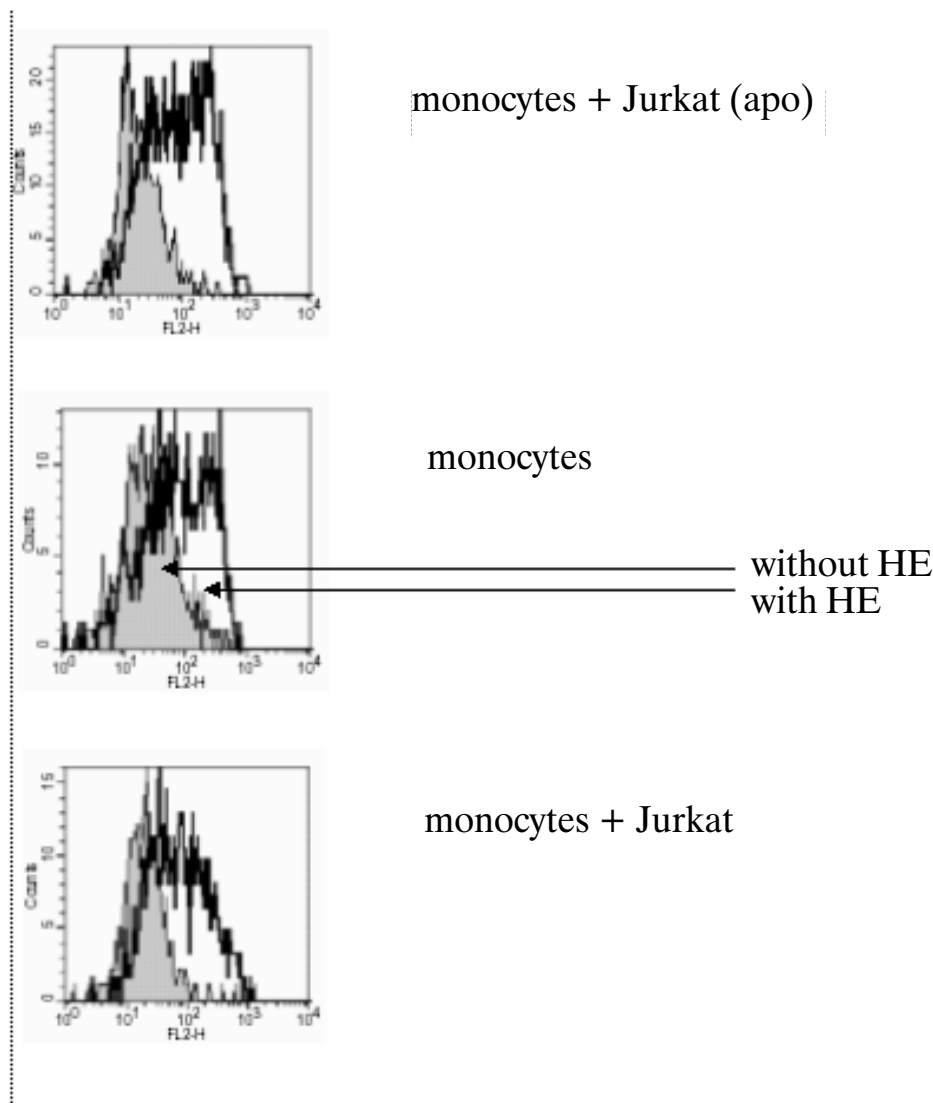


Figure 4. Production of superoxide by monocytes associated with bacteria. Histogram analysis of monocytes cultured alone or with Jurkat cells and exposed for 30 min to FITC-labeled *S. aureus*. Fluorogenic probe hydroethidine was added to each sample for the last 15 min of incubation. Thereafter, samples were analyzed by flow cytometry. FL2 (red fluorescence) was measured in monocyte gate of samples with (open histograms) and without (filled histogram) hydroethidine, as indicated. Figure shows representative patterns. The proportion of annexin V-positive cells was respectively 5.3 and 57.8 % in fresh and UV- treated Jurkat cells.

ry gating of the monocyte population (Fig. 1). Using this approach, the proportion of green-labeled monocytes (due to FITC-labeled bacteria) could be determined. As shown (Fig. 2), the presence of apoptotic or nonapoptotic PMNs had no significant influence on the proportion of monocytes that associated with labeled bacteria. Furthermore, the presence of apoptotic (as well as non-apoptotic) PMNs had no influence on engulfment of bacteria as judged from the measurements performed in the presence of crystal violet to quench extracellular fluorescence. The results were not changed upon 2 h of preincubation of monocytes with apoptotic cells before addition of the bacteria. The proportion of monocytes associated with FITC-labeled bacteria was also not affected by the

presence of apoptotic Jurkat cells (Fig. 3). However, when the number of Jurkat cells exceeded monocytes by a factor of 10, the percentage of monocytes with bacteria was lower in some experiments, although the reduction was not statistically significant. Similar results were observed when samples were analyzed in the presence of crystal violet and when monocytes were co-cultured with nonapoptotic Jurkat cells (data not shown).

*The presence of apoptotic cells has no influence on ROI produced by phagocytes in response to *S. aureus**

ROI are essential for the bactericidal effect of phagocytes². Therefore we wanted to know whether or not the

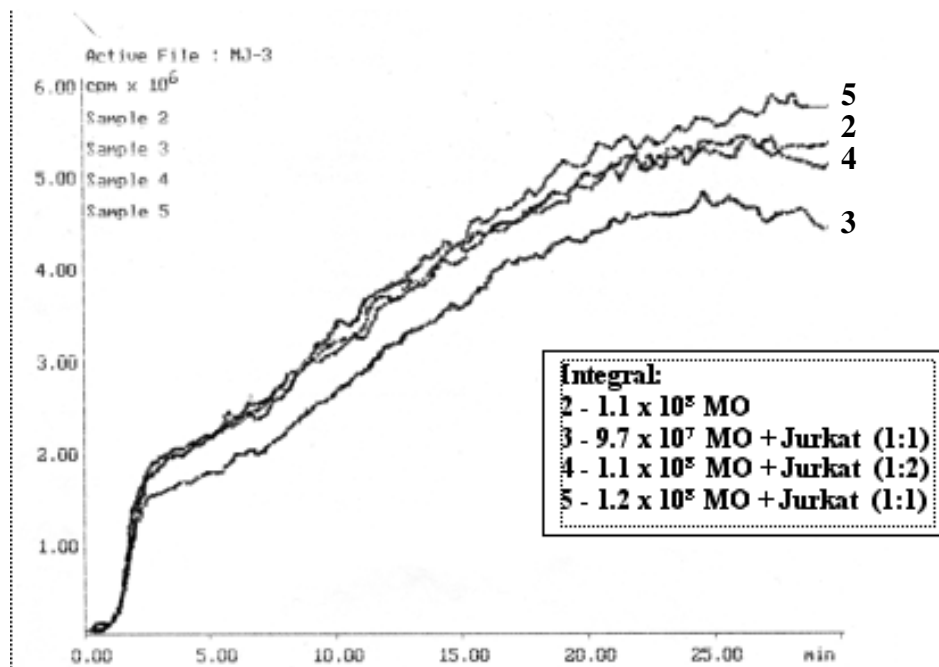


Figure 5. Luminol-dependent chemiluminescence of monocytes (MO) in response to bacteria. Monocytes alone or pre-cultured (1 h, at indicated ratio) with apoptotic Jurkat cells were stimulated with opsonized *S. aureus* (monocyte:bacteria ratio 1:100). The response was monitored over 30 min of incubation. Calculated cumulative response (integral) is given as an insert. The picture shows a typical experiment out of 5 performed. The proportion of annexin V-positive cells in UV-treated Jurkat cells was 62.5%.

presence of apoptotic cells had any impact on monocytes' ability to produce ROI in response to bacteria. To answer this question, monocytes cultured (1–2 h) alone or in the presence of apoptotic or nonapoptotic Jurkat cells were challenged with bacteria and loaded with HE. This approach allowed the detection of intracellular superoxide production¹⁷. As shown (Fig. 4), a comparable proportion of monocytes was positively stained, indicating a comparable production of superoxide by monocytes regardless of the presence or the absence of apoptotic (or nonapoptotic) Jurkat cells. Similar data were obtained when monocytes were cultured in the presence of apoptotic PMNs (not shown). The generation of ROI by monocytes cultured with or without Jurkat cells and challenged with opsonized *S. aureus* was also analyzed in the chemiluminometer. The measured luminol-dependent chemiluminescence was exclusively derived from monocytes, as responses of Jurkat cells alone, with or without bacteria, were the background level (data not shown). As shown (Fig. 5), the presence of apoptotic Jurkat cells had no influence on the chemiluminescence triggered by phagocytosis of the bacteria. Similarly, the presence of Jurkat cells had no influence on latex-triggered chemiluminescence (not shown).

Apoptotic Jurkat cells do not influence monocytes' ability to kill S. aureus

To evaluate monocytes ability to kill *S. aureus* in the presence and absence of apoptotic cells, the standard c.f.u. assay was employed. When monocytes were co-cultured with apoptotic PMNs, the efficiency of killing bacteria was usually even greater than the killing by monocytes cultured alone (data not shown). As the proportion of annexin V-positive cells within apoptotic PMNs varied 50–80%, a contribution of PMNs to bactericidal activity could not be excluded. Therefore, we focused on co-cultures of monocytes with apoptotic Jurkat cells. As shown (Fig. 6), the killing of bacteria by a mixture of monocytes and Jurkat cells was as efficient as by monocytes cultured alone. Similarly, when non-apoptotic Jurkat cells were added to monocytes, the killing efficiency was not affected. Finally, the bactericidal efficiency of monocytes did not change after 18 h of preculture with apoptotic Jurkat cells (data not shown).

DISCUSSION

The apoptosis of cells comprising inflammation leukocyte infiltration enables their clearance by neighboring

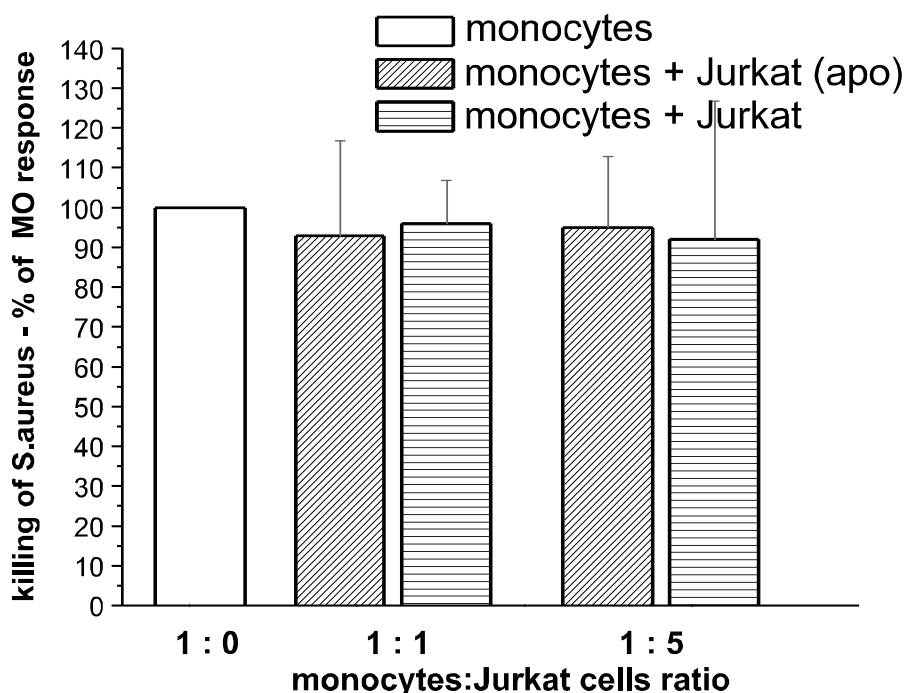


Figure 6. Killing of *S. aureus* by monocytes co-cultured with Jurkat cells. Fresh human serum-opsonized *S. aureus* bacteria were added to monocytes cultured alone (empty bar) or monocytes co-cultured for 1 h with Jurkat cells (apoptotic or non-apoptotic, stripped bars as indicated). The ratio of phagocytes/bacteria was 1:20. The killing of bacteria was calculated as reduction of c.f.u. after 60 min in comparison with the number of viable bacteria after 30 min (see Materials and Methods). To compare data from different experiments, the bactericidal effect of monocytes cultured alone was always considered as 100%. Figure shows the mean $\pm$ SD of 5 independent experiments

cells, mostly macrophages. This prevents the release of noxious agents from dying cells that otherwise might exacerbate or prolong inflammation^{17,18,21}. Furthermore, the recognition and phagocytosis of apoptotic cells triggers the secretion of anti-inflammatory mediators which help in the resolution of the inflammation and healing^{7,19}. Although initially only macrophages were recognized as responsible for the production of anti-inflammatory mediators^{7,12}, two groups including ours, recently made the observation that monocytes recognize apoptotic neutrophils^{4,5}. They showed an increased production of IL-10 and, in parallel, the reduced secretion of some pro-inflammatory cytokines by LPS-stimulated monocytes cultured in the presence of apoptotic PMNs. More recently we found a similar response of monocytes to apoptotic lymphocytes (Frączek et al., in preparation). Moreover, we have found that the increased IL-10 production by monocytes requires direct cell-cell contact between monocyte and apoptotic cell⁵. The above-mentioned findings indicate that monocytes receive and react to signals provided by apoptotic cells directly by cell surface contact. This justified the question whether the cell-cell contact of monocytes with apoptotic cells influences the monocytes' ability to cope with pathogens. Apparently, as shown in this study, these signals do not

influence monocytes' ability to phagocytose and kill *S. aureus*. When co-cultured with monocytes, neither PMNs nor Jurkat apoptotic cells influenced uptake of bacteria. The reduced phagocytic activity recorded in the presence of an excessive number of Jurkat cells probably reflected the difficulty in accurate gating during flow cytometry analysis. Although FSC/SSC signals allowed separation of monocytes and other cell populations (compare Fig. 1), these clusters were partially overlapping. Therefore, in the presence of an excessive number of Jurkat cells, the "monocyte gate" was likely to be diluted by contaminating non-phagocytic cells. Furthermore, this phenomenon was not related to apoptotic cells, as it was seen in the presence of excessive numbers of nonapoptotic cells as well. The presence of apoptotic cells also had no influence on the generation of ROI and the killing of *S. aureus* by monocytes.

The above findings strongly suggest that signals received by monocytes during recognition of apoptotic cells do not change their antibacterial efficiency. This conclusion is not in variance with previous reports that showed impairment of phagocyte antibacterial function by IL-10, as in our studies phagocytes had only minimal contact with cytokines, if any. The majority of the above-report-

ed experiments were of short duration and incubation with bacteria was only 30–90 min, depending on whether phagocytosis or killing was assessed. Although in one type of experiment monocytes before bactericidal assay were co-cultured with apoptotic cells overnight, cells were not additionally activated, and we have already shown that cell-cell contact of monocytes with apoptotic cells by itself does not trigger IL-10 production⁵. In addition, although IL-10 suppresses antibacterial activity of PMNs and macrophages^{11, 22}, under some conditions it may even increase monocyte phagocytic capacity⁶.

Altogether, these data suggest that monocytes newly arriving to the inflammatory site are not impaired in their antibacterial activity by contact with apoptotic cells. This explains why immune protection against pathogens is usually maintained at later stages of inflammation despite the local presence of apoptotic cells and likely production of anti-inflammatory cytokines. By contrast, when apoptotic cells appear in substantial numbers in the circulation, which correlates with an increased plasma IL-10 level, the ability of circulating phagocytes to cope with pathogens is impaired²².

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