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Letter to the Editor

Apoptotic cells quench reactive oxygen and nitrogen species and modulate TNF-α/TGF-β1 balance in activated macrophages: involvement of phosphatidylserine-dependent and -independent pathways

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Dear Editor,

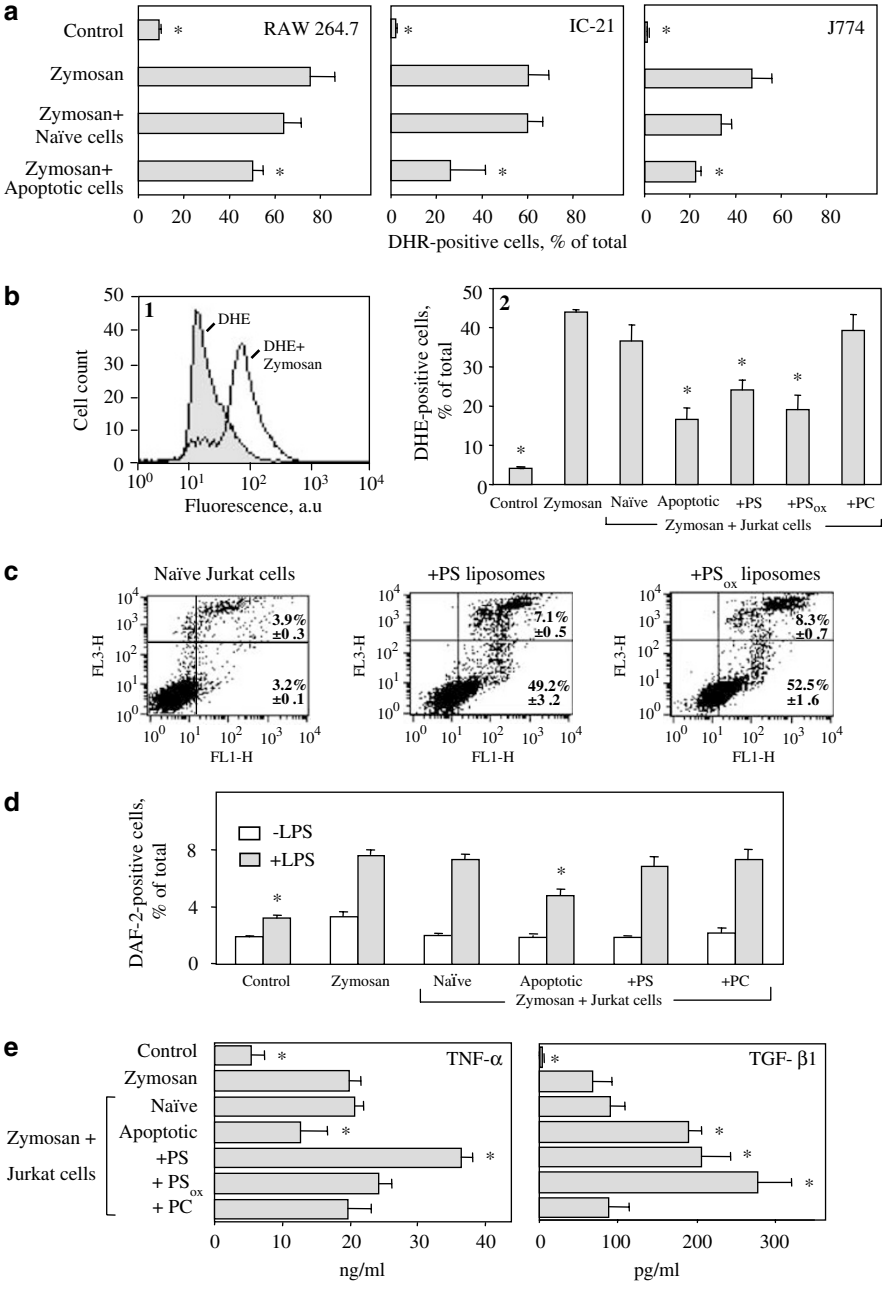
Professional phagocytes are essential for host defense. The functional responses of the phagocytes to infections include chemotaxis, actin assembly, migration, adhesion, aggregation, phagocytosis, degranulation, and production of reactive oxygen species (ROS) and reactive nitrogen species (RNS). Superoxide generation by phagocytic NADPH oxidase as well as production of NO· by nitric oxide synthase (iNOS) are necessary steps toward effective innate immune response. In addition to their principal task to eliminate bacteria, phagocytes are engaged in removing damaged, senescent, and apoptotic cells.[1] During clearance of invading pathogens, proinflammatory processes ensure maximal immunological activation in the early phase but are followed by resolution and active inflammatory suppression. Both of these stages rely heavily on interrelated and redox-regulated pathways in macrophages. Initial activation is governed by the release of cytokines, eicosanoids, and chemokines whose actions orchestrate vasodilatation and increased vascular permeability and amplify further recruitment of leukocytes to aid in microbial clearance.

Importantly, the response is self-limited by anti-inflammatory mechanisms driven by the release of anti-inflammatory mediators (IL-10 and transforming growth factor-β (TGF-β)).[2] It is likely that ROS and RNS produced as a part of innate immune response are directed not only toward killing pathogens but also cause appearance of apoptotic cells whose anti-inflammatory effects are well described.[3] The clearance of apoptotic cells is mediated via their surface phospholipid changes and the engagement of recognition receptors on macrophages. In particular, phosphatidylserine (PS) externalization from the inner leaflet of the apoptotic cell membrane and its recognition by macrophage receptor(s) is a prerequisite for effective clearance.[3] Our previous work has identified oxidized PS (PS_{ox}) as a more effective 'eat-me' signal for macrophages than PS.[4] Several reports note that PS-induced cytokine-dependent pathways are involved in the switch from pro- to anti-inflammatory profiles of macrophages. However, the ability of apoptotic cells and their externalized PS signals to regulate generation of ROS and RNS is not well defined.[5] This is important because macrophage production of ROS/RNS may (1) govern the magnitude of apoptosis (*versus* necrosis), hence modulate the anti-inflammatory signal in macrophages, and (2) change the intracellular redox environment of macrophages, hence affect their specific signal transduction pathways. Here, we report that apoptotic cells quench ROS/RNS production by activated macrophages and the extent to which this effect is dependent on PS or PS_{ox}.

To determine the effects of apoptotic cells in ROS/RNS production, we preincubated macrophage cell lines (RAW 264.7, IC-21, J774) with normal and apoptotic (Fas-triggered) Jurkat cells followed by activation with zymosan (Figure 1a). We used dihydrorhodamine (DHR) as a fluorogenic probe sensitive to both ROS (H_2O_2) and RNS (peroxynitrite). In all three macrophage cell lines, zymosan-induced DHR fluorescence was markedly suppressed by apoptotic cells but not control cells. In the absence of zymosan, neither control nor apoptotic cells altered DHR fluorescence (data not shown). We next utilized dihydroethidium (DHE) and 4,5-diaminofluorescein diacetate (DAF-2DA) to specifically assess effects of apoptotic cells on superoxide and NO· production, respectively (Figure 1b and d). Apoptotic cells (but not control cells) effectively suppressed superoxide production by zymosan-activated macrophages (Figure 1b). The effect of apoptotic cells could also be mimicked by normal cells engineered to contain PS in the outer leaflet of their plasma membrane. To accomplish this, we delivered specific phospholipids by liposomes and prevented PS internalization by *N*-ethylmaleimide (NEM) treatment. As shown on Figure 1c, thus treated cells elicited annexin V-positivity and minimal cell damage (low PI response). PS-enriched cells were effective inhibitors of zymosan-induced superoxide production. Interestingly, PS_{ox} integrated into Jurkat cells to approximately the same level as PS, was even more efficacious than PS (Figure 1b and c). Zymosan alone was not sufficient to induce appreciable NO· production in macrophages (Figure 1d). Additional stimulation as provided by lipopolysaccharide (LPS) was required to cause a significant NO· production (DAF-2 response). The LPS/zymosan response was effectively inhibited by apoptotic cells. In contrast to the

effect of apoptotic cells on DHE response, Jurkat cells with integrated PS did not significantly affect NO· production (DAF-2 fluorescence in macrophages). Therefore, the release of superoxide and NO· differed not only in their inducing stimuli requirements (i.e., need for zymosan alone or zymosan/LPS) but also in their sensitivity to inhibition by PS-enriched nonapoptotic cells. In other words, ROS could be initiated solely by a toll-like receptor 2 (TLR-2) agonist like zymosan; however, NO· required stimulation via the TLR-4 pathway with LPS exposure. Thus, it is not surprising that different signaling pathways would also govern their down-regulation, and more work will be required to determine the role of apoptotic cells in selective responses to specific TLR stimuli of innate immunity.

We were further interested in determining the extent to which apoptotic cell quenching of ROS and RNS production is associated with the switch from pro- to anti-inflammatory macrophage phenotypes. To this end, we used TNF-α as a marker of proinflammatory activation, and TGF-β1 as a marker of anti-inflammatory response (Figure 1e). In line with previously reported results[3,5] preincubation of RAW 264.7 macrophages with apoptotic Jurkat cells (but not normal cells) resulted in a significant inhibition of zymosan-induced TNF-α production. The presence of externalized PS on the cells



surface was insufficient to attenuate TNF-α release. In fact, PS-enriched cells significantly increased TNF-α production above that seen with zymosan alone. Interestingly, Lucas *et al*.[6] observed that the combination of apoptotic cells with LPS regulated TNF-α in a bimodal pattern with early enhancement of release followed by later suppression. We next attempted to elucidate the association between TNF-α suppression with enhanced elaboration of TGF-β1 as reported earlier. Expectedly, apoptotic cells doubled the amount of TGF-β1 released by zymosan-activated RAW 264.7 macrophages. In contrast to TNF-α, PS-enriched Jurkat cells were sufficient to stimulate TGF-β1 to a magnitude similar or exceeding that seen with apoptotic cells. Further, PS_{ox}-enriched nonapoptotic Jurkat cells were slightly more efficacious relative to apoptotic cells or normal PS-enriched cells.

Macrophages utilize the toxic ROS and RNS to control microbial pathogens as part of the innate immune response. The combination of two major activities – NADPH oxidase and iNOS – is synergistic in the successful containment of invading pathogens.[7] In addition, ROS and RNS have been recently identified as important mediators and signaling molecules/pathways in macrophages.[8,9] As a result, antioxidant interventions not only serve to protect macrophages from direct damage but are also instrumental in regulating their pro- *versus* anti-inflammatory responses.[10] Proinflammatory cytokines (IL-1, TNF-α, and IFN-γ) produced by macrophages are upregulated in response to a variety of redox manipulations *in vivo* or *in vitro* particularly those that increase superoxide formation.[11–13] The common mechanism in upregulation of these inflammatory cytokines is believed to be the activation of redox-sensitive signaling pathways (MAP-Kinases) and transcription factors (NF-κB).[8,9] We report here for the first time that apoptotic cells markedly attenuate production of ROS/RNS by activated macrophages. We further established that the presence qof PS on plasma membrane surface of nonapoptotic cells – a signal that alerts macrophages about the imminent apoptotic death – was sufficient to quench production of superoxide by activated macrophages but was insufficient to inhibit LPS-induced NO$\cdot$ production in zymosan-activated macrophages. This suggests that regulation of NADPH oxidase-dependent pathways for superoxide production and iNOS-dependent mechanisms of NO$\cdot$ generation are differentially sensitive to PS-specific signaling. It is possible that suppression of superoxide production is realized, at least in part, via PS receptor.[3,14] However, other receptor(s) of apoptotic signals may be involved as PS_{ox} was even more efficacious as an inhibitor of superoxide generation which is likely recognized not only by the PS receptor.[4] It is also clear that inhibition of NO$\cdot$ production by apoptotic cells does not rely on PS signaling and requires additional recognition signals present on the apoptotic cell surface. This separation of ROS- and RNS-dependent pathways may be important in preventing indiscriminative damage to macrophages via the formation of peroxynitrite, while ROS production by NADPH oxidase is sufficient for phagosomal digestion of bacteria.

The apoptotic cell-dependent quenching of ROS/RNS should inevitably affect intracellular redox status of macrophages, hence their pro-/anti-inflammatory phenotype. Accordingly, apoptotic cells decreased release of proinflammatory TNF-α while increasing anti-inflammatory TGF-β1 production. Our findings revealed differences in the effects of apoptotic cells *versus* PS-enriched nonapoptotic cells on the production of TNF-α but not of TGF-β1. Assuming that TGF-β1 expression is associated with suppression of TNF-α,[15] it is at least safe to suggest that PS-dependent signaling is sufficient for upregulation of TGF-β1 and may be required but is insufficient for TNF-α downregulation. Thus, effects of

Figure 1 Effects of apoptotic Jurkat cells and nonapoptotic PS-enriched Jurkat cells on ROS/RNS generation and cytokine production by macrophages. (**a**) Apoptotic Jurkat cells decrease intracellular ROS production in macrophage cell lines. RAW 264.7, IC-21, and J774 macrophages (0.3×10^6/well) were preincubated with DHR (Molecular Probes, Eugene, OR, USA) (10 μg/ml for 15 min at 37°C). DHR-loaded macrophages were then exposed to either 2.5×10^6 of naïve or Fas-triggered (250 ng/10^6 cells) (MBL, Nagoya, Japan) apoptotic Jurkat cells or left untreated for 1 h. Cells were then stimulated with zymosan (0.25 mg/ml for 30 min at 37°C). In all experiments, cells were exposed to stimuli in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen Corp., Carlsbad, CA, USA). DHR-positive macrophages were assessed by flow cytometry (FACScan, Becton-Dickinson, San Jose, CA, USA) by monitoring fluorescence (ex. 485 nm, em. 530 nm, channel FL1). In total, 10 000 events were collected and analyzed using the CellQuest software (Becton-Dickinson, San Jose, CA, USA). Data for this and all succeeding figures are displayed as mean$\pm$S.D., collected from five separate experiments, except in panels (**b2**) and (**e**), where $n=6$ and 4, respectively. All statistical comparisons here and below were made relative to zymosan treatment alone using one-way ANOVA and Tukey–Kramer method for all pairwise differences between level means (*denotes $P<0.05$). (**b**) Apoptotic and PS-, PS_{ox}-enriched naïve Jurkat cells inhibit superoxide production in zymosan-activated macrophages. RAW 264.7 macrophages were preincubated for 10 min at 37°C with 10 μM DHE (Molecular Probes, Eugene, OR, USA) and then coincubated with apoptotic or naïve Jurkat cells under conditions similar to (**a**). PS-, PS_{ox}- or PC-enriched naïve Jurkat cells were also prepared by prior incorporation of liposomes containing these phospholipids (Avanti Polar Lipids, Alabaster, AL, USA) as described in (**c**). Zymosan-activated superoxide production was assessed by flow cytometry (ex. 488 nm, em. 565–585 nm, channel FL-2). Panel 1 shows a typical response of RAW 264.7 to zymosan stimulation alone. Panel 2 shows the quenching effects of the various preparations of apoptotic, naïve, and lipid-enriched Jurkat cells. (**c**) Incorporation of PS and PS_{ox} into plasma membrane of Jurkat cells. Naïve Jurkat cells were preincubated for 10 min with 10 μM NEM (Sigma-Aldrich, St. Louis, MO, USA) followed by incubation with liposomes containing either PC, PS : PC (1 : 1) or PS_{ox} : PC (1 : 1) (150 nmol total phospholipid/10^6 cells). Treated cells were stained with annexin V-FITC (0.5 μg/ml) and propidium iodide (0.6 μg/ml) (BioVision Research Products, Mountain View, CA, USA) for 5 min at room temperature and immediately analyzed by flow cytometry with simultaneous monitoring of green fluorescence (530 nm, 30 nm band-pass filter) for annexin V-FITC (FL-1) and red fluorescence (long-pass emission filter that transmits light >650 nm) for propidium iodide (FL-3). (**d**) Inhibition of macrophage production of nitric oxide by apoptotic Jurkat cells is not mimicked by nonapoptotic PS-enriched cells. Naïve or LPS-primed (0.1 μg/ml for 12 h at 37°C) RAW 264.7 macrophages were preincubated for 1 h at 37°C with 2 μM DAF-2A (Calbiochem, San Diego, CA, USA). Macrophages were then incubated with either apoptotic, PS- or PC-enriched naive Jurkat cells for 1 h. RAW 264.7 macrophages were stimulated by zymosan (0.25 mg/ml for 1 h at 37°C) and positive cells scored by fluorescence microscopy (Nikon, Eclipse TE200, FITC filter). (**e**) Differential effects of apoptotic and PS-, PS_{ox}-enriched naïve Jurkat cells on the production of cytokines (TNF-α and TGF-β1) by RAW 264.7 macrophages. Apoptotic and lipid-enriched Jurkat cells prepared as described above were incubated with RAW 264.7 macrophages for 1 h; nonphagocytosed Jurkat cells were removed by washing. Macrophages were then stimulated with zymosan (for 30 min), supernatants were collected 18 h later and cytokines measured by ELISA (R&D Systems (Minneapolis, MN, USA)

apoptotic cells on inhibition of TNF-α production cannot be assigned exclusively to PS-dependent mechanisms. Our results, however, are consistent with the known critical role of PS in TGF-β1 enhancement by apoptotic cells. Since increased levels of TGF-β1 prevent superoxide generation by NADPH oxidase in activated macrophages,[16] it is possible that PS(PS_{ox})-induced TGF-β1 expression is an important pathway contributing to PS-dependent quenching of ROS production. In conclusion, multiple signals on the surface of apoptotic cells each initiate different responses including production of ROS/RNS and change the cytokine profile to ultimately optimize anti-inflammatory state.

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