What can we learn from highly purified neutrophils?

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Abstract

Neutrophil purification has traditionally been performed by centrifugation of leucocytes through density gradients. These reliable methods produce populations that are typically >95% pure neutrophils, and have allowed the widespread study of the function of these cells. Our recent work has suggested that residual monocytes may play a more important role than has been previously realized, and suggest that for some functional experiments, further purification of cells is required to understand fully the neutrophil phenotype.

Neutrophils play essential roles in host defence through their ability to clear bacterial infections. Their inappropriate activation contributes to a variety of inflammatory diseases, from the acute respiratory distress syndrome, to asthma, chronic obstructive pulmonary disease and rheumatoid arthritis. Resolution of neutrophilic inflammation depends on the induction of neutrophil apoptosis, programmed cell death, limiting release of inflammatory mediators and toxic granule constituents. The study of the mechanisms involved in neutrophil recruitment, activation and apoptosis has been of considerable interest for many years, and has necessitated the purification of these cells *in vitro*, usually from peripheral blood. Techniques for purifying neutrophils almost universally involve the separation of granulocytes (comprising eosinophils and neutrophils) from PBMC (peripheral blood mononuclear cells) over discontinuous density gradients. Some early purification methods were associated with neutrophil activation, and a method developed by Haslett et al. [1] in the 1980s achieved widespread acceptance for its utility in producing pure granulocyte preparations that did not show an activated phenotype. This method was based on discontinuous gradients of plasma/Percoll^{TM} mixes, and results in granulocyte purities of 95–99% in experienced hands. Although still in use in many groups, other preparative techniques based on reagents such as Histopaque^{TM} also produce pure neutrophils without an activated phenotype, but still often with a few residual PBMC [2].

Our group undertook an investigation of the mechanisms and consequences of LPS (lipopolysaccharide) signalling in primary human neutrophils. LPS activates neutrophils via engagement of TLR4 (Toll-like receptor 4) [3], resulting in the induction of a characteristic proinflammatory phenotype and prolongation of cell lifespan [4]. During the course of our studies, we noted that eosinophils, which are supposed to respond to LPS with increased lifespan, did not express TLR4 [5]. Resolution of this conflict was provided by an elegant study from Meerschaert et al. [6], who showed that survival of eosinophils in response to LPS depends on the presence of CD14+ cells contaminating the eosinophil preparations. These studies prompted us to determine the level to which contaminating PBMC contributed to the regulation of neutrophil responses to LPS.

Our initial experiments used negative magnetic selection to deplete granulocyte preparations of contaminating CD14+ cells. These studies showed that CD14-depleted populations, in which the monocyte was the primary cell type to be removed, resulted in reduced LPS-driven neutrophil activation as measured by changes in the expression of adhesion molecules [5]. Moreover, CD14-depleted neutrophil populations failed to show the expected prolongation of lifespan after treatment with LPS: survival of neutrophils was enhanced over short, but not long, time courses, contradicting the results of Lee et al. [4]. The antibody used to CD14-deplete the cell populations does not block CD14 signalling. However, to exclude an effect of this antibody on the neutrophils, we repeated the experiments and found similar results with a custom-made negative selection cocktail that removed almost all PBMC including monocytes; the latter with anti-CD36, but we did not use anti-CD14 [2,5].

These experiments have revealed an important role for contaminating cells, quite probably monocytes, in regulating neutrophil survival. Re-addition of a 5% PBMC 'contamination' restores prolonged neutrophil survival in response to LPS. This is not the only surprise thrown up using these purified cells. It has been proposed that stimulation of neutrophils by LPS results in the generation of IL-1β (interleukin-1β), which, acting in an autocrine fashion, is the principal mediator of prolonged neutrophil survival [7]. We found that IL-1β did not cause activation or inhibition of apoptosis in highly purified granulocytes, and this suggests that the actions of IL-1β may be mediated by bystander cells (L. Prince, M. White and I. Sabroe, unpublished work), a

Key words: apoptosis, lipopolysaccharide, mononuclear cell, neutrophil, neutrophil purification.
Abbreviations used: IL-1β, interleukin-1β; LPS, lipopolysaccharide; PBMC, peripheral blood mononuclear cells; TLR4, Toll-like receptor 4.
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Figure 1 | Published constitutive rates of neutrophil apoptosis

Each point represents the rate of constitutive apoptosis at the indicated time point, taken from a published study (extrapolated from graphs or directly from quoted constitutive apoptosis rates). Where a study contained more than one time point, the results for all time points are shown.

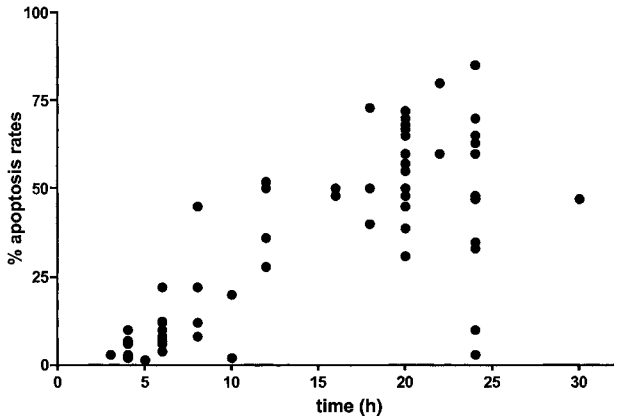
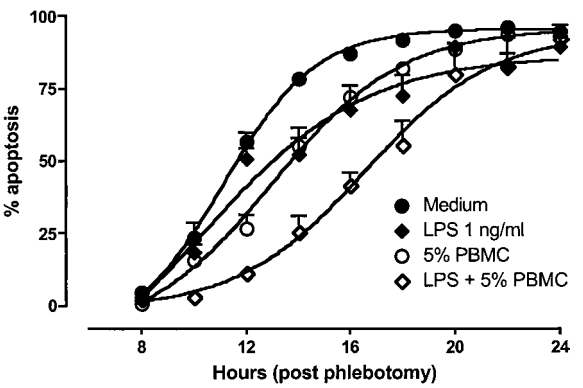


Figure 2 | Constitutive neutrophil apoptosis is abrogated by LPS and by the presence of mononuclear cells

Neutrophils were highly purified by negative magnetic selection and cultured in RPMI + 10% FCS in Falcon Flexiwell plates as described in [2]. Cells were cultured with the medium alone or with purified LPS (1 ng/ml). In other wells, autologous PBMC or autologous PBMC + LPS (1 ng/ml) was added. Apoptosis was measured by morphology at the indicated time points post phlebotomy. Results are $n = 4$, each from a separate donor, with all donors being studied in a single experiment. There was no loss of cell viability as indicated by Trypan Blue staining over the course of the experiment.



result consistent with the predominant expression of the IL-1 decoy receptor by human neutrophils [8]. Another paper [9], appearing in this volume, has reported that the fractional increase in the survival effect of TNFα observed when this cytokine is added at 6 h rather than at 0 h is also PBMC-dependent.

We note with interest that constitutive neutrophil apoptosis rates published in the literature show marked variations (Figure 1). We hypothesize that these variations in apoptosis rates may be, in part, due to different levels of monocyte contamination and cell activation. To explore the effects of PBMC on constitutive neutrophil apoptosis, we conducted an experiment where cell death was measured at frequent intervals over a 24 h period in neutrophils prepared from four individual donors and studied in parallel (Figure 2). These experiments used a concentration of LPS which we have shown to be an effective stimulator of L-selectin shedding and to induce prolongation of neutrophil lifespan over short time courses [2]. The experiment showed that the addition of an efficacious dose of LPS was no more effective at prolonging lifespan than was the presence of unstimulated PBMC, but that the combination of the two had a synergistic interaction resulting in a very significant increase in cell survival. These results would be consistent with a model in which control of cell activation is at least partially separated from regulation of cell survival. Such a system could provide a control on the process of inflammation, potentially preventing a resolving local infection from generating a long-lived population of highly activated and potentially destructive neutrophils that could cause host damage. If this were the case, it would provide another role for monocytes/macrophages in the regulation of inflammation, facilitating/determining the resolution of infection as well as its initiation.

In conclusion, studies with highly purified cells have revealed an important role for contaminating PBMC in a number of responses previously assumed to be solely due to direct activation of the neutrophil. Monocytes respond to many inflammatory mediators by the release of a panoply of neutrophil-activating molecules, and by extension, PBMC contamination may be playing roles in other responses as yet not investigated.

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