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The Role of Interleukin-1β in Direct and Toll-Like Receptor 4-Mediated Neutrophil Activation and Survival

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The regulation of systemic and local neutrophil activation is crucial to the clearance of infections and the successful resolution of inflammation without progress to tissue damage or disseminated inflammatory reactions. Using purified lipopolysaccharide (pLPS) and highly purified neutrophils, we have previously shown that Toll-like receptor 4 signaling is a potent neutrophil activator, but a poor stimulator of survival. In the presence of peripheral blood mononuclear cells (PBMCs), however, pLPS becomes a potent neutrophil survival factor. Interleukin (IL)-1β has been identified as an important neutrophil activator and prosurvival cytokine, and is produced in abundance by LPS-stimulated PBMCs. We now show that IL-1β fails to activate highly purified neutrophils or enhance their survival, but in the presence of PBMCs, IL-1β induces neutrophil survival. We hypothesized that LPS-primed neutrophils might become responsive to IL-1β, but were unable to demonstrate this. Moreover, IL-1ra failed to prevent pLPS + PBMC-dependent neutrophil survival. In studies of IL-1R1$^{-/-}$ mice, we found that LPS was still able to mediate neutrophil survival, and neutrophil survival was enhanced by the addition of monocytic cells. Thus an important paradigm of neutrophil regulation needs to be viewed in the context of a cellular network in which actions of IL-1β on neutrophils are indirect and mediated by other cells. ***(Am J Pathol 2004, 165:1819–1826)***

The regulation of neutrophil function in the circulation and at tissue sites is essential to allow clearance of infections without persistent damaging inflammation. Neutrophils rapidly undergo apoptosis (programmed cell death[1]) facilitating their clearance by tissue macrophages[2] in a process considered to be an effective injury-limiting way of removing potentially dangerous inflammatory cells from sites of inflammation. Previous studies have shown that neutrophil apoptosis is sensitive to inhibition by proinflammatory mediators such as lipopolysaccharide (LPS). Therapies to prevent this prolonged survival at sites of inflammation may be useful treatments for the many diseases characterized by neutrophilic inflammation, ranging from arthritis to asthma to chronic obstructive pulmonary disease and acute respiratory distress.[3] Interleukin (IL)-1β has also been demonstrated to delay constitutive neutrophil apoptosis,[4–7] and to be important in LPS-mediated cell survival, in which it is thought to act as an autocrine prosurvival cytokine released from LPS-treated neutrophils.[7] This IL-1β-mediated delay of apoptosis has been associated with the up-regulation of anti-apoptotic members of the Bcl-2 family, eg, Mcl-1.[8]

As well as its roles in the regulation of apoptosis, IL-1β plays a key role in the regulation of neutrophil recruitment through the induction of adhesion molecule expression on endothelium and local chemokine production (including IL-8). Thus IL-1β signaling has been considered a target for the treatment of neutrophilic inflammation. The promising anti-inflammatory actions of IL-1ra in, for example, human arthritis[9] might in part be mediated by a reduction in the recruitment to the joint, and the subsequent survival of, inflammatory neutrophils. IL-18 has also been implicated in the development of acute pulmonary

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inflammation by increasing lung vascular permeability, cytokine production, and neutrophil infiltration.[10] IL-18 uses similar intracellular signaling pathways to IL-1β and Toll-like receptors (TLRs),[11] and it has been shown to activate peripheral blood neutrophils by stimulating cytokine and chemokine release, degranulation, respiratory burst, and the up-regulation of CD11b expression.[12] We also found previously that IL-18 caused a relatively minor up-regulation of CD11b expression.[13] Despite its potency in other areas of neutrophil activation, IL-18 was unable to modulate apoptosis.[12]

In recent studies, we examined LPS responses in highly purified neutrophils, depleted of contaminating peripheral blood mononuclear cells (PBMCs) by negative magnetic selection. In contrast to the published literature, we have shown that pLPS, acting on TLR4, is a potent stimulator of neutrophil activation, but not of neutrophil survival.[13,14] Addition of low numbers of PBMCs markedly enhance the survival of neutrophils in response to LPS,[13,15] presumed to be through the release of survival cytokines.[13,15–17] We believe that this separation of control of activation and apoptosis may provide an important level of regulation of neutrophil function, so that potentially damaging populations of neutrophils activated by LPS do not continue to live beyond a time when the infection has been cleared and the inflammation resolved.[14]

Our data showing that TLR4 signals were poor stimulators of neutrophil survival, together with that of Leung and colleagues[12] and colleagues showing that IL-18 shared a similar response phenotype to LPS (inducing neutrophil activation but not delaying apoptosis), prompted us to re-examine the role of IL-1β in the regulation of neutrophil life span and activation, to determine how antagonism of IL-1 signaling might modulate neutrophilic inflammatory disease.

Materials and Methods

Reagents

General laboratory reagents were obtained from Sigma-Aldrich (Poole, UK) unless otherwise stated. Phosphate-buffered saline (PBS) and tissue culture reagents were from Invitrogen (Paisley, UK), except for fetal calf serum (FCS) (containing <0.5 EU/ml endotoxin), which was purchased from BioWhittaker (Cambrex Bioscience, Wokingham, UK). The selective TLR4 agonist, purified LPS (pLPS) from *Escherichia coli* strain K235 was prepared as described[18] and was a generous gift from Dr. S. Vogel (University of Baltimore, Baltimore, MD). IL-1β was obtained from ImmunoKontact (Abingdon, UK) and Peprotech EC (London, UK), and tumor necrosis factor-α was purchased from ImmunoKontact. GM-CSF was obtained from Sigma-Aldrich. The anti-L-selectin and anti-CD11b monoclonal antibodies were obtained from eBioscience (San Diego, CA). Anti-IL-1R2 antibody was obtained from R&D Systems (Abingdon, UK).

Animals

IL-1R1$^{-/-}$ (C57BL/6 background) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All experiments were performed in accordance with the Home Office Animal (Scientific Procedures) Act 1986.

Leukocyte Preparation

Human neutrophils and PBMCs were isolated from fresh peripheral blood by dextran sedimentation followed by either Histopaque 1077 (Sigma-Aldrich) or plasma/Percoll gradient centrifugation as described.[13,15] All human studies were approved by the Local Ethics Committee. Both methods give cells with identical functional responses.[13,15] Neutrophils were further purified by negative magnetic selection as described,[15] using a custom antibody cocktail containing antibodies to CD36, CD2, CD3, CD19, CD56, and glycophorin A (StemCell Technologies, Vancouver, Canada). The resulting highly pure cells contained small numbers of contaminating eosinophils only, with PBMC contamination being virtually undetectable.

Murine neutrophils were prepared as previously described.[19,20] Briefly, 1 ml of blood per mouse was collected from anesthetized mice by cardiac puncture. After dextran sedimentation, leukocyte-rich populations underwent magnetic purification (using antibodies to CD2, CD5, CD45R, F4/80, and ICAM-1) and neutrophils were isolated by negative selection.

Cell Culture

Unless otherwise stated, human neutrophils were cultured at a density of 2.5×10^6/ml in RPMI 1640 supplemented with 10% FCS and 100 U/L penicillin and streptomycin in Falcon Flexiwell plates (BD Pharmingen, Oxford, UK). Where indicated, neutrophils were cultured in the presence of PBMCs that were added at a final density of 1.25×10^5/ml (5% PBMCs, ie ~0.5 to 1% monocytes). Murine neutrophils were cultured as above at a density of 1×10^6/ml in RPMI with 10% FCS. In some experiments, murine neutrophils were cultured in the presence of the murine monocytic RAW 264.7 cell line, which were added at an initial density of 10%.

L-Selectin and CD11b Expression

Neutrophils were stimulated with the indicated agonists in assay buffer (Dulbecco's modified PBS containing Ca^{2+} and Mg^{2+}, supplemented with 2% FCS, 10 mmol/L HEPES, and 0.18% glucose, pH 7.3 to 7.4) for 1 hour at 37°C. Cells were washed in ice-cold fluorescence-activated cell-sorting buffer (PBS without Ca^{2+} and Mg^{2+}, supplemented with 10 mmol/L HEPES and 0.25% bovine serum albumin, pH 7.3 to 7.4) and L-selectin and CD11b expressions were determined by dual staining and flow cytometry using a FacsCalibur flow cytometer (BD Immunocytometry Systems, San Jose, CA) as described,[13,15] with appropriate isotype controls, and single-stained samples for setting of compensation. Data were quanti-

fied as percent change in geometric mean fluorescence intensity of buffer-treated cells,[13] using consistent gates between experiments.

Measurement of Apoptosis

Neutrophils were removed from culture and apoptosis was determined by light microscopical examination of duplicate cytospin preparations, as previously described.[21] Samples were typically counted blinded, and previous studies[22–24] have shown a very close correlation between apoptosis scored by this methodology and that scored by staining for Annexin V binding and loss of surface CD16. In additional experiments, samples were stained with a fluorescent Annexin V conjugate and apoptosis measured by flow cytometry as previously described,[15] using consistent gates between experiments.

Cytokine Generation

In duplicate wells, highly purified neutrophils (150 μl at a density of 5×10^6/ml) were cultured in the presence or absence of monocytes (added to achieve a final density of 2.5×10^5/ml), for 24 hours with IL-1β or pLPS. Cell-free supernatants were prepared and IL-8 measured by enzyme-linked immunosorbent assay (National Institute of Biological Standards and Control, Potters Bar, UK) as previously described.[13]

Statistics

When two data sets were compared, Student's *t*-test was used. In all cases in which more than two data sets were compared, data were analyzed using analysis of variance, and specific comparisons between indicated data points performed using an appropriate post test (detailed in the figure legends). Data were analyzed using Prism 4 software (GraphPad Software, San Diego, CA).

Results

IL-1β Is Unable to Cause Neutrophil Survival in the Absence of PBMCs

Previous reports have described an anti-apoptotic effect of IL-1β on neutrophils,[4,7] but these experiments studied cells purified by methods that typically leave a detectable level of contaminating PBMCs. To examine the effects of IL-1β on neutrophil apoptosis in the absence of PBMCs, highly purified neutrophils were prepared by negative selection and were treated with IL-1β for 4 and 22 hours. Figure 1A shows that a concentration range of IL-1β or IL-18 failed to modulate neutrophil apoptosis at either time point when apoptosis was quantified by morphology. In a further series of experiments, similar results were seen when apoptosis was quantified by detection of Annexin V binding (Figure 1B). In contrast, pLPS (1 ng/ml) caused neutrophil survival at 4 hours, consistent with previous observations.[13] IL-1β from two other sources

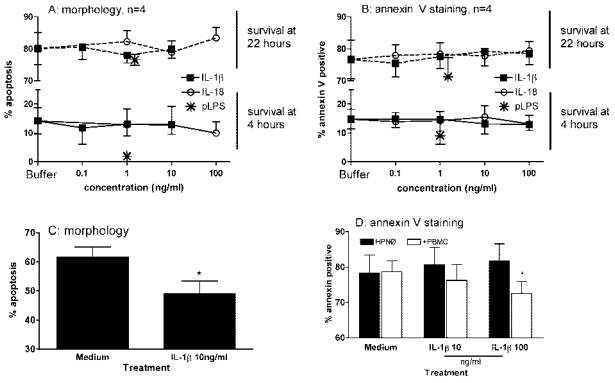

Figure 1. IL-1β does not modulate neutrophil apoptosis in the absence of PBMCs. **A:** Highly purified neutrophils were stimulated with IL-1β (0.1 to 10 ng/ml; **filled squares**), IL-18 (0.1 to 100 ng/ml; **open circles**), or pLPS (1 ng/ml; **asterisks**) for 4 and 22 hours, and rates of apoptosis were determined by morphology. **B:** In a separate series of experiments cells were stimulated with these agonists and rates of apoptosis were determined by binding of Annexin V, measured by flow cytometry. Charts show mean ± SEM data from four independent experiments. **C:** PBMCs (5%) were added back to populations of highly purified neutrophils and treated with IL-1β for 22 hours. Apoptosis was quantified by morphology ($n = 7$, ±SEM, analyzed versus buffer-treated control using a paired *t*-test in which an **asterisk** indicates $P < 0.05$). **D:** Highly pure neutrophils (HRN0) were treated with IL-1β in the presence or absence of 5% PBMCs, and apoptosis quantified by Annexin V binding ($n = 4$, ±SEM; *, $P < 0.05$ for effect of PBMCs on responses to 100 ng/ml of IL-1β, two-way analysis of variance with Bonferroni's post test).

was also tested and were verified for their ability to induce activation of the IL-8 promoter in transfection-based reporter assays in our laboratories, but both of these preparations were also unable to cause neutrophil survival (data not shown).

In contrast, in the presence of PBMCs (5% of total cell number), IL-1β modestly delayed neutrophil apoptosis at 22 hours (mean apoptosis in control populations at 22 hours, 61.8 ± 3.0% compared to 49.1 ± 4.3% in populations treated with 10 ng/ml of IL-1β), at levels in keeping with previously published IL-1β-mediated neutrophil survival data[8] (Figure 1C). Similar results were seen in a further set of experiments when apoptosis was quantified by Annexin V binding, in which IL-1β failed to induce neutrophil survival in the absence of PBMCs, but induced a neutrophil survival response in the presence of PBMCs (Figure 1D).

Neutrophils Do Not Modulate Adhesion Molecule Expression after IL-1β Treatment

Classical activators of neutrophil function such as LPS, fMLP, and chemokines induce L-selectin shedding,[15] and mediators such as LPS and IL-1β also activate NF-κB and MAPKs, and hence proinflammatory gene transcription.[25] To determine the effects of IL-1β on L-selectin shedding of highly purified neutrophils, cells were treated with two concentrations of IL-1β, in comparison with pLPS and fMLP. L-selectin and CD11b expressions were determined by flow cytometry. Unlike pLPS and fMLP that caused L-selectin shedding and up-regulation of CD11b expression, IL-1β had little effect on expression of either of these adhesion molecules (Figure 2).

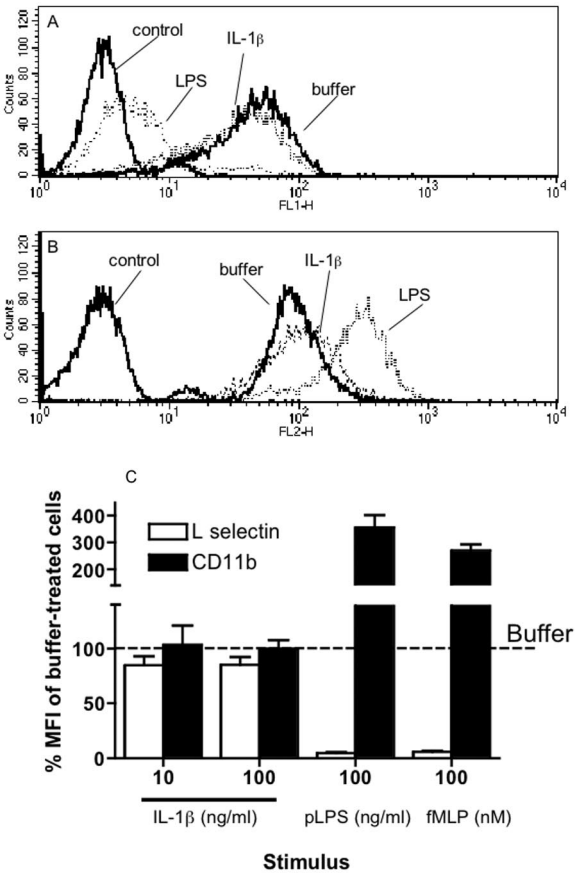

Figure 2. IL-1β is unable to modulate neutrophil adhesion molecule expression. Highly purified neutrophils were treated with the indicated concentrations of IL-1β, pLPS, or fMLP. Expression of L-selectin and CD11b was determined by flow cytometry and data was expressed relative to the geometric mean fluorescence intensity of buffer-treated controls. **Top**: Representative histograms of L-selectin (**A**) and CD11b (**B**) expression after activation by the indicated agonists. The control histogram is binding of an isotype-matched control antibody; buffer indicates cells treated with buffer at 37°C before measurement of CD11b and L-selectin expression. **C:** Mean data (n = 3, ±SEM).

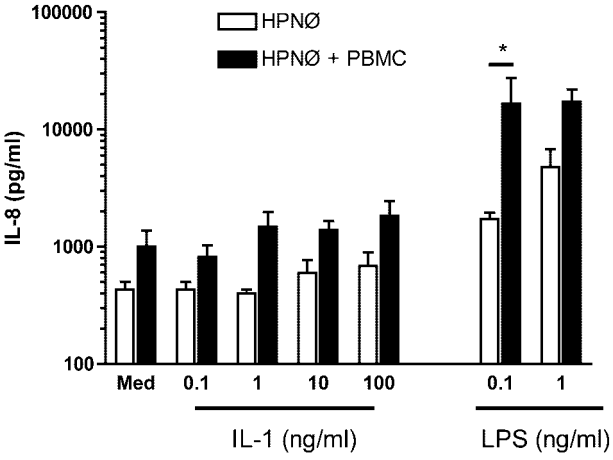

Figure 3. IL-1β is unable to enhance neutrophil IL-8 generation. Highly purified neutrophils were stimulated for 24 hours with a concentration range of IL-1β (0.1 to 100 ng/ml), pLPS (0.1 to 1 ng/ml), or medium alone, either in the presence (**filled bars**) or absence (**open bars**) of 5% PBMCs. Cell-free supernatants were prepared and IL-8 measured by enzyme-linked immunosorbent assay. IL-1β was unable to significantly enhance IL-8 generation in any condition tested. Data shown are mean ± SEM from three independent experiments. *, $P < 0.05$ indicates statistically significant change in IL-8 production within indicated pair, analyzed by two-way analysis of variance and Bonferroni's post test.

Neutrophils Do Not Secrete IL-8 in Response to IL-1β Treatment

In separate experiments, highly purified neutrophils were treated with IL-1β for 24 hours in the presence or absence of 5% PBMCs. Identical wells were also set up for pLPS as a positive control. Purified LPS induced neutrophil IL-8 generation, which was amplified by co-incubation with PBMCs (Figure 3). In contrast, IL-1β failed to induce a concentration-dependent increase in IL-8 generation, either in the absence or presence of monocytes.

Priming Neutrophils with LPS Does Not Render Them Sensitive to Anti-Apoptotic Effects of IL-1β

Neutrophils express the decoy, nonsignaling IL-1 receptor (IL-1R2), which is shed from LPS-stimulated cells.[26] We therefore hypothesized that LPS-primed cells might become more sensitive to the actions of IL-1β. Highly purified neutrophils were pretreated with medium alone or pLPS (at a concentration causing effective activation of neutrophils as shown by assays of L-selectin shedding) for 4 hours before the addition of IL-1β, IL-18, or GM-CSF for a further 18 hours. GM-CSF proved to be a potent neutrophil survival factor, whose actions were enhanced by pLPS pretreatment, but in contrast, IL-1β and IL-18 were unable to delay apoptosis even after pLPS pretreatment (Figure 4). In further experiments, we attempted to reduce availability of IL-1R2 on the neutrophil surface, proposing that this would enhance interactions of IL-1β with any cell surface IL-1R1. We therefore pretreated neutrophils with a high concentration (≤100 μg/ml) of a blocking anti-IL-1R2 antibody before stimulation, but this

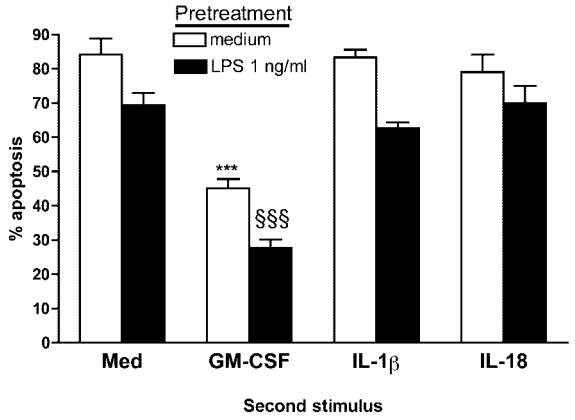

Figure 4. pLPS pretreatment does not render neutrophils sensitive to anti-apoptotic effects of IL-1β. Highly purified neutrophils were pretreated with medium alone or pLPS (1 ng/ml) for 4 hours before the addition of a selection of cytokines comprising of IL-1β (10 ng/ml), GM-CSF (50 U/ml), and IL-18 (100 ng/ml) for a further 18 hours. Apoptosis was assessed by light microscopy. Data shown are means (±SEM) from four independent experiments (***, $P < 0.001$ indicates change compared to medium alone; §§§, $P < 0.001$ indicates change in populations pretreated with pLPS, analyzed by analysis of variance with Tukey's post test).

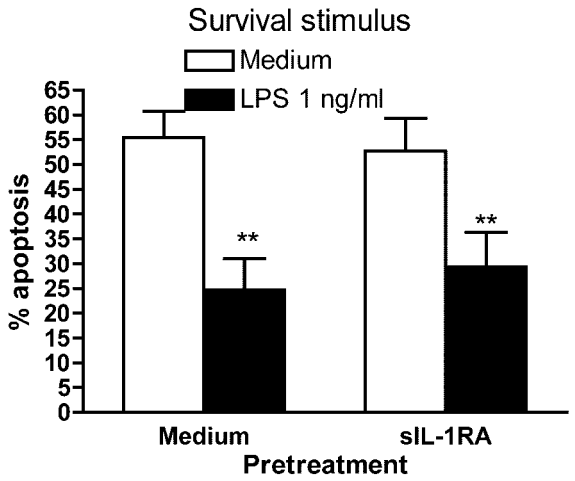

Figure 5. Soluble IL-1ra does not prevent pLPS-mediated survival. Highly purified neutrophils were pretreated with medium alone or IL-1ra (1 mg/ml shown here, lower concentrations were also found to be ineffective) for 4 hours before the addition of pLPS (1 ng/ml) for a further 22 hours. Apoptosis was assessed by light microscopy. Data shown are means (±SEM) from three independent experiments (**, $P < 0.01$ indicates change compared to medium alone, analyzed by two-way analysis of variance with Bonferroni's post test).

failed to allow IL-1β (10 ng/ml) to induce neutrophil survival after 22 hours ($n = 4$, data not shown).

Soluble IL-1ra Does Not Block LPS-Mediated Survival

Our data showed that IL-1β was inactive on purified neutrophils, but had some ability to delay constitutive apoptosis when 5% PBMCs were present. We therefore proposed that IL-1β might be relevant in neutrophil survival, but that this would be through a more complex mechanism in which it could serve to enhance monocyte-dependent release of neutrophil prosurvival cytokines. To investigate this possibility we treated neutrophil/5% PBMC co-cultures with pLPS, after pretreatment with IL-1ra for 30 minutes. Apoptosis was measured at 22 hours. Figure 5 shows that IL-1ra was unable to inhibit the LPS-mediated, monocyte-dependent delay of apoptosis seen at this time point. IL-1ra was shown to be biologically active in several other laboratory assays (data not shown).

IL-1R1$^{-/-}$ Murine Neutrophils Exhibit Delayed Apoptosis with LPS Treatment

To further investigate the role of IL-1β in LPS-mediated delay of apoptosis, we obtained mice deficient in the IL-1 signaling receptor, IL-1R1. Neutrophils (typically 90% pure) prepared from mice deficient in IL-1R1 were examined for their responsiveness to cLPS. Commercial LPS was able to delay constitutive apoptosis at both 6 (Figure 6A, $P < 0.001$) and 16 (Figure 6B, $P < 0.05$) hours, showing that LPS-mediated neutrophil survival could be effectively driven in the absence of IL-1 signaling. The prominent survival response to LPS at late time points almost certainly relates to the presence of PBMCs in the

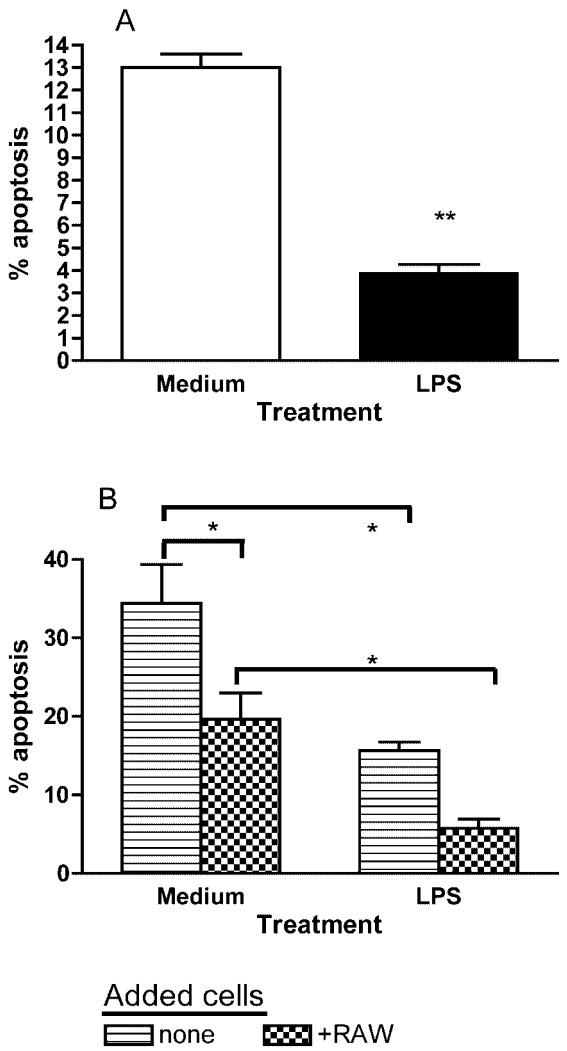

Figure 6. IL-1R1$^{-/-}$ neutrophils exhibit delayed apoptosis with LPS. Neutrophils isolated from IL-1R1$^{-/-}$ mice were cultured with cLPS (1 μg/ml) for 6 (**A**) and 16 (**B**) hours. RAW 264.7 cells (10%) were added to 16-hour cultures. Apoptosis was assessed by light microscopy. Data shown are means from three independent experiments. **A:** **, Significant difference of $P < 0.01$ between medium and LPS-treated cells, determined by *t*-test. **B:** *, Significant difference of $P < 0.05$ between indicated conditions, analyzed by analysis of variance and Tukey's post test.

cell preparations, which cannot be purified to the same degree as human cells. The addition of a murine monocytic cell line (RAW 264.7) that is IL-1R1 sufficient further delayed both constitutive and LPS-inhibited apoptosis (Figure 6B, $P < 0.05$).

Discussion

The proinflammatory cytokine, IL-1β, has a critical role in the development of numerous local and systemic inflammatory diseases,[27] and its receptors and signaling pathways represent major targets for the development of new therapeutics targeting the many pathologies characterized by marked neutrophil infiltration. It is thought that the actions of IL-1β are mediated both by increased cellular activation and by the delay of apoptosis because many cell types have been demonstrated to be susceptible to

the anti-apoptotic effects of IL-1β, including neutrophils.[4,7,8,28] These studies now require reinterpretation because they were performed using populations of neutrophils purified in such a way that leaves low but significant numbers of contaminating PBMCs. We previously demonstrated that signaling via TLR4, a member of the IL-1 receptor superfamily, resulted in a moderate delay in apoptosis of highly purified neutrophils that was greatly amplified by levels of PBMCs (5%), similar to those found in neutrophil populations prepared by standard gradient purification techniques.[13,14] In these experiments, we observed that TLR4 activation caused PBMC-dependent neutrophil survival, whereas TLR2 activation did not. In keeping with these data, TLR4 activation of PBMCs caused a marked generation of IL-1β that was not evident in PBMCs treated with a TLR2 agonist, potentially implicating IL-1β in PBMC-dependent, TLR4-mediated, neutrophil survival. However, in keeping with other published data,[12] we also found that IL-18 was unable to induce neutrophil survival. Because TLR4 and IL-18 activate not dissimilar signaling pathways, but are poor regulators of neutrophil survival, we revisited the paradigm of IL-1-mediated regulation of neutrophil apoptosis.

We found that populations of highly purified neutrophils that were depleted of contaminating monocytes were generally unresponsive to the effects of IL-1β, with respect to both apoptosis and other markers of cellular activation, including IL-8 generation and L-selectin shedding. These data, and other work published previously,[13,15] show that cells retained their responses to other prosurvival and activation stimuli, making it unlikely that the neutrophils had become unresponsive to IL-1β as an artifact of their preparation. Interestingly, when low numbers (5%) of mononuclear cells were added back to neutrophil suspensions, IL-1β was able to moderately inhibit neutrophil apoptosis at late time points.

Similar to our observations in the presence of PBMCs, Moulding and colleagues[8] showed IL-1β had a modest yet statistically significant effect on neutrophil apoptosis at 12 hours, which they associated with an increase in Mcl-1 expression. An elegant study by Watson and colleagues[7] supported the significant body of literature showing a role for IL-1β in neutrophil responses, and found that LPS mediated anti-apoptotic effects on neutrophils via the autocrine production of IL-1β after caspase-1 activation. Furthermore, this group demonstrated that LPS-mediated delay of apoptosis could be partially blocked using soluble IL-1ra and a blocking antibody to IL-1β. Our data, showing that IL-1β exerts a modest survival effect only when PBMCs are present, suggest that the effect observed by Watson and colleagues[7] may have been moderated by PBMCs present in cell preparations. In our co-cultures of neutrophils and 5% PBMCs, we found that soluble IL-1ra was unable to abrogate the survival effects induced by pLPS, suggesting IL-1β was not the major factor responsible for the observed delay in apoptosis, although it could still have been playing a modest but redundant role in amplifying the actions of monocyte-derived neutrophil survival factors. These effects are summarized in Figure 7. The identity of the neutrophil survival factor released from LPS-stimulated PBMCs in co-cultures remains uncertain. A likely candidate would be GM-CSF, but we have previously shown that LPS, but not Pam_3CSK_4, can cause PBMC-dependent neutrophil survival, yet these agonists caused the generation of similar (and low) quantities of GM-CSF from PBMCs.[13] Thus, GM-CSF is unlikely to be the survival mediator, and the identity of these mediator(s) is the subject of further work in our group.

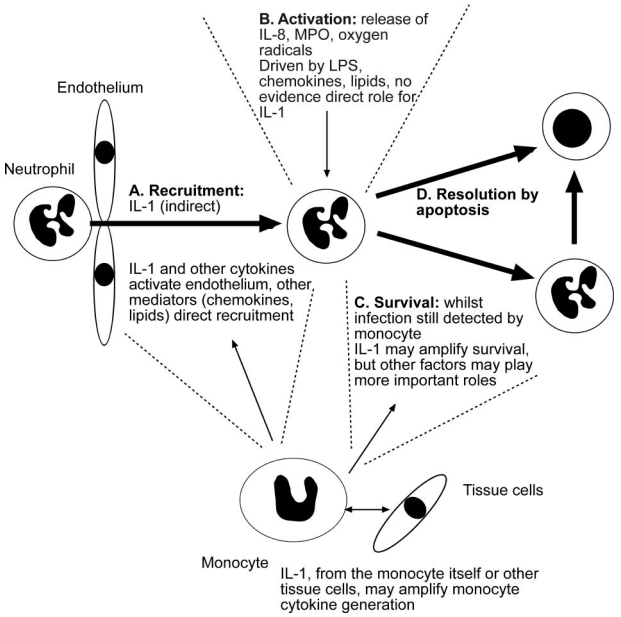

Figure 7. Compartmentalization of neutrophil regulation. Neutrophil recruitment is under the control of tissue cells, whose cytokines cause up-regulation of endothelial adhesion molecule expression and provide directional migration cues (**A**). At sites of inflammation, mediators such as LPS activate the cell (**B**), but do not prolong life span to the same degree. Thus, the ongoing need for a surviving population of neutrophils may again be determined by tissue cells or monocytes (**C**), whose activation is in turn regulated. Ultimately, resolution of neutrophilic inflammation proceeds by apoptosis (**D**). IL-1 may play roles in aiding neutrophil recruitment by actions on endothelium, and as a mediator activating cells such as monocytes to produce neutrophil survival factors, perhaps in autocrine loops, but does not appear to have a major direct role in regulating neutrophil survival.

Using negative magnetic selection techniques[19] we were able to examine neutrophil apoptosis rates in cells purified from the peripheral blood of IL-1R1 knockout mice. Neutrophils treated with LPS showed delayed apoptosis with LPS at both early (6 hours) and late time points (16 hours, Figure 6). These cell preparations were ~90% pure neutrophils, and it is likely that the 16-hour, LPS-induced neutrophil survival is again a reflection of amplification of LPS signaling via mononuclear cell contaminants. The addition of LPS-responsive cells, in the form of an IL-1R-sufficient murine monocytic line, further amplified the survival effect of LPS. These actions of LPS could not be mediated by IL-1β actions on the neutrophils, because they did not possess the functional type 1 IL-1R, excluding a major direct role for IL-1β in LPS-mediated delay of apoptosis. Interestingly, these data are slightly different from work performed in the caspase-1 (interleukin-1-converting enzyme, ICE) knockout mouse. In this strain, we found that neutrophils (in populations also containing PBMC contaminants) displayed significantly lower rates of constitutive apoptosis compared to

wild-type mice, which were not significantly further delayed by LPS.[20] Overall, it is likely that IL-1β has a small role to play in regulating indirect neutrophil apoptosis, by acting on the mononuclear cells to enhance release of prosurvival cytokines. This would be consistent with our data showing some survival effect of IL-1β in the presence of PBMCs, and would suggest that in the caspase-1 knockout mouse, this relatively modest IL-1β contribution to neutrophil survival could not be seen because of the greater effect of this knockout on constitutive apoptosis rates.

Other information from *in vivo* studies also supports only a minor role for IL-1β in neutrophil activation and survival. Our recent study showed that caspase-1 knockout mice had an enhanced neutrophilic infiltrate in a lung inflammation model despite lower levels of IL-1β.[20] A study by Mizgerd and colleagues[29] showed that TNFR1/IL-1R1-deficient mice demonstrated compromised neutrophil emigration on *E. coli* challenge, but show normal levels of neutrophil numbers in the circulation and in alveolar septae. Furthermore, Parsey and colleagues[30] found that both neutrophil recruitment and levels of neutrophil apoptosis after hemorrhage or endotoxemia were unaffected in IL-1β-deficient mice, compared to wild-type strains.

Neutrophils produce IL-1β[31,32] in response to LPS, but in addition, it has recently been shown that they can rapidly internalize IL-1β via IL-1R2, allowing the cells to scavenge and clear IL-1β.[33] Moreover, soluble IL-1R2 is released from the cell surface in a regulated manner (induced by dexamethasone or IL-4), resulting in neutralization of IL-1 responses.[34,35] Freshly isolated human neutrophils also express low levels of IL-1ra that are augmented by treatment with LPS,[36,37] although IL-1ra protein is not induced by IL-1β treatment.[37] Thus, the peripheral blood neutrophil possesses many mechanisms to limit IL-1 responses and fails to respond to IL-1β directly. It remains possible that tissue neutrophils regulate their expression of IL-1Rs in a manner enabling direct proinflammatory responses, but various knockout mice also suggest that the role for IL-1β in neutrophilic inflammation may be less than previously thought.[20,29,30]

Our discovery that neutrophils are poorly responsive to IL-1β, and the evidence that they can act as a sink for IL-1β,[33] potentially has great physiological significance. Monocytic cells respond to pathogens by the induction of IL-1β generation, which in co-cultures can activate tissue cell types to facilitate proinflammatory responses.[38,39] IL-1β is a major stimulator of leukocyte recruitment, through its ability to up-regulate adhesion to endothelial cells,[40] and is important in host defense.[41] It is likely that monocyte/macrophage-derived IL-1β has a role in the induction of leukocyte recruitment and proinflammatory responses. We have also shown that PBMCs regulate and amplify many neutrophil responses to LPS,[13–15] yet these responses are independent of IL-1β, to which we have shown neutrophils are unresponsive. These data could be consistent with a degree of compartmentalization of regulation of neutrophil recruitment and inflammation, facilitating specific regulation of each phase of the inflammatory response (Figure 7). Our data suggest that targeting of IL-1 may be effective in neutrophilic inflammation more by reducing neutrophil recruitment than inhibiting neutrophil activation and enhancing apoptosis. A therapeutic IL-1 receptor antagonist is showing promise in diseases such as rheumatoid arthritis in which neutrophil recruitment is ongoing.[42] Targeting IL-1 therapeutically may perhaps be less effective in facilitating resolution of established acute neutrophilic activation such as occurs in the acute respiratory distress syndrome, which can occur secondarily to systemic sepsis. In the light of previous data demonstrating a role for PBMC contamination in neutrophil TLR responses,[13] we also wonder what other responses previously attributed exclusively to direct actions of soluble mediators on human neutrophils may also, in fact, be amplified through PBMCs.

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