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# Balance between autocrine interleukin-1b and caspases defines life versus death of polymorphonuclear cells.

## Abstract

The role of endogenous IL-1b in regulating spontaneous and Fas-triggered apoptosis of human PMN has been studied in relation to the activity of the IL-1b-generating enzyme ICE (caspase-1), an enzyme also involved in the mechanism of cell death. Upon in vitro culture, PMN undergo spontaneous apoptosis and express increasing levels of IL-1b, caspase-1- and caspase-3-like enzymes.

Endogenous IL-1b protects PMN from apoptosis, since inhibition of either IL-1b or caspase-1 activity can accelerate PMN apoptotic death. Thus, in spontaneous PMN apoptosis caspase-1 essentially plays an anti-apoptotic role by inducing maturation of protective IL-1b, whereas other molecules are responsible of driving apoptosis. Upon Fas triggering, PMN apoptosis is greatly accelerated, in correlation with increased caspase activity, whereas IL-1b production is not augmented. Inhibition of IL-1b activity can increase Fas-induced apoptosis, whereas caspase-1 inhibitors are without significant effect. It is hypothesized that in Fas-induced PMN apoptosis caspase-1 has a double role: it can protect from apoptosis through generation of protective IL-1b, as in spontaneous apoptosis, and it can also exert pro-apoptotic activity which counterbalances the protective effect and allows accelerated apoptosis.

## Full text

### INTRODUCTION

Polymorphonuclear leukocytes (PMN), the major cell population involved in acute inflammatory response, represent the first line of defence against infection and injury. In response to chemotactic stimuli, PMN are quickly recruited into the inflammed site where they eliminate invaders thanks to their potent phagocytic and degradative abilities. Having accomplished their defence function, ageing PMN are removed upon recognition and engulfment by macrophages. The process of recognition by scavenger macrophages correlates with the apoptotic state of senescent PMN [1, 2]. In fact, among human blood leukocytes PMN have the shortest life span (6-10 hours in circulation and 1-2 days in the tissue after margination), a feature of critical importance for the resolution of inflammatory reactions and for re-establishment of physiological conditions [2]. Thus, PMN apoptosis appears to be a crucial event for *in vivo* homeostasis of inflammatory processes and must be subjected to tight regulation to avoid pathological derangements.

The life span of PMN can be increased by inflammatory mediators and cytokines, thus allowing prolonged PMN persistence at the inflammatory site [3, 4]. Of particular interest in this context is IL-1, one of the earliest cytokines responsible for the development of inflammatory responses, which can prolong PMN survival in culture by delaying spontaneous apoptosis [4]. IL-1 is a family of cytokines which includes at least three members: two IL-1 isoforms with comparable

agonist biological activities (IL-1alpha and IL-1beta) and one receptor antagonist with inhibitory functions (IL-1Ra) [5]. IL-1beta is the main type of IL-1 produced during inflammatory processes and the one mainly responsible for inflammatory events. The mature 17 kDa active form IL-1beta is derived from an inactive precursor of 31 kDa (proIL-1beta). ProIL-1beta cleavage is carried out by a peculiar cysteine protease named ICE (interleukin-1 converting enzyme) or caspase-1 [6]. Beside its role in IL-1beta maturation, a large body of evidence in recent years has also attributed to caspase-1 and to several other caspases, a crucial role in the post-receptor cascade of events leading to cell apoptosis, in particular in the Fas/APO-1 triggered apoptotic mechanism [7-9]. PMN express Fas on their membrane and are thus very sensitive to Fas-triggered apoptosis [10-12]. Both spontaneous and Fas-triggered PMN apoptosis apparently involve the activity of pro-apoptotic caspases [13, 14].

On the basis of these observations, the effect of the balance between endogenous IL-1beta production and caspase activity in determining human PMN apoptosis *versus* survival has been examined in this study. Both caspase activity (caspase-1- and caspase-3-like) and bioactive IL-1beta are detectable in unstimulated PMN cultures. Endogenous IL-1beta is apparently responsible for prolonging cell survival in an autocrine fashion, since blocking of IL-1 with either caspase-1 or IL-1 inhibitors increases PMN apoptosis. On the other hand, when PMN undergo Fas triggering, their accelerated apoptosis correlates with increased caspase activity but not with augmentation of mature IL-1beta. It is thus proposed that the constant levels of mature IL-1beta in PMN play an anti-apoptotic role and that survival *versus* death is determined by the balance between IL-1beta and pro-apoptotic caspases, both of which are upregulated by apoptotic stimuli.

## MATERIALS AND METHODS

### *Blood cell isolation and culture*

PMN were purified in heparinised human blood obtained from healthy adult volunteers (Italian Red Cross, and National Research Council, L'Aquila, Italy). Briefly, 10 ml of blood were diluted 1:5 with sterile saline solution, centrifuged at 350 x g for 10 min at RT without braking, resuspended with 35 ml saline, layered onto 10 ml of Ficoll-Paque density gradient (Pharmacia, Uppsala, Sweden) and centrifuged at 400 x g for 20 min at RT.

In order to obtain purified PMN, the pellet was washed twice with saline and resuspended with 1.5% Dextran T-500 (Pharmacia). After RBC were allowed to settle at RT for 30 min, PMN were recovered from the supernatant and washed with saline. Contaminating RBC were lysed with lysing buffer (8.29 g/l $NH_4Cl$, 3.7 mg/l $Na_2EDTA$, 1 g/l $KHCO_3$). Purity and viability of PMN preparations were greater than 95% as determined by Diff-Quik staining followed by morphological analysis and trypan blue dye exclusion, respectively. Preparations with detectable platelet contamination were discarded.

PMN were suspended in RPMI-1640 (Life Technologies, Paisley, UK) supplemented with 2 mM L-glutamine (Life Technologies), 50 mug/ml gentamicin sulfate (Sigma Chemical Co., St. Louis, MO, USA) and 5% heat-inactivated human AB serum (Life Technologies). Cells were kept in suspension culture in 15 ml polypropylene tubes (Falcon, Oxnard, CA, USA) in order to minimise cell adhesion and activation, and were incubated at 37° C in a 5% $CO_2$ humidified atmosphere for different times, as indicated. Depending on the experimental conditions, PMN in culture were exposed to the following reagents: human recombinant IL-1beta (mature fragment 117-269, expressed in *E. coli*, sp. act. $1 \times 10^8$ IU/mg) [15], human recombinant IL-1Ra (expressed in *E. coli*, inhibitory activity $1.6 \times 10^6$ inhibitory units/mg, where one inhibitory unit is the amount of IL-1Ra which inhibits 50% of optimal IL-1-induced thymocyte proliferation) [16], neutralizing polyclonal antibody to human IL-1beta (produced in rabbits), blocking anti-human IL-$1R_I$ monoclonal antibody M1 and blocking anti-human IL-$1R_{II}$ monoclonal antibody M22 (kindly provided by J.E. Sims, Immunex Corp., Seattle, WA, USA through the courtesy of A. Mantovani, University of Milano, Italy), reversible ICE inhibitor Ac-Tyr-Val-Ala-Asp-aldehyde (YVAD-CHO; Calbiochem, San Diego, CA, USA), irreversible ICE inhibitor YVAD-chloromethylketone (YVAD-CMK, Calbiochem; stock solution 10 mM in DMSO), elastase inhibitor elastatinal (Calbiochem). All tissue culture media, additives and reagents were tested for endotoxin contamination. Only reagents found free of endotoxin (< 0.1 EU/ml for 1 x media/additives and < 10 EU/mg for solid reagents) were used.

### In situ *hybridisation*

cDNA fragments of human IL-1beta (fragment between EcoR/and Pst/sites or fragment between Sec and BamH/sites) and ICE (900 bp fragment between the Hind///and BamH/sites) cloned in Bluescript $KS^+$ were used as probes. Probes

were labelled with $^{35}$SdCTP using the random primers method. *In situ* hybridisation was performed as previously reported [17]. Briefly, PMN cytosmears were fixed with 4% buffered formaldehyde, dehydrated in ethanol, then sequentially rehydrated in 1x PBS with 0.1 M glycine, washed in 0.1 M Tris-HCl pH 7.5 with 50 mM $MgCl_2$, acetylated in 2x SSC, with 0.1 M triethanolamine and 0.5% acetic anhydride pH 8.0 and dehydrated in ethanol. Cytosmears were prehybridised for 10 min at 70° C with 50% formamide and 2x SSC immediately followed by o/n hybridisation at 42° C with radiolabelled probes ($10^6$ cpm/slide) in 2x SSC with 10 mM DTT, 5x Denhardt's solution, 500 mug/ml salmon sperm DNA and 10% w/v dextran sulphate. Unbound or nonspecifically bound probe was removed by washing with 2x SSC, 50% formamide and 0.1% SSC. Slides were dipped in NBT2 autoradiographic emulsion (Eastman Kodak, Rochester, NY, USA) and exposed for 10 days at 4° C in a light-tight box, then developed in D19 (Eastman Kodak), fixed in Rapid Fixer (Eastman Kodak) and counterstained with haematoxylin.

Specific binding to cellular RNA was assessed by prior RNA digestion with 100 mug/ml ribonuclease A and 10 U/ml ribonuclease T (Sigma). Nonspecific cDNA binding was evaluated by hybridisation with $^{35}$S-labeled Pst-1-digested pUC9 plasmid. The specificity of the hybridisation was assessed by cold probe inhibition by adding 50 ng/slide of cold probes in the hybridisation solutions. Cytosmears of LPS-stimulated macrophages and AT-20 cell line were included in each experiment as positive and negative controls, respectively (data not shown).

### *IL-1*beta *assays*

IL-1beta biological activity was assessed in supernatants obtained from PMN cultures. PMN preparations devoid of contaminant monocytes were used in these experiments. Endotoxin contamination of culture medium was below 0.1 EU/ml (LAL chromogenic assay). Supernatants were collected at different times as indicated, filtered through 0.22 mum Millex GV filters (Millipore, Bedford, MA, USA) and stored at - 20° C until use. One hundred mul of different dilutions of supernatants were added to 2 x $10^4$ D10.G4.1 T cells in wells of Cluster$^{96}$ plates (Costar, Cambridge, MA, USA) and proliferation was evaluated after 48 hours of incubation by measurement of $^3$H-thymidine incorporation [18]. The amount of bioactive IL-1 present in samples was determined by comparing proliferation induced by samples to that induced by a laboratory standard of purified human

recombinant IL-1beta titrated against the international reference human IL-1beta standard (86/680, 1 x $10^5$ IU/mug; NIBSC, Potters Bar, UK). All the D10.G4.1 experiments were performed in parallel with, or without a neutralising concentration of rabbit polyclonal anti-IL-1beta antibody. Assays of IL-1beta protein were performed with the use of commercially available ELISA kits (Endogen, Woburn, MA, USA).

### *Assessment of apoptosis*

PMN apoptosis was assessed on the basis of DNA loss measured by propidium iodide staining of nuclei followed by cytofluorimetric analysis. Briefly, 5 x $10^6$ cells in 12 x 75 mm polystyrene tubes were fixed by adding dropwise 5 ml of ice-cold 80% ethanol, mixing by pipetting and incubating for 30 min at 4° C. Cells were then washed once with PBS, resuspended in 1.25 ml of PBS and further incubated for 20 min at RT. After addition of an equal volume of propidium iodide solution (100 mug/ml; Sigma) containing 100 mug/ml of RNase and overnight incubation in the dark at 4° C, cells were washed with PBS and analysed by flow cytometry (FACScan, Becton-Dickinson, Mountain View, CA, USA). A minimum of 10,000 events per sample were counted and debris was excluded by gating on the basis of physical parameters. Apoptosis is expressed as the percentage of hypodiploid nuclei counted over the total cell population. Estimation of apoptotic cells by flow cytometry was always confirmed by morphological analysis of cytospin smears stained with Diff-Quik (not shown).

The variation of PMN apoptosis among 44 tested donors ranged from 0 to 32% at time zero (29 donors), from 4.5 to 39.5% at 6 hours (19 donors), from 3.6 to 71.2% at 18 hours (38 donors), from 31.5 to 88.5% at 48 hours (16 donors) and from 47.5 to 91.4% at 72 hours (4 donors), with variability experienced also among different preparations from the same donor (*e.g.* apoptosis from 4.7 to 71.2% at 18 hours in six samples from the same donor over seven months). Despite this background variability, the effects observed upon cell treatment were consistently reproducible.

### *Analysis of caspase activity*

PMN were resuspended at a concentration of 1 x $10^8$ cells/ml in hypotonic buffer containing 25 mM HEPES pH 7.5, 5 mM $MgCl_2$, 1 mM EDTA and the protease inhibitors PMSF (1 mM), pepstatin (10 mug/ml) and leupeptin (10 mug/ml) (all

from Calbiochem). After homogenisation in Dounce and microscopic examination of cell disruption, nuclei were removed by centrifugation at 2,000 x g for 20 min at 4° C. Supernatant (S2) was recovered and centrifuged again at 18,000 x g for 20 min at 4° C. On this final supernatant preparation (S20) caspase-1-like activity (caspase-1, -4 and -14) was measured by kinetic detection of fluorescent cleavage product using the selective substrate tetrapeptide YVAD-7-amino-4-methylcoumarin (YVAD-AMC; Calbiochem), whereas caspase-3-like activity (caspase-3, -6, -7, -8 and -10) was similarly assessed on the selective substrate Ac-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (DEVD-AMC; Calbiochem). Specificity controls were run by adding the protease inhibitors YVAD-CHO (caspase-1 inhibitor), DEVD-CHO (caspase-3 inhibitor; Calbiochem) and elastatinal (elastase inhibitor, negative control) together with the substrate.

## RESULTS

### *PMN express ICE and produce bioactive IL-1*beta

It is known that human PMN undergo spontaneous apoptosis in culture and that IL-1beta can decrease and delay their death (data not shown) [3]. As a first step in investigating the involvement of endogenous IL-1beta in the survival of human PMN in culture, the presence of IL-1beta and of its maturing enzyme caspase-1 was assessed in PMN cultures both as mRNA and as biologically active proteins. As shown in Figure 1, mRNA for both proIL-1beta and caspase-1 was barely detectable in fresh-ly isolated PMN but it was significantly induced upon 18 hour culture, as determined by *in situ* hybridisation. Morphological examination confirmed that IL-1beta- and caspase-1-expressing cells were in fact PMN, rather than residual monocytes. To assess the production of bioactive IL-1beta, supernatants from 18 hour PMN cultures were collected and their ability to induce proliferation of the IL-1-responsive D10.G4.1 cell line was analysed. For supernatant generation, only PMN preparations with a high purity, devoid of contaminant monocytes, were chosen and used in rigorously endotoxin-free conditions. As shown in Figure 2 (upper panel), PMN supernatants could significantly stimulate D10.G4.1 proliferation, which was completely abrogated by a neutralising anti-IL-1beta antibody. Similarly, the IL-1beta-like activity in 18 hour PMN supernatants could be significantly suppressed (> 80%) by the caspase-1 inhibitor YVAD-CHO (Figure 2, lower panel). By comparison with the standard IL-1beta proliferation curve, it was possible to calculate that $1 \times 10^6$ PMN

synthesized about 130-300 pg of bioactive IL-1beta upon 18 hours of culture in the absence of stimulation. ELISA determinations confirmed both the presence of IL-1beta protein in PMN supernatants (448 pg/$10^6$ PMN) and the inhibitory effect of 100 muM YVAD-CHO (24 pg IL-1beta/$10^6$ PMN; 95% inhibition). On the other hand, the elastase inhibitor elastatinal (100 muM) had only marginal effect on IL-1beta production from PMN (380 pg IL-1beta/$10^6$ PMN; 15% inhibition). The expression of bioactive caspase-1 in PMN lysates was confirmed by means of enzymatic cleavage of the selective substrate YVAD-AMC. Caspase-1-like activity in freshly isolated PMN was barely measurable and not significant above assay background (0.8 ± 0.8 pmoles/min/mg), but it was clearly detectable upon 18 hour culture (4.9 ± 0.3 pmoles/min/mg).

### *Endogenous IL-1*beta *inhibition increases PMN apoptosis*

In order to define the autocrine role of IL-1beta in the regulation of PMN survival, purified human PMN were cultured *in vitro* for 6-48 hours and their ability to undergo apoptosis was measured in conventional conditions and upon inhibition of endogenous IL-1beta production or activity.

As reported in Figure 3 (left panel), the apoptosis of PMN cultured for 18 hours is increased by the presence of the IL-1 receptor antagonist IL-1Ra, in a dose-dependent manner. The highest level of apoptosis was reached with 100 ng/ml of IL-1Ra, with no further significant increase observed at higher doses of antagonist (data not shown). In time course experiments, the pro-apoptotic effect of IL-1Ra could be observed over 6-48 hours of culture (data not shown). The importance of endogenous IL-1beta for the survival of cultured PMN was further supported by results obtained by other means of IL-1beta inhibition. A neutralising concentration of anti-IL-1beta polyclonal antibody could induce an increase of apoptosis comparable to that induced by 100 ng/ml of IL-1Ra (Figure 3, right panel). In parallel, a blocking antibody for type I IL-1R (M1) could also increase PMN apoptosis, whereas a control antibody recognizing the decoy type II IL-1R (M22) was without effect. These data confirm that the presence of endogenously produced IL-1beta in cultured PMN can promote cell survival by inhibiting spontaneous apoptosis.

### *Endogenous caspase-1 inhibition increases PMN apoptosis*

The role of caspase-1 in the spontaneous apoptosis of human PMN was assessed by adding reversible or irreversible inhibitors to the cells in culture. As shown in a representative experiment in Figure 4, increasing doses of the irreversible caspase-1 inhibitor YVAD-CMK could progressively increase spontaneous PMN apoptosis after 18 hours of culture. Superimposable results were obtained with the reversible inhibitor YVAD-CHO and at different periods of culture (data not shown). These results confirm the hypothesis of a rescuing role for the caspase-1 enzymatic activity in spontaneous PMN apoptosis through maturation of anti-apoptotic IL-1beta.

### *Fas triggering increases apoptosis and caspase-1 activity but not mature IL-1*beta *production*

The role of caspase-1 and IL-1beta in PMN apoptosis induced by Fas triggering was analysed. PMN were exposed to an optimal dose of an agonist anti-Fas IgM antibody, which mimics FasL and induces Fas-mediated apoptosis. As shown in Figure 5, Fas triggering induces a significant increase of PMN apoptosis. This increase in apoptosis was not accompanied by any variation in mature IL-1beta production, IL-1beta remaining at the same level as in untriggered PMN. When the activity of caspases was assayed, a significant increase in enzymatic activity could be noticed both for caspase-1-like enzymes, which however is detectable at very low levels, and for caspase-3-like enzymes, measurable only at much higher levels. Thus, caspase activities apparently correlate with PMN apoptosis, as they increased with time in culture and upon Fas triggering.

### *IL-1*beta *but not caspase-1 protects PMN from Fas-induced apoptosis*

The anti-apoptotic role of endogenous IL-1beta has been also assessed in PMN apoptosis induced by Fas triggering. Data in Table 1 show that Fas-induced apoptosis of PMN can be inhibited by addition of recombinant IL-1beta, whereas inhibition of endogenous IL-1beta with IL-1Ra can accelerate Fas-triggered apoptosis. However, the caspase-1 inhibitor YVAD-CHO did not show any significant effect on Fas-triggered apoptosis. This suggests that, whereas caspase-1 exclusively plays an anti-apoptotic role in spontaneous PMN apoptosis through generation of protective IL-1beta, in Fas-triggered apoptosis caspase-1 also becomes involved in the enzymatic cascade leading to cell death, thus counterbalancing its IL-1beta-dependent anti-apoptotic effect.

## DISCUSSION

Among leukocytes, the most peculiar biological feature of PMN is their very short life span, their ability to readily undergo apoptosis, and to be subsequently recognised and cleared by scavenging macrophages in a non-phlogistic manner. The rapid clearance of PMN, apparently without requirement for inductive stimuli, can occur both in the circulation, where they have a half-life of < 10 hours, and in inflammed tissues, where recruited PMN can survive 1-2 days [19]. Rapid removal of activated PMN by apoptosis is a feature of key importance to limit tissue injury and leads to the safe resolution of inflammation [1, 2]. During the inflammatory reaction, PMN are recruited to the reaction site and are activated by inflammatory mediators present at the site [19]. In order to be fully effective in their defensive functions (degranulation with release of proteases, production of reactive oxygen species, phagocytosis), PMN survival should be sufficiently prolonged. Indeed, in parallel to activation, inflammatory mediators such as bacterial lipopolysaccharide and the cytokine IL-1 can effectively delay PMN apoptosis, thus allowing the inflammatory reaction to fully develop [3, 4]. This prolongation in PMN life span during the development of the inflammatory reaction should however be tightly controlled and limited both topically and temporally to avoid tissue damage and unwanted pathological consequences. Due to their distinct biological function, it is therefore conceivable that different mechanisms could regulate spontaneous *versus* triggered PMN apoptosis.

Several studies have addressed the issue of spontaneous and induced PMN apoptosis. Experimental evidence indicates that the Fas/FasL system could be partially involved in the phenomenon of spontaneous PMN apoptosis [10-12]. In fact, PMN constitutively express high levels of Fas on their membrane and are therefore sensitive to apoptosis induced by Fas triggering [10, 11]. In addition, PMN also express FasL and could thus induce auto-apoptosis. Indeed, partial inhibition of spontaneous PMN apoptosis could be achieved with inhibitors of Fas/FasL binding [10]. However, other data indicate that spontaneous apoptosis could occur independently of Fas triggering. For instance, activation of p38 MAPK, which plays an important role in spontaneous PMN apoptosis, is not induced in response to Fas triggering [20]. Caspases, a large family of cysteine proteases with a major role in the mechanism of programmed cell death [7], are activated during spontaneous and Fas-triggered PMN apoptosis [9, 13]. Among caspases, caspase-3 (CPP32) is one of the pivotal enzymes involved in spontaneous and Fas-mediated apoptosis of haematopoietic cells [9, 21-23],

and it also plays a major role in the programmed cell death of PMN [14]. On the other hand, the involvement of caspase-1 (the interleukin-1-converting enzyme ICE) in the mechanism of cell death is controversial. Since the initial observations of the pro-apoptotic role of ICE, the mammalian homologue of the *C. elegans* death gene *ced-3* [8], a wealth of data have revealed that the family of ICE-like proteases (caspases) is very large and that other enzymes rather than caspase-1 itself are more relevant to the mechanism of apoptotic cell death. The participation of caspase-1 in the apoptotic mechanism has been however clearly indicated by data obtained from caspase-1 knock-out mice, whose thymocytes become resistant to Fas-mediated apoptosis [24], and from cells overexpressing caspase-1, which become more sensitive to Fas-triggered apoptotic death [25]. Moreover, recent data suggest that nuclear translocation of the caspase-1 prodomain could directly induce apoptosis, without the need for the active enzyme to be present [26].

Indirectly, caspase-1 might be able to modulate cell apoptosis through maturation of IL-1. In fact, the inflammatory cytokine IL-1beta is synthesized as a 31 kDa inactive precursor which needs to be proteolytically cleaved by the specific enzyme caspase-1 to give rise to the mature active form [6]. The role of mature IL-1beta in the regulation of apoptotic cell death is complex, since in many cellular systems IL-1beta has a well-defined pro-apoptotic capacity, with possible pathological consequences such as in autoimmune thyroiditis and diabetes [27-31], whereas in other instances IL-1 could rescue cells from apoptosis, as in the case of inflammatory responses [3, 29, 32-35]. On PMN, as well as on other inflammatory cells, IL-1beta has a clear-cut protective role in spontaneous apoptosis, as it can significantly delay cell death in culture [3, 32, 34]. This effect of IL-1beta is shared by other inflammatory mediators (LPS, GM-CSF, in part
TNF-alpha) and is thought to be one of the regulatory mechanisms which allow the full development of the inflammatory reaction [3, 4, 14, 36]. PMN could produce IL-1beta, which is released at variable but significant levels upon *in vitro* culture [34, 37-40; this paper]. That IL-1beta is indeed produced by PMN and not by contaminating monocytes is suggested by a series of results, *e.g.* the positivity of PMN for IL-1beta mRNA in *in situ* hybridisation experiments (Figure 1) and the detection of significant amounts of bioactive IL-1beta in the LPS-free supernatants of highly purified PMN preparations, in the absence of detectable residual monocytes and endotoxin contamination. The amount of IL-1beta produced by PMN in the experiments described here, although high as

compared to previous observations, is not outside the range of expected variability due to variation of donors and culture conditions. Moreover, the fact that IL-1 can induce its own synthesis [39] could also be considered as an additional cause of the high level of IL-1beta found in supernatants of PMN cultured for 18 hours. It can be hypothesized that PMN-derived IL-1beta could have an autocrine anti-apoptotic role. This is suggested by the observation that in PMN cultures, spontaneous apoptosis can be significantly accelerated by inhibitors of the IL-1 action (the antagonist IL-1Ra or blocking antibodies to IL-1 or to the $IL\text{-}1R_I$). In addition, caspase-1 mRNA and activity are found in cultured PMN and caspase-1 inhibition could concomitantly block IL-1beta production and accelerate spontaneous apoptosis. These observations thus confirm that in PMN, endogenous IL-1beta acts as an autocrine anti-apoptotic rescue mechanism and indicate that the role of caspase-1 in spontaneous PMN apoptosis is essentially that of generating the protective mature IL-1beta, with no evidence for any direct involvement in the pro-apoptotic mechanism. The detection of high levels of caspase-3-like activity during spontaneous PMN apoptosis would suggest that in fact, enzymes of the caspase-3 family could be directly involved in PMN programmed death [13, 14]. Thus, spontaneous PMN apoptosis, possibly dependent on caspase-3 activity, can be counterbalanced by caspase-1-dependent production of protective IL-1beta. In inflammatory sites, where high levels of IL-1beta are induced by inflammatory or infectious agents, the presence of IL-1 would prolong the life span of recruited PMN by shifting the balance between pro-apoptotic and anti-apoptotic autocrine mechanisms towards survival. This would allow PMN to effectively participate in the inflammatory reaction.

Fas-triggered PMN apoptosis on the other hand is apparently based on distinct mechanisms. It could be hypothesized that Fas-induced apoptosis may intervene as a late mechanism of PMN removal to resolve the inflammatory response, once the specific immune response has been initiated. In fact, Fas-mediated apoptosis is one of the main mechanisms of activated T cell regulation, and triggered T lymphocytes are the main effectors of Fas-dependent cell death through their capacity to produce FasL. Thus, during resolution of the inflammatory reaction Fas-positive PMN could be rapidly disposed of by activation of their Fas-dependent apoptotic program induced by FasL-producing immune cells. Analysis of the role of caspases and of IL-1beta in Fas-triggered PMN apoptosis reveals that endogenous IL-1beta is present at the same levels as in unstimulated cells and maintains its protective anti-apoptotic

effect. Accelerated PMN apoptosis at the end of the inflammatory reaction could therefore depend on the discontinuation of IL-1beta stimulation consequent to successful removal of the inducing agents and/or to the late synthesis of the IL-1beta antagonist IL-1Ra. However, inhibitors of caspase-1 activity do not have any pro-apoptotic effect on Fas-stimulated apoptosis, suggesting that the role of caspase-1 in this situation is not exclusively anti-apoptotic as in the case of spontaneous apoptosis. Indeed, caspase-1-like enzymatic activity is increased by Fas triggering, even though the mature IL-1beta levels are not increased. This suggests that Fas triggering could induce the synthesis of other proteins which are targets of the caspase-1 enzymatic activity and which participate in the apoptotic process, such as other caspases or PARP. Thus, the anti-apoptotic effect of caspase-1-dependent endogenous IL-1beta would be counterbalanced and outweighed by the pro-apoptotic activity of Fas-induced and caspase-1-activated mediators. In addition, Fas triggering appears to increase the activity of caspase-3-like enzymes, which are of major importance in PMN apoptosis [13, 14].

Thus, the balance between pro- and anti-apoptotic molecules determines survival *versus* death in PMN. Spontaneously, PMN undergo rapid apoptosis, which is the outcome of the balance between the pro-apoptotic activity of caspase-3 and the rescuing role of endogenous IL-1beta. In inflammatory situations, IL-1 and other inflammatory mediators produced at the inflammatory site prolong PMN survival by shifting the survival *versus* death balance in favour of survival. When the inflammatory reaction must be terminated and inflammatory mediators are no longer present or are inhibited, PMN return to rapid apoptosis. In this circumstance, Fas stimulation (*e.g.*, by FasL-producing activated $CD8^+$ cells generated during the ensuing immune response) will further accelerate PMN apoptosis. Fas-triggered PMN in fact do not show any increase in endogenous protective IL-1 production, but they show a significant increase in both caspase-3 (mainly involved in apoptosis) and in caspase-1, which conceivably cleaves Fas-induced pro-apoptotic substrates, further shifting the balance toward death.

## CONCLUSION

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## Figures

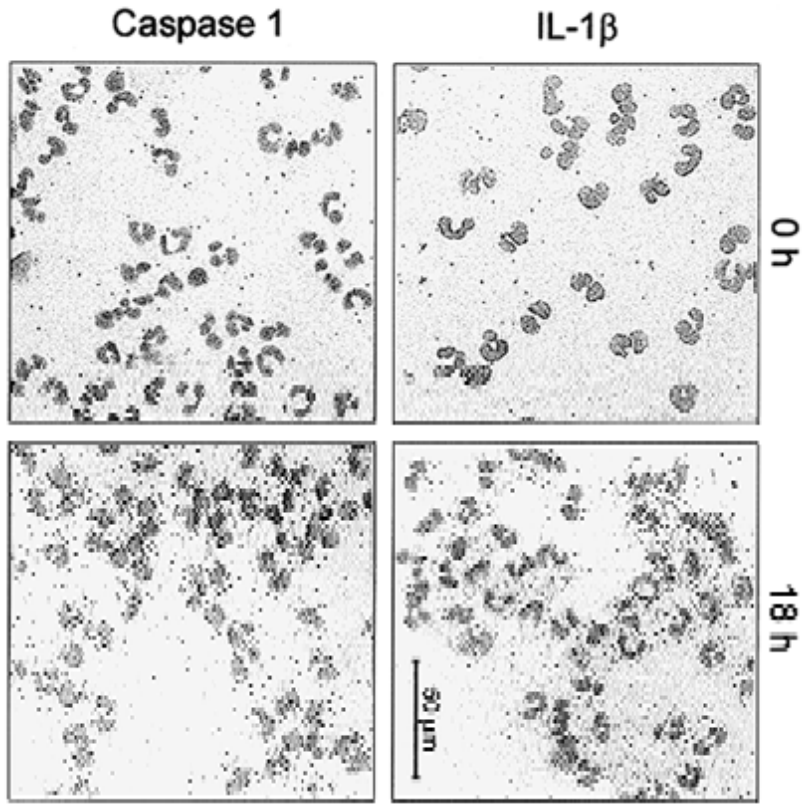
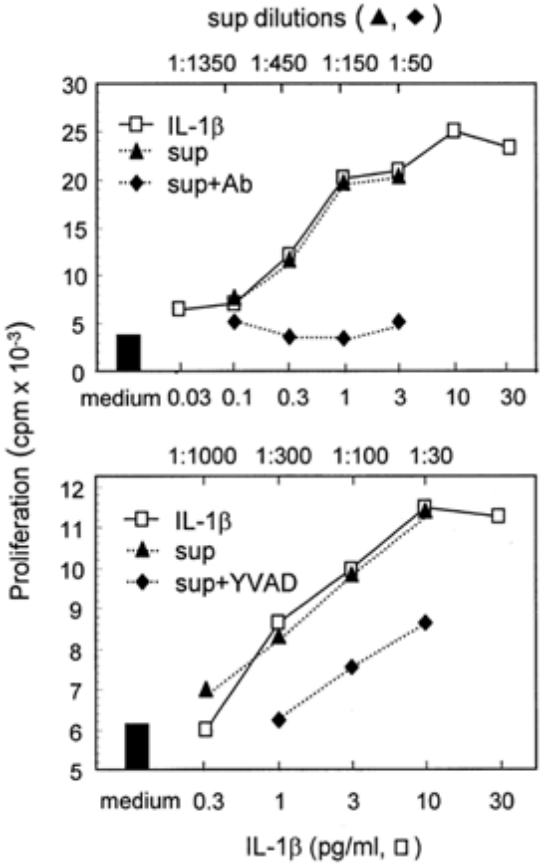



**Figure 1**

**PMN express proIL-1**beta **and caspase-1 mRNA.**

Upper panels: expression of IL-1beta mRNA on fresh PMN (left) and on PMN cultured for 18 hours (right). Lower panels: expression of caspase-1 mRNA on fresh PMN (left) and on PMN cultured for 18 hours (right). *In situ* hybridisation on PMN cytosmears was performed as described in Materials and Methods.




## Figure 2

**PMN produce bioactive mature IL-1**beta.

Upper panel: proliferation of D10.G4.1 cells in response to human recombinant IL-1beta (square) or to different dilutions of 18 hour PMN supernatant, in the absence (triangle) or in the presence (diamond) of a polyclonal anti-IL-1beta antibody (diluted 1/3,000). Lower panel: proliferation of D10.G4.1 cells in response to human

recombinant IL-1beta (square) or to different dilutions of 18 hour PMN supernatant, in the absence (triangle) or in the presence (diamond) of the reversible caspase-1 inhibitor YVAD-CHO (100 muM). Data are the mean of triplicate determinations with errors below 5% (not shown). Results are from single experiments and are representative of data obtained with cells from 7 different donors.

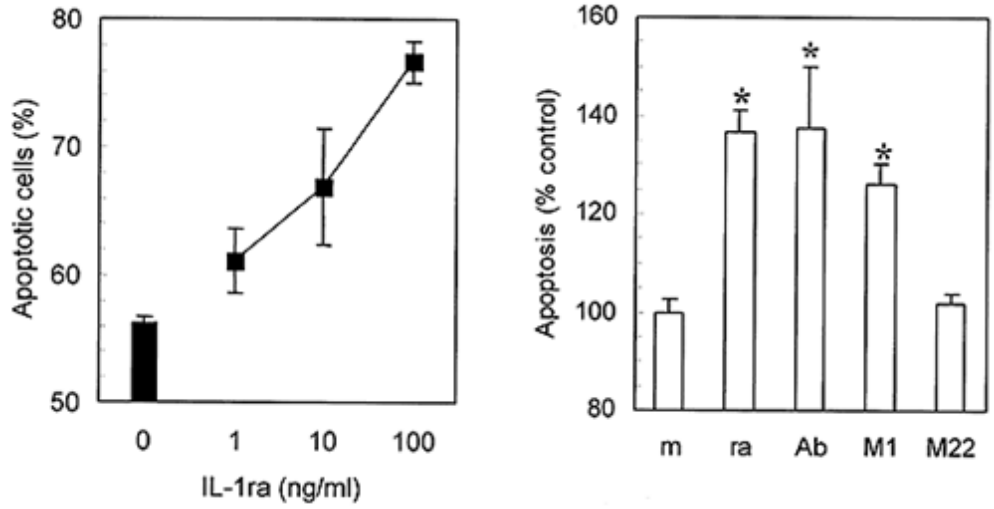



**Figure 3**

**Spontaneous PMN apoptosis is increased by IL-1**beta **blockade.**

Left panel: PMN apoptosis was evaluated after 18 hours of culture in the absence or in the presence of increasing concentrations of human recombinant IL-1Ra. Data are the mean ± SD of results from two experiments, representative of 9 performed. For each IL-1Ra point, $p < 0.05$ *versus* control without IL-1Ra. Right panel: PMN were cultured for 18 hours in the absence (first column, taken as 100% reference apoptosis; m) or in the presence of different IL-1beta inhibitors: IL-1Ra (100 ng/ml; ra), a polyclonal neutralizing

antibody anti-IL-1beta (diluted 1/3000; Ab), the monoclonal anti-IL-$1R_I$ antibody M1 (10 mug/ml), the monoclonal anti-IL-$1R_{II}$ control antibody M22 (10 mug/ml). Data reported are the mean ± SEM of 3-8 replicate experiments and are expressed as percentage apoptosis relative to that of control untreated PMN. * $p < 0.05$ *versus* medium control.

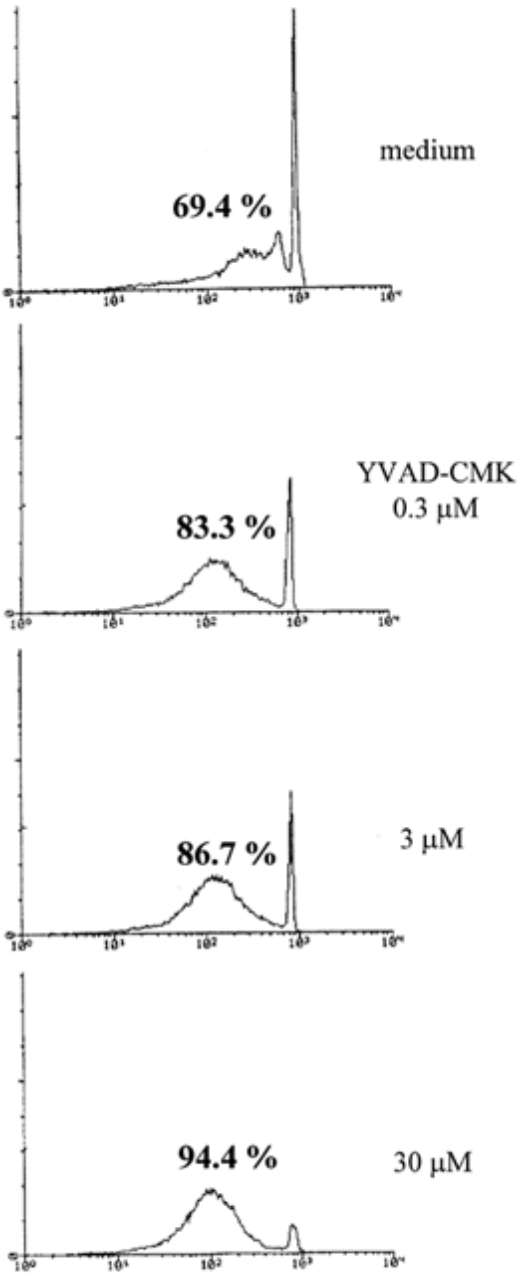
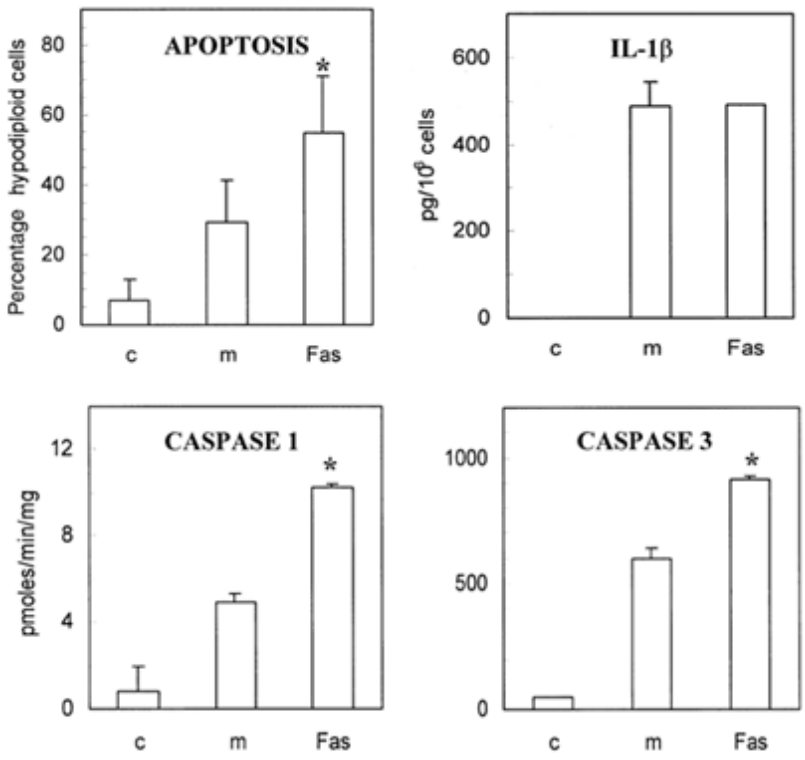



**Figure 4**

**Spontaneous PMN apoptosis is increased by caspase-1 blockade.**

Propidium iodide staining of PMN cultured for 18 hours in culture medium alone (upper trace; medium) or containing increasing concentrations of the irreversible caspase-1 inhibitor YVAD-CMK (0.3-30 muM). In each trace, diploid nuclei are represented by the far right peak, whereas hypodiploid apoptotic nuclei are located on the left of the diploid peak. The percentage of apoptotic cells in each condition is reported above each trace. Results are from a single experiment representative of 12 performed. In control samples, cells were exposed to the vehicle DMSO, at concentrations corresponding to those present in the YVAD-CMK dilutions at 3, 10 and 30 muM. Apoptosis of PMN treated with DMSO (expressed as % of the apoptosis of medium-treated cells) did not vary significantly as compared to that of cells exposed to medium alone (= 100%): 95.3 ± 4.8% for the 3 muM control (2 experiments), 98.5 ± 10.6% for the 10 muM control (7 experiments), 104.0 ± 6.3% for the 30 muM control (3 experiments).




## Figure 5

**Fas triggering increases PMN apoptosis and caspase-1 activity but not IL-1**beta **production.**

PMN were cultured for 18 hours in medium alone (m) or containing 300 ng/ml of an agonist IgM monoclonal antibody against Fas (Fas). On these cells, apoptosis was measured by cytofluorimetric analysis of hypodiploid cells after propidium iodide staining (upper left), IL-1beta production by ELISA on cell supernatants (upper right), caspase-1- (lower left) and caspase-3-like activity (lower right) by fluorimetric measurement of selective substrate degradation. A control of fresh cells (C) was included in each experiment for comparison purposes. Data are the mean ± SD of results from 2-16 separate experiments. n.d. not determined. * $p < 0.01$ *versus* medium control.

**Table 1**
**Role of IL-1beta and caspase-1 in Fas-triggered PMN apoptosis**

| Stimulus | PMN apoptosis (%) | | |
|---|---|---|---|
| | Exp. 1 | Exp. 2 | Exp. 3 |
| Medium | 28.9 | 18.8 | 23.6 |
| Anti-Fas IgM | 39.5* | 32.1* | 47.3* |
| Anti-Fas + IL-1β | 28.8' | – | – |
| Anti-Fas + IL-1Ra | – | 47.8*' | – |
| Anti-Fas + YVAD | – | – | 49.2* |

Data reported are results from a single experiment, representative of 4 performed (exp. 1), or the mean of 4 separate experiments with superimposable results. Apoptosis was measured after 6-18 hours of culture. Anti-Fas antibody was used at 300 ng/ml, IL-1β and IL-1Ra at 100 ng/ml and YVAD-CHO at 100 μM. Similar results were obtained at different times of culture and with a range of concentrations of the inhibitory reagents (data not shown). * $p < 0.05$ *versus* medium control. ' $p < 0.05$ *versus* anti-Fas.

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