Neutrophils process interleukin-1β and interleukin-18 precursors in a caspase-1-like fashion – processing is inhibited by human vascular smooth muscle cells

Elena Westphal[1,*], Mona Herzberg[2,*], Ingo Neumann[1,**], Li Beibei[1], Claudia Pilowski[1], Chen Li[1], Karl Werdan[1], Harald Loppnow[1,2]

[1] Universitätsklinik und Poliklinik für Innere Medizin III, Martin-Luther-Universität Halle-Wittenberg, 06097 Halle (Saale)
[2] Abteilung Immunologie und Zellbiologie, Forschungszentrum Borstel, 23845 Borstel, Germany

Correspondence : H. Loppnow
<Harald.Loppnow@medizin.uni-halle.de>
Accepted for publication February 20, 2006

ABSTRACT. Inflammation contributes to the pathogenesis of atherosclerosis. Proinflammatory cytokines, including interleukin-1 (IL-1), may be involved in the local inflammation occurring in the vessel wall. Vascular smooth muscle cells express the unprocessed IL-1β precursor molecule. Invading leukocytes, such as monocytes or polymorphonuclear granulocytes (PMN) may activate the IL-1β precursor during atherogenesis. Thus, we investigated the capacity of PMN to process IL-1β and IL-18 precursors. Processing was analyzed using Western blot and bioassay for IL-1-activity was performed. As few as 80 to 400 PMN/mL detectably processed preIL-1β. PMN also cleaved the caspase-1 substrate preIL-18. The preIL-1β and preIL-18 cleavage products were located at the same apparent molecular weight as those resulting from cleavage by monocyte-derived caspase-1. PMN expressed caspase-1 mRNA and immunoreactive protein. The N-terminus of the preIL-1β cleavage product expressed the sequence expected for caspase-1 cleavage. The cleavage product was active in the bioassay for IL-1 activity, and the caspase-1 inhibitor YVAD blocked processing. We have shown previously that SMC can block processing of preIL-1 by caspase-1. In contrast, SMC do not block processing of PARP by caspase-3. Here, we show that SMC also inhibited the PMN-mediated processing of recombinant and native preIL-1β or preIL-18 depending on the cell number, whereas EC or fibroblasts did not block processing. Our results indicate that PMN can activate preIL-1β in a caspase-1-like fashion. During inflammatory processes, PMN may activate preIL-1β released from SMC, thereby altering IL-1-mediated cardiovascular functions, including contractility, apoptosis, and cytokine production.

Keywords: human, neutrophils, monocytes/macrophages, cytokines, inflammation

Cytokines derived from cardiovascular cells, including cardiomyocytes, endothelial cells (EC) or smooth muscle cells (SMC) contribute to the pathogenesis of cardiovascular diseases by regulating local inflammatory responses [1, 2]. Among the cytokines interleukin-1 (IL-1) is a key mediator in inflammation [3] and may contribute to development of atherosclerosis [4]. IL-1 has been detected in coronary artery SMC [5] and in its precursor form in rabbit arteries [6], but not in proliferating arterial SMC [7, 8]. In addition to a number of other cell types, cultured SMC also produce both IL-1 isoforms, but only their precursors can be detected in western blot [9, 10]. In contrast to the IL-1α precursor (preIL-1α), the preIL-1β is biologically inactive and proteolytic activity is necessary to obtain biological activity.

Proteolytic cleavage of preIL-1β into its biologically active, 17 kDa form is performed by the IL-1β-converting enzyme (ICE; caspase-1; [11, 12]). This enzyme was originally identified by its capacity to process the IL-1β precursor [13, 11, 12]. It was the first member of the caspase family. Caspases contain a cysteine in their active site and cleave behind aspartic acid. In the 31 kDa IL-1β precursor, this position is Asp^{116}-Ala^{117}, which results in the mature 17 kDa IL-1β. An additional cleavage position at Asp^{27}-Gly^{28} may result in formation of a 28 kDa protein. Besides IL-1β, other substrates of caspase-1, such as IL-18 or IL-33, have been described [14-16]. At present, 14 caspases are known. Some of them, caspase-4 and -5, are only expressed in human, in mice (caspase-11) or in bovine (caspase-13). Most of the caspases, such as the initiator caspases-8, -9, and -10, as well as the effector caspases-3, -6, and -7, are preferentially involved in the

* E.W. and M.H. contributed equally to this work.
** Current address: Marthin-Luther-Universität Halle-Wittenberg, Klinikum der Medizinischen Fakultät, Universitätsklinik und Poliklinik für Kinder- und Jugendmedizin, Forschungslabor für krebskranke Kinder, Ernst-Grube-Straße 40, 06120 Halle (Saale)

regulation of apoptosis. The caspases-1, -4, and -5 in human, and caspase-1, -11, and -12 in mice, are thought to be important for the regulation of inflammatory processes [17]. The presence of caspase-3, -8, -9, and -10 in PMN has been reported recently [18] and caspase-1 (ICE) expression in murine or human PMN has been shown [19, 20]. Although caspase-1 is not a key caspase in apoptosis, since mice lacking caspase-1 are not apoptosis-defective [21], its involvement in various aspects of apoptotic cell death has been shown for example in fibroblast cell culture [22], in human neurons [23], as well as in ovarian cancer [24]. Caspases-12 and -14 are believed to be phylogenetically related to the inflammatory caspases. The caspases are activated in protein complexes composed of various proteins. The assembly of these complexes depends on the stimulus and the caspase to be activated. One of the first examples for such a complex was the apoptosome involved in the activation of caspase-9 [25]. Some of the numerous proteins involved in the activation of the inflammatory caspases in the inflammasome necessary for the activation of caspase-1 have been summarized in recent reviews [26, 27, 17]. Evidence for the presence of the inflammasome protein ASC in PMN was obtained using PMN cDNA for two hybrid yeast experiments [28], and it has been shown that ASC is upregulated upon stimulation in PMN [29]. These complexes are thought to be present in the cytosolic compartment where they are assembled from monomeric proteins. The activation of these complexes is unclear, but it appears as if pathogens, via their conserved pathogen-associated molecular patterns (PAMPs) or *via* Toll-like receptor activation, induce the assembly of the inflammasome. More recently, other inflammasome activators have been described, including uric acid crystals [30], which act in an ASC/NALP3-dependent fashion. On the other hand intracellular pathogens may activate the inflammasome in an ASC/IPAF-dependant pathway [31]. Upon activation of caspase-1 in the inflammasome, biologically active IL-1β is formed.

IL-1 can stimulate or modulate many functions of cardiovascular cells, including contractility of heart [32] or vessel wall cells [33], apoptosis [34], migration [35], as well as production of proteases [36, 37], NO [38] or inflammatory mediators [39]. We have shown previously that SMC produce IL-1-activity, but do not release it [10], SMC express the IL-1β precursor cell-associated [40], but do not detectably process it, however block processing of preIL-1β by caspase-1 or monocytes [41]. In addition to SMC, cultured heart muscle cells, but not fibroblasts or endothelial cells, also inhibited processing of preIL-1β (our unpublished data). In order to contribute to the regulation of cell function and/or the pathogenesis of cardiovascular diseases, SMC-derived preIL-1β has to be converted into its biologically active form, despite the presence of an inhibitory activity.

During inflammatory processes in the heart or the vessel wall, leukocytes and cardiovascular cells may interact [42], and monocytes or, to some degree, polymorphonuclear cells (PMN) may invade these tissues [43]. It has been reported that PMN can express IL-1 mRNAs [44] and that murine PMN can express caspase-1 RNA [19, 20]. However, it remained unclear whether caspase-1 in human PMN is active and cleaves preIL-1β or preIL-18, two major caspase-1 substrates. Production and release of functional caspase-1 by PMN invading cardiovascular tissues may contribute to the activation of biologically inactive SMC-derived preIL-1β, released from injured and/or necrotic SMC. Thus, we investigated the expression and function of caspase-1 in human PMN and the modification of PMN-mediated preIL-1β cleavage by SMC.

We show here that PMN express caspase-1 mRNA and immunoreactive protein, and that they process preIL-1β and preIL-18. The cleavage product of preIL-1β expressed the expected N-terminal sequence unique to caspase-1 cleavage. The processing products were biologically active and a caspase-1 inhibitor blocked the processing. Processing of precursor IL-1β by PMN was inhibited by SMC preparations in a cell ratio (SMC/PMN)-dependent manner. Thus, PMN may activate preIL-1β in a caspase-1-like fashion, resulting in modification of cardiovascular cell functions, including contractility, apoptosis and inflammatory processes (*i.e.* cytokine production).

METHODS

Materials

Cell culture media were purchased from Biochrom (Berlin, Germany). Fetal calf serum (FCS; endotoxin < 50 pg/mL) was obtained from Serva (Heidelberg, Germany). Heparin was from Sigma (Deisenhofen, Germany), ficoll-hypaque was from Pharmacia (Uppsala, Sweden) and polyvinyl alcohol (PVA; 10 g/L; 0.9% NaCl) from Merck (Darmstadt, Germany). Rabbit polyclonal antiserum directed against recombinant IL-1β was generated in rabbits. The anti-IL-$1\beta_{251\text{-}269}$ antibody (Fib 3) was raised as described previously [45]. The anti-caspase-1 antibody (sc-622) was obtained from Santa Cruz (Heidelberg, Germany), and the IL-18 antibody was obtained from R&D (MAB318; Wiesbaden). Peroxidase-conjugated goat-anti-rabbit and goat-anti-mouse antibodies were obtained from Dianova (Hamburg, Germany). Recombinant caspase-1 was purchased at Biomol (Hamburg, Germany).

Isolation and culture of human vascular cells and leukocytes

Human vascular smooth muscle cells (SMC) were cultured in DMEM containing 1 g/L glucose, 2 % antibiotics, L-glutamine, and 10 % fetal calf serum and were characterized as described previously [46]. Polymorphonuclear cells (PMN) were isolated from heparinized blood of healthy donors following ficoll-hypaque density gradient centrifugation (40 min, 500 x g) and further sedimentation through PVA (50 % in HBSS) [47]. The remaining eryth-

Abbreviations:

EC	human vascular endothelial cells
ICE	IL-1β-converting enzyme
IL	interleukin
MNC	human mononuclear cells
PARP	polyADP ribose polymerase
PMN	polymorphonuclear granulocytes
preIL	precursor molecule of interleukin
SMC	human vascular smooth muscle cells

rocytes were hypotonically lysed and the PMN were washed with PBS. The resulting cells were > 98 % PMN, as determined by forward and side scatter FACS analysis. The use of the cells has been approved by the local ethical committee.

Preparation of recombinant proteins

Recombinant proteins (preIL-1α, preIL-1β, and preIL-18) were cloned (pQE-30 vector) and expressed in *Escherichia coli* as described [41, 48]. The recombinant proteins were purified by Ni-NTA chromatography (Quiagen, Chatsworth, USA).

PreIL-1β and preIL-18 cleavage by recombinant caspase-1 or PMN and Western blot analysis

For processing experiments, recombinant preIL-1β (15 µg/mL = 225 ng/lane), preIL-18 (27 µg/mL = 400 ng/lane) or native SMC-derived IL-1β-precursor was incubated with caspase-1, monocytes or PMN in processing buffer (10 mM HEPES, 1 mM DTT, 10 % glycerol).

We investigated the blocking capacity of SMC using SMC lysates added to processing experiments. SMC lysates were prepared from cultured cells following trypsinization, centrifugation and washing in PBS. The cells were adjusted to 50,000 cells/µL in water and were subjected to 4 freeze/thaw cycles. PreIL-1β was then preincubated (37 °C) with the lysates for 10 minutes. After this period, caspase-1 or cells (as a source of cellular caspase-1) were added (30 min, 37 °C).

Both the processing and the inhibition experiments were stopped by addition of SDS-PAGE sample buffer (1 M Tris, 25 % glycerol, 0.5 % SDS, 15 % 2-mercaptoethanol, 0.1 mg/mL bromophenol blue) and heat-inactivation (95 °C, 10 min). Finally, the samples were separated by standard SDS-PAGE under reducing conditions and blotted to polyvinylidene-difluoride membranes (0.8 mA/cm^2, 6 V, 30 min; Immobilon-TM-P, Millipore, Eschborn, Germany).

After blocking the blots with dilution buffer (15 % bovine serum, 0.01 % thimerosal, 0.05 % Tween-20, 150 mM NaCl, 10 mM NaH_2PO_4), the blots were incubated (1 h) with the respective antibody in dilution buffer. Subsequently, a peroxidase-conjugated second antibody (1:4,000; 1 h) was added. After each incubation, the blots were washed three times (1 mM Tris-HCl, 0.05 % Tween-20 and 0.01 % thimerosal). Finally, substrate solution was added (diaminobenzidine (5 mg/mL) in 17 mM citric acid, 65 mM NaH_2PO_4, 0.1% H_2O_2, 0.01% thimerosal).

Fibroblast assay for detection of biological IL-1 activity in processed samples

In order to show, that PMN cleave the IL-1β precursor specifically and sufficiently to obtain biologically active material, we analyzed the processing samples for biological IL-1-activity in the fibroblast assay [49, 50]. We also investigated the caspase-1 inhibitor YVAD (Bachem, Weil a.R., Germany) in the biological processing assay. Briefly, stimulated heparinized whole blood, as well as PMN (0.1×10^6) or MNC incubated with the precursor, were cultured in the presence or absence of the caspase-1 inhibitor YVAD. After overnight culture, the supernatants were harvested and analyzed in the fibroblast assay. Briefly, human dermal fibroblasts were cultured in DMEM containing 4.5 g/L glucose, 2 % antibiotics, L-glutamine and 10 % fetal calf serum. The fibroblasts (5,000/well) were plated onto flat-bottomed, 96-well plates, cultured overnight, washed, and samples or standard applied [49, 50]. After incubation for 96 hours, the cells were stained with crystal violet and measured in a multi-well reader at 570 nm. This assay is much more sensitive than the Western blot and can detect IL-1 activity at 10 to 40 pg/mL.

Isolation of total RNA and reverse transcriptase polymerase chain reaction (PCR)

Total RNA of the cultured cells was isolated according to Chomczynski *et al.* [51]. cDNA was prepared by reverse transcription (RT) of total RNA (1 µg) with oligo $(dT)_{12\text{-}18}$ using reverse transcriptase (Superscript™, Gibco-BRL, Karlsruhe, Germany) and diluted in 100 µL double-distilled water. The cDNA (10 µL) was mixed on ice with 10 µL of primers, 80 µL of reaction mix (10 mM Tris, pH 8; 50 mM KCl, 2.5 mM $MgCl_2$, 0.01 % gelatin, 4 µL dNTPs (200 µM)) and 0.5 µL Taq-polymerase (2.5 U, Gibco BRL, Karlsruhe, Germany). The following specific primers were used for the analysis of caspase-1 expression: sense, ATAT GGA TCC GAC AAC CCA GCT ATG CCC; anti-sense ATAT CTG CAG ATG TCC TGG GAA GAG GTA (20 µM each). PCR was started in the first cycle with melting (2 min, 94 °C), annealing (2 min, 58 °C) and extension (3 min, 72 °C). The PCR was performed for 35 cycles with 2 seconds prolongation of extension per cycle. The PCR-products were separated in 1.3 % agarose and visualized by UV-trans-illumination. In order to exclude contamination with genomic DNA, PCR was performed with non-reverse-transcribed mRNA.

Statistics

All experiments were performed at least three times and representative experiments are shown. The mean and standard deviation of the IL-1-activity were calculated from triplicate cultures.

RESULTS

Polymorphonuclear neutrophils cleave preIL-1β and preIL-18 in a caspase-1-like fashion

Polymorphonuclear neutrophils (PMN) may activate unprocessed, inactive IL-1β precursor (preIL-1β) in a caspase-1-like fashion. In order to evaluate this hypothesis we first investigated the capacity of PMN to process the preIL-1β using recombinant preIL-1β (15 µg/mL). PMN processed preIL-1β depending on cell number (*figure 1*). The lowest cell concentrations generating processed IL-1β detectable in Western blot were 80 to 400 PMN/mL, whereas more than 6.25×10^6 cells/mL completely processed the preIL-1β. PMN did not contain endogenous IL-1β detectable in Western blot even at the highest PMN concentration tested (*i.e.* 75×10^6/mL; -). The preIL-1β cleavage products obtained following processing by PMN ran at the same apparent molecular weight as those ob-

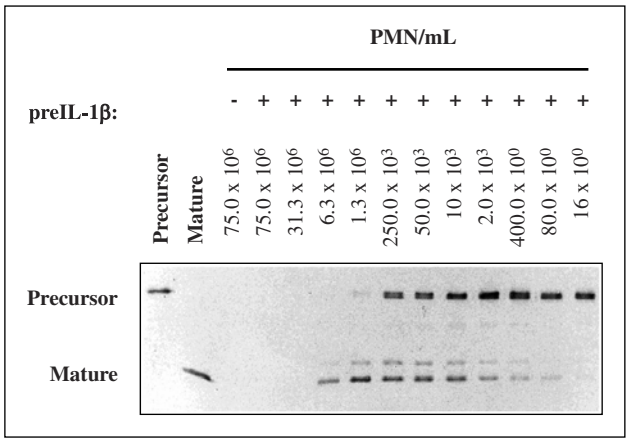

Figure 1
PMN process recombinant IL-1β precursor in a cell number-dependent fashion.
PMN were isolated from heparinized blood and washed with PBS. Various cell numbers of PMN were incubated for 30 minutes at 37°C with recombinant preIL-1β (+; 15 μg/mL = 225 ng/lane). The processing products were separated by SDS-PAGE and analyzed in Western blot (FIB-3; 1 μg/mL). For control, preIL-1β (Precursor; 225 ng/lane) and mature IL-1β (Mature; 40 ng/lane) were included. No endogenous IL-1β was detected in the highest PMN concentration (-). Five experiments with comparable results were performed.

tained following monocyte-derived caspase-1 cleavage (*figure 2A*) and expressed the N-terminal sequence expected for caspase-1 cleavage. Further experiments showed that the PMN released a part of the caspase-1-like activity into the supernatant (data not shown). Taken together these data indicate that PMN can cleave preIL-1β in a caspase-1-like fashion.

A further caspase-1 substrate is preIL-18. SMC express IL-18 mRNA, but do not release IL-18 protein detectable on Western blot (our unpublished results). Thus, we analyzed the capacity of the PMN to process this caspase-1 substrate. These experiments may provide further evidence for the caspase-1-like character of the processing

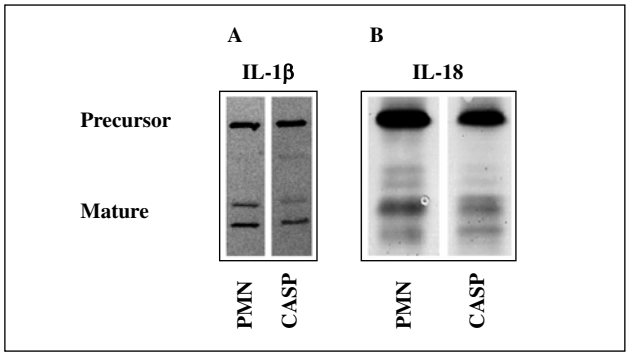

Figure 2
PMN process recombinant preIL-1β and preIL-18 in a caspase-1-like fashion.
A) *PMN and caspase-1 produce similar IL-1β processing products.* PMN (10^5 cells/mL) or caspase-1 (20 ng/mL; CASP) were incubated for 30 minutes at 37 °C with recombinant IL-1β precursor (15 μg/mL) in processing buffer and analyzed as described in *figure 1*. Three experiments showed similar results.
B) *PMN and caspase-1 produce similar IL-18 processing products.* PreIL-18 (27 μg/mL) was incubated with PMN (3.3×10^6 cells/mL) or caspase-1 (33 ng/mL) in processing buffer for 15 minutes. The products were separated by SDS-PAGE and analyzed in Western blot. Similar results were obtained in three experiments.

activity in the PMN. For this purpose we synthesized recombinant preIL-18 and investigated cleavage of this protein by PMN. *Figure 2B* shows that monocyte-derived-caspase-1 and PMN cleaved preIL-18 and that the cleavage products ran at the same apparent molecular weights.

PMN express caspase-1 mRNA and protein, and generate biologically active IL-1β from recombinant preIL-1β

The above data indicate that PMN process IL-1β precursor in a caspase-1-like fashion. In order to further substantiate this hypothesis, we performed RT-PCR with total RNA isolated from monocytes (Mø) or PMN preparations (*figure 3A*). The experiments showed that highly purified PMN constitutively express caspase-1 mRNA. PCR without prior RT (-) did not contain specific products, indicating the absence of detectable DNA contamination. In line with the expression of caspase-1 mRNA, Western blot experiments with a caspase-1 antiserum showed that PMN express immunoreactive caspase-1 protein (*figure 3B*). As expected, the major band was detected at 45 kDa. Compared to monocytes, the PMN contained a higher amount of caspase-1 propart, as indicated by the additional band at 14 kDa.

We obtained further evidence for caspase-1-like cleavage by analyzing the biological activity of the processed samples in the IL-1-assay with human dermal fibroblasts. IL-1β is not active in its precursor form, but needs to be cleaved by caspase-1, in order to become biologically active. Removal of a few amino acids from the C-terminus or proteolysis results in loss of biological activity. Thus, examination of the biological activity may provide information about the intactness of the processed IL-1β. We incubated recombinant IL-1β precursor with three concentrations of PMN and harvested the supernatants. Part of the supernatant was tested using Western blot and showed the same results as presented above. The other part of the sample was tested in the bioassay for IL-1 activity. The experiments showed that the PMN produced biologically active IL-1β from the inactive precursor in a cell-number dependent fashion (*figure 4A*). The supernatants of all three PMN cultures expressed biological IL-1 activity significantly higher than the control (none), which contained recombinant IL-1 precursor but no cells. The specificity of the resulting biological activity was confirmed by inhibition with a monospecific IL-1β antibody, showing that the antibody blocked the IL-1 activity (light grey bars) to reach control levels, *i.e*. the activity in the inhibited samples was not significantly higher than in the control. Finally, we investigated a synthetic caspase-1 inhibitor peptide (YVAD) in the processing assay for biological IL-1 activity (*figure 4B*). As expected, stimulated whole blood produced IL-1 activity. The caspase-specific peptide blocked the generation of IL-1 activity in whole blood by up to 39 %. Stimulated PMN and MNC (100 ng LPS/mL) were incubated with precursor IL-1β and produced biologically active IL-1. In the presence of the caspase-1 inhibitor, the generation of biological activity by PMN was reduced to 44 %. This inhibition provides further evidence for the presence of enzymatically active caspase-1 activity in the PMN.

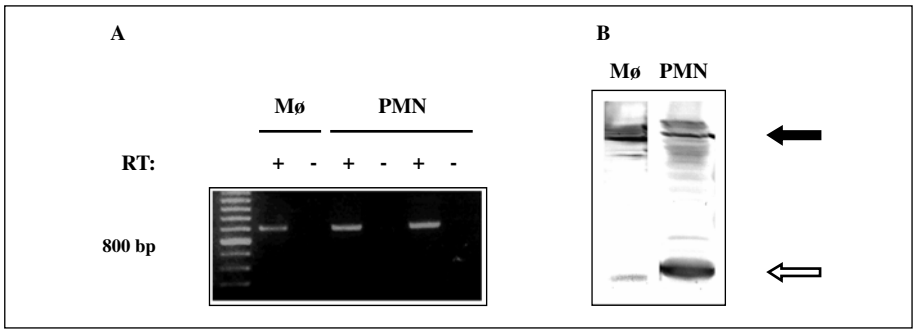

Figure 3

PMN express caspase-1 mRNA and immunoreactive protein.

A) *PMN express caspase-1 mRNA.* RT-PCR was performed using total mRNA isolated from monocytes (Mø) or PMN from two different donors. These preparations were incubated with (+) or without (-) reverse transcriptase and PCR was performed (35 cycles) using primers specific for caspase-1. Samples were run on a 1.3 % agarose gel and visualized by UV-trans-illumination. A 100 base pair ladder was included as marker. The expected size of the PCR product was 880 bp. Two separate experiments provided the same result.

B) *PMN express caspase-1 protein.* PMN and as the control the monocytic cell line HL60 (Mø), were applied to SDS-PAGE, blotted and stained with an antibody detecting the 45 form of the caspase-1 (filled arrow). The 14 kDa protein (open arrow) corresponds to the N-terminal propart of caspase-1 zymogen, which was used as antigen (*i.e.* the antibody detects the complete caspase-1, containing the propart, and the propart itself).

SMC block the PMN-mediated processing of recombinant preIL-1β, preIL-18 and native SMC-derived IL-1β precursor

We have shown previously that SMC can inhibit recombinant caspase-1. Above we have presented data showing that PMN can process caspase-1 substrates in a caspase-1-like fashion. Consequently, we proposed that SMC may inhibit processing by PMN also. In order to verify this proposal, we investigated the effect of SMC on the caspase-1-like activity of PMN. SMC preparations blocked the processing of preIL-1β by PMN depending on the cell number (*figure 5A*). At the ratios of 3.1 or 6.3 SMC to 1 PMN, we detected processing, whereas at the ratios of 25 or 50 SMC to 1 PMN we measured complete inhibition of processing. In contrast, endothelial cells at the highest concentration (50 EC to 1 PMN) did not inhibit the PMN-mediated IL-1β processing. SMC preparations also blocked the processing of preIL-18 by PMN. The left lane of *figure 5B* shows that PMN processed preIL-18, whereas SMC did not process IL-18 (right lane). However, in the presence of SMC, the PMN no longer processed the preIL-18 (middle lane). In separate control experiments (data not shown), we found that EC did not block the processing of preIL-18, indicating the specificity of the inhibition.

In the above experiments we used recombinant caspase-1 substrates. The next experiments were performed to investigate the processing of native SMC-derived preIL-1β in-

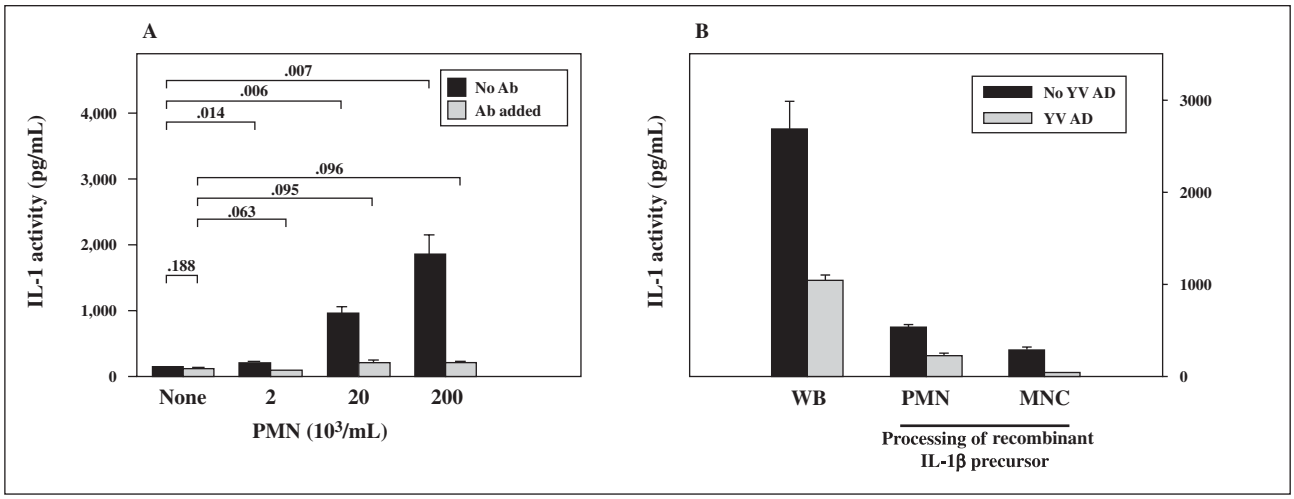

Figure 4

PMN release biologically active IL-1 from recombinant IL-1β precursor in a caspase-1-like fashion.

A) *Processing of recombinant IL-1β precursor by PMN results in biologically active IL-1β.* Biologically inactive recombinant preIL-1β was incubated without (None) or with PMN (2×10^3, 2×10^4, and 2×10^5/mL, respectively) for 30 minutes (125 µL; 1,665 ng preIL-1β). Samples (25 µL) were analyzed in Western blot (data not shown) and fibroblast assay (100 µL) for biological IL-1-activity. To parallel cultures in the fibroblast assay, a specific IL-1β antibody was added ("Ab added"; light grey bars; final Ab concentration 1:400). The p-values are presented above the columns. Three experiments with similar results were performed.

B) *Activation of recombinant IL-1β precursor by PMN is inhibited by the caspase-1 inhibitor YVAD.* Heparinized whole blood, as well as isolated MNC (5×10^5/mL) or PMN (1×10^5/mL) were stimulated with LPS (100 ng/mL) and cultured overnight (37°C, 7.5% CO_2) in the presence or absence of the inhibitor YVAD (Ac-Tyr-Val-Ala-Asp-aldehyde; Bachem). This peptide preferentially inhibits caspase-1 and, 100-fold less, other inflammatory caspases, such as caspase-4 or -5. The supernatants were harvested and analyzed in the fibroblast assay for IL-1 activity. Three experiments showed similar results.

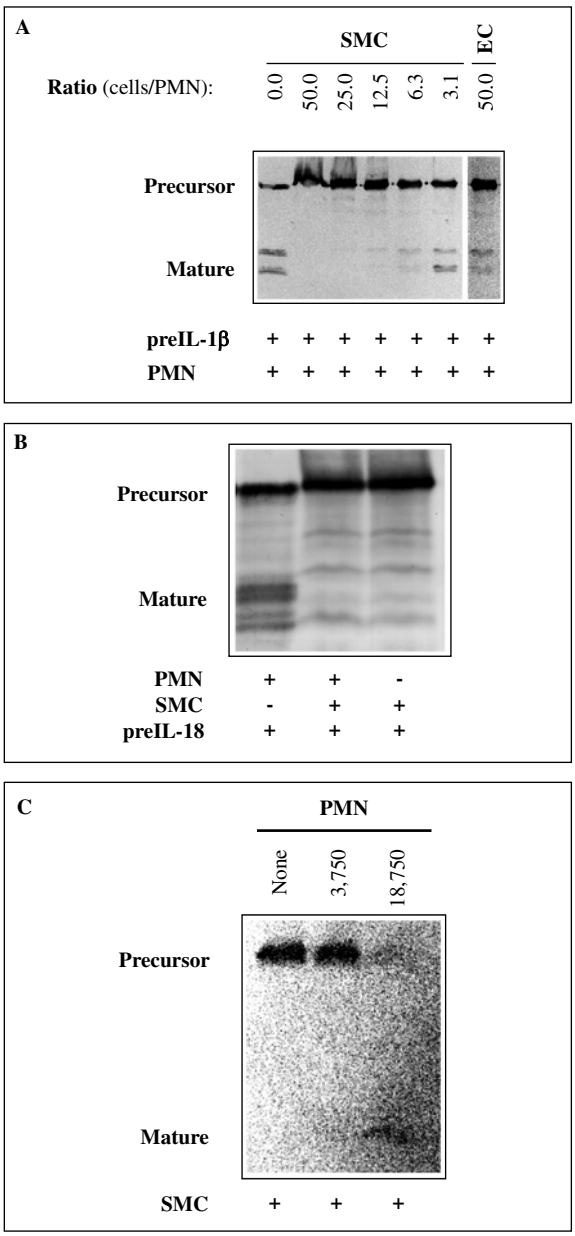

Figure 5
SMC inhibit processing of recombinant and native caspase-1 substrates.
A) *Increasing numbers of SMC inhibit processing of recombinant preIL-1β by PMN.* First, preIL-1β (15 µg/mL = 225 ng/lane) was preincubated for 10 minutes (37°C) with processing buffer (0.0) or processing buffer containing lysates of different numbers of SMC or EC, corresponding to the ratios presented in the figure (*i.e.* 25 = 25 SMC per 1 PMN). Subsequently, defined numbers of PMN were added to these preparations (ratio of 1/50 = 2.5×10^5 PMN/mL + 12.5 × 10^6 SMC/mL), incubated for 30 minutes and analyzed as described in *figure 1*. Similar results were obtained in four experiments.
B) *Processing of recombinant preIL-18 by PMN is inhibited by SMC.* preIL-18 (27 µg/mL) was preincubated for 10 minutes (37°C) with or without SMC lysates (16.7 × 10^6 cells/mL; SMC), subsequently incubated for 30 minutes with isolated PMN (3.3 × 10^6 cells/mL) and blotted. Similar results were obtained in three experiments.
C) *Increasing numbers of PMN overcome the inhibition of processing of native SMC-derived IL-1β precursor.* Lysates of interleukin-α/TNF-α-stimulated (10/20 ng/mL) SMC (187,500/lane) were used as a source of native IL-1β precursor (None; 0/50). Two concentrations of PMN were added to the native SMC-derived IL-1β precursor: A) 3750 = 3750 PMN to 187 500 SMC (1/50); B) 18 750 = 18 750 PMN to 187 500 SMC (5/50). In parallel samples (data not shown), the same PMN concentrations, exposed to preIL-1β in the absence of SMC, readily processed the recombinant preIL-1β. Similar results were obtained in three experiments

stead of recombinant preIL-1β (*figure 5C*). SMC stimulated with TNF and IL-1α contained native IL-1β precursor detectable in Western blot, but no mature IL-1β. This native preIL-1β of SMC was incubated without PMN (none), or with PMN at the ratio of 1 PMN per 50 SMC (*i.e.* 3,750 PMN) or 5 PMN per 50 SMC (*i.e.* 18,750 PMN). At the ratio of 1 to 50, the PMN did not process the native IL-1β precursor, although controls, in the absence of SMC, showed that the same concentration of PMN readily processed recombinant preIL-1β. This finding indicates that the processing is blocked at the ratio of 1 PMN per 50 SMC. However, at the five-fold higher ratio of 5 PMN to 50 SMC the PMN could overcome inhibition, and processed the native SMC-derived IL-1β precursor, as shown by the disappearance of the precursor and the appearance of a faint IL-1β processing product.
Summarizing the data of *figure 5A* and *C*, we concluded that PMN processed native, SMC-derived IL-1β precursor and that SMC inhibited the processing of this native, as well as the processing of the recombinant IL-1β precursor at a ratio of > 10 SMC to 1 PMN.
Taken together, we show that PMN process recombinant and native caspase-1 substrates, the caspase-1 inhibitor YVAD inhibits processing by PMN, and that processing of IL-1β precursor results in biological activity and the correct N-terminal sequence. Furthermore, as shown previously by us for caspase-1, SMC preparations also inhibited processing by PMN. Thus, we conclude that PMN can process IL-1β and IL-18 precursors in a caspase-1-like fashion.

DISCUSSION

It has been reported previously that PMN express IL-1α and IL-1β mRNA [52, 44], and that murine PMN express ICE mRNA [19]. However, it has also been shown that caspase-1 in oral and circulating PMN is inactive [53]. Thus, it remained unclear whether or not PMN can cleave caspase-1 substrates, such as preIL-1β or preIL-18. In order to answer this question, we analyzed the expression and function of caspase-1 in isolated human PMN. In this report, we provide evidence that polymorphonuclear granulocytes (PMN) cleave the IL-1β precursor (preIL-1β) in a caspase-1-like fashion, and may overcome the previously described IL-1β processing inhibitory activity present in SMC [41].
The IL-1β processing activity of PMN was cell-number dependent. Already a PMN-concentration of 80 to 400 PMN/mL was capable of processing the recombinant IL-1β precursor. This result and the purity of PMN of more than 98 % indicates that the IL-1β processing was not caused by contamination of the PMN preparation by monocytes or other cells. Other enzymes, besides caspase-1, may also contribute to the processing of the preIL-1β. Such enzymes include lysosomal proteases, such as elastase or cathepsin G, which are capable of processing preIL-1β at different cleavage sites [54-56], thereby resulting in N-termini different from those resulting from caspase-1 cleavage. However, the PMN processing product expressed the N-terminal sequence expected for caspase-1 cleavage. The experiments showing biological activity of the processing products added further evidence for a specific (physiological) cleavage, since unspe-

cific protein breakdown would also alter the C-terminus. However, removal of as few as 4 to 7 amino acids at the C-terminus would result in loss of biological activity [57]. We obtained further evidence for the presence of a caspase-1-like cleavage from inhibition experiments with a synthetic caspase-1 inhibitor (YVAD). This inhibitor blocked the production of IL-1 activity in stimulated whole blood used for control. It also inhibited the development of biologically active IL-1 from the recombinant IL-1β precursor by monocytes and PMN. Many efforts to show inhibition in Western blot failed, probably due to the lower sensitivity of this method or the time-dependence of the inhibitor reported previously [58], *i.e;* the processing assay uses a very short incubation period of 30 minutes, however, in the biological inhibition assay the inhibitor was present overnight. PMN also processed IL-18 in the same manner as recombinant caspase-1 or monocytes. Taken together, these data suggest that PMN express caspase-1 or a caspase-1-like enzyme with a comparable specificity. PCR experiments, showing that PMN expressed caspase-1 mRNA, and Western blot experiments showing that PMN expressed immunoreactive caspase-1 further support this suggestion.

Our Western blots indicated that PMN do not release endogenous IL-1β, although others have reported the presence of IL-1-activity in human or murine PMN culture supernatants [59-61]. The detection limit of Western blot may have contributed to this result. However, even in the thousand-fold more sensitive biological fibroblast-assay, we did not detect significant IL-1-activity in PMN. One explanation for these different results is that the cited authors used the thymocyte assay [59], which measures other cytokines, such as IL-6 and IL-7, in addition to IL-1. Furthermore, the different isolation methods [60] may have contributed to the different results. On the other hand, the authors described the presence of 2 to 6 pg IL-1β/10^6 PMN supernatants, as measured by ELISA [20]. This is a very small amount of IL-1β protein, which is at the threshold of the ELISA system, and just below the detection limit of the bioassay. In experiments with wild type and caspase-$1^{-/-}$ mice low concentrations of IL-1β were also detected. However, the purity of the PMN in these experiments was only 90 to 95 %. Thus, the low IL-1β measured may have been derived from contaminating cells [61]. Furthermore, it is possible that the ELISA may have detected inactive IL-1β cleavage products, rather than biologically active IL-1. Another explanation for the lack of bioactivity in the fibroblast assay could be the presence of IL-1 receptor antagonist, which may be produced by PMN [62]. However, we did not obtain evidence for an inhibitory effect of the PMN samples in the biological IL-1 assay. On the other hand, it has been reported that oral and circulating PMN express active caspase-3, but not active caspase-1 [53], which is in line with our results that PMN express no endogenous IL-1β.

Compared to monocyte IL-1β production, which can release 500 to 2,000 pg/mL into culture supernatants, the highest IL-1β measurement of 40 pg/mL in PMN found by Rowe *et al.* [61] appears low. Thus, since PMN can express and release active caspase-1 activity, as we have shown in this report, we may speculate that the PMN-derived caspase-1 preferentially activates external substrates provided by other cells in the inflammatory environment. Such an environment may be present in the inflamed vessel wall or heart tissue with invading PMN (compare below). On the other hand, a further activation by pathogens or inflammatory activators could be sufficient to enhance IL-1β production and release by PMN. Such a stimulus could include interaction of external ATP with P2X receptors, which for example potently triggers superoxide production in PMN [63] and which is necessary for IL-1β release in monocytes [64-67].

Previously, we have shown that SMC release IL-6 [46], whereas they retain proIL-1β cell-associated [10, 40, 39], possibly due to inhibition of the processing of the IL-1β precursor [41]. Recent investigations (our unpublished results) have shown the specificity of the inhibition, since SMC preparations blocked cleavage of preIL-1β and preIL-18 by caspase-1, but not processing of PARP by caspase-3. In the present report we show that SMC lysates also inhibited the PMN-mediated processing of preIL-1β and preIL-18. We believe that this inhibitory activity, which still awaits further characterization, is different from the previously described ICEBERG [68], since it is located primarily in the membrane fraction, rather than in the cytosolic fraction of the SMC, as reported for ICEBERG. For the same reason we suggest that the serpin proteinase inhibitor PI-9, which can inhibit caspase-1 [69, 70], is probably not a candidate for the inhibitory activity described here. Other inhibitory pathways for inflammatory caspases like caspase-1, may interfere with the inflammasome. This multi-protein complex, where the enzymes are brought together in close proximity with other proteins, activates caspases by cleaving them at specific sites [71, 17, 72]. Thus, the blockade of the inflammasome may result in the blockade of IL-1 processing. However, the inflammasome proteins are probably not involved in the inhibitory mechanism described by us, because they are components of a caspase-activating protein complex. However, the inhibition observed in our system also takes place in the presence of active (*i.e*. already cleaved) recombinant caspase-1 in a cell-free system, where no further activation or "complexation" of the caspase-1 inflammasome is needed. Thus, in addition to the discussed pathways of inhibition of preIL-1β processing, we assume that additional inhibitory mechanisms may exist in the SMC.

The interaction of PMN and SMC suggested by us may take place during various inflammatory conditions observed during atherosclerosis, which is thought to be influenced potently by inflammatory processes [1, 2], but also during other types of pathogenesis, which involves inflammation. There is evidence that after balloon angioplasty or shunt implantation, proliferation of SMC, accumulation of monocytes and apoptosis in both cell types takes place in parallel [73], raising the possibility of multiple interactions as suggested in this paper. On the other hand, in coronary artery bypass grafting SMC loss, probably mediated by accumulating monocytes, has been considered [74]. Also, in atheromata, apoptosis of SMC as well as monocytes, and the presence of caspase-1 have been reported [75]. In PDE-inhibitor-mediated vasculitis, SMC associated with inflammation showed caspase-3 expression and TUNEL staining, and PMN depletion reduced the vasculitis and, to some degree, caspase-3 [76]. Even more interestingly, in experimentally-induced intima thickening in rabbit, direct interaction and engulfment has been shown. Surprisingly, PMN, but not monocytes, were engulfed by SMC [77], a process that may result in further activation of inflamma-

tory mediators. In balloon-injured arteries it has been shown that PMN precede monocyte infiltration [78] and therefore may well have a role in the vessel wall response. Thus, it appears possible that after injury, PMN infiltration may result in development of synthetic SMC [79]. In summary, a variety of cellular interactions, which may contribute to local inflammation, may take place in the vessel wall for example by enhanced IL-1β maturation, as shown here.

Taken together, we propose that PMN can process and activate preIL-1β in a caspase-1-like fashion. In the event of accumulation of leukocytes in cardiovascular tissues, the leukocytes may overcome the IL-1β processing inhibitory activity caused by SMC and cleave unprocessed preIL-1β from injured and/or necrotic SMC. Enhanced levels of activated IL-1 may contribute to inflammatory processes in cardiovascular tissues by altering contractility, apoptosis or cytokine production by cardiovascular cells.

Acknowledgements. *We are grateful to PD Dr. Arndt Petersen (Forschungszentrum Borstel) for the amino terminal sequencing and Professor Dr. Helmut Brade (Forschungszentrum Borstel) for the kind gift of S. friedenau LPS. This work was supported by grants from the Deutsche Forschungsgemeinschaft to HL (Lo385/4-1; Lo385/5-1), to HL and KW (SFB598-A7), and by a grant from the BMBF to HL and KW.*
E. Westphal and M. Herzberg contributed equally to this work.

REFERENCES

1. Libby P. Inflammation in atherosclerosis. *Nature* 2002; 420: 868.
2. Hansson GK. Inflammation, atherosclerosis, and coronary artery disease. *N Engl J Med* 2005; 352: 1685.
3. Dinarello CA. Biologic basis for interleukin-1 in disease. *Blood* 1996; 87: 2095.
4. Kirii H, Niwa T, Yamada Y, Wada H, Saito K, Iwakura Y, Asano M, Moriwaki H, Seishima M. Lack of interleukin-1β decreases the severity of atherosclerosis in ApoE-deficient mice. *Arterioscler Thromb Vasc Biol* 2003; 23: 656.
5. Galea J, Armstrong J, Gadsdon P, Holden H, Francis SE, Holt CM. Interleukin-1β in coronary arteries of patients with ischemic heart disease. *Arterioscler Thromb Vasc Biol* 1996; 16: 1000.
6. Clinton SK, Fleet JC, Loppnow H, Salomon RN, Clark B, Cannon JG, Shaw AR, Dinarello CA, Libby P. Interleukin-1 gene expression in rabbit tissue *in vivo*. *Am J Pathol* 1991; 158: 1005.
7. Clausell N, Molossi S, Rabinovitch M. Increased interleukin-1β and fibronectin expression are early features of the development of the postcardiac transplant coronary arteriopathy in piglets. *Am J Pathol* 1993; 142: 1772.
8. Tanaka H, Sukhova G, Schwartz D, Libby P. Proliferating arterial smooth muscle cells after balloon injury express TNF-alpha but not interleukin-1 or basic fibroblast growth factor. *Arterioscler Thromb Vasc Biol* 1996; 16: 12.
9. Libby P, Ordovas JM, Birinyi LK, Auger KS, Dinarello CA. Inducible interleukin-1 gene expression in human vascular smooth muscle cells. *J Clin Invest* 1986; 78: 1432.
10. Loppnow H, Libby P. Functional significance of human vascular smooth muscle cell-derived interleukin-1 in paracrine and autocrine regulation pathways. *Exp Cell Res* 1992; 198: 283.
11. Cerretti DP, Kozlosky CJ, Mosley B, Nelson N, van Ness K, Greenstreet TA, March CC, Kronheim SR, Druck T, Cannizzaro LA, Huebner K, Black RA. Molecular cloning of the interleukin-1ß-converting enzyme. *Science* 1992; 256: 97.
12. Thornberry NA, Bull HG, Calaycay JR, Chapman KT, Howard AD, Kostura MJ, Miller DK, Molineaux SM, Weidner JR, Aunins J, Elliston KO, Ayala JM, Casano FJ, Chin J, Ding G-F, Egger LA, Gaffney EP, Limjuco G, Palyha OC, Raju SM, Rolando AM, Salley JP, Yamin TT, Lee TD, Shively JE, MacCross M, Mumford RA, Schmidt JA, Tocci MJ. A novel heterodimeric cysteine protease is required for interleukin-1β processing in monocytes. *Nature* 1992; 356: 768.
13. Kostura MJ, Tocci MJ, Limjuco G, Chin J, Cameron P, Hillman AG, Chartrain NA, Schmidt JA. Identification of a monocyte specific pre-interleukin-1ß convertase activity. *Proc Natl Acad Sci USA* 1989; 86: 5227.
14. Gu Y, Kuida K, Tsutsui H, Ku G, Hsiao K, Fleming MA, Hayashi N, Higashino K, Okamura H, Nakanishi K, Kurimoto M, Tanimoto T, Flavell RA, Sato V, Harding MW, Livingston DJ, Su MSS. Activation of interferon-γ-inducing factor mediated by interleukin-1β-converting enzyme. *Science* 1997; 275: 206.
15. Ghayur T, Banerjee S, Hugunin M, Butler D, Herzog L, Carter A, Quintal L, Sekut L, Talanian R, Paskind M, Wong W, Kamen R, Tracey D, Allen H. Caspase-1 processes IFN-γ-inducing factor and regulates LPS-induced IFN-γ production. *Nature* 1997; 386: 619.
16. Schmitz J, Owyang A, Oldham E, Song Y, Murphy E, McClanahan TK, Zurawski G, Moshrefi M, Qin J, Li X, Gorman DM, Bazan JF, Kastelein RA. IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. *Immunity* 2005; 23: 479.
17. Martinon F, Tschopp J. Inflammatory caspases: linking an intracellular innate immune system to autoinflammatory diseases. *Cell* 2004; 117: 561.
18. Derouet M, Thomas L, Moulding DA, Akgul C, Cross A, Moots RJ, Edwards SW. Sodium salicylate promotes neutrophil apoptosis by stimulating caspase-dependent turnover of Mcl-1. *J Immunol* 2006; 176: 957.
19. Watson RW, Rotstein OD, Parodo J, Jimenez M, Soric I, Bitar R, Marshall JC. Impaired apoptotic death signaling in inflammatory lung neutrophils is associated with decreased expression of interleukin-1β-converting enzyme family proteases (caspases). *Surgery* 1997; 122: 163.
20. Watson RW, Rotstein OD, Parodo J, Bitar R, Marshall JC. The IL-1β-converting enzyme (caspase-1) inhibits apoptosis of inflammatory neutrophils through activation of IL-1β. *J Immunol* 1998; 161: 957.
21. Kuida K, Lippke JA, Ku G, Harding MW, Livingston DJ, Su M-S, Flavell RA. Altered cytokine export and apoptosis in mice deficient in interleukin-1ß-converting enzyme. *Science* 1995; 267: 2000.
22. Miura M, Zhu H, Rotello R, Hartwieg EA, Yuan J. Induction of apoptosis in fibroblasts by IL-1β-converting enzyme, a mammalian homolog of the *C. elegans* cell death gene *ced*-3. *Cell* 1993; 75: 653.
23. Guo H, Petrin D, Zhang Y, Bergeron C, Goodyer CG, LeBlanc AC. Caspase-1 activation of caspase-6 in human apoptotic neurons. *Cell Death Differ* 2006; 13: 285.
24. Feng Q, Li P, Salamanca C, Huntsman D, Leung PC, Auersperg N. Caspase-1α is down-regulated in human ovarian cancer cells and the overexpression of caspase-1α induces apoptosis. *Cancer Res* 2005; 65: 8591.
25. Zou H, Li Y, Liu X, Wang X. An APAF-1 cytochrome c multimeric complex is a functional apoptosome that activates procaspase-9. *J Biol Chem* 1999; 274: 11549.
26. Tschopp J, Martinon F, Burns K. NALPs: a novel protein family involved in inflammation. *Nat Rev Mol Cell Biol* 2003; 4: 95.
27. Inohara N, Nunez G. NODs: intracellular proteins involved in inflammation and apoptosis. *Nat Rev Immunol* 2003; 3: 371.

28. Richards N, Schaner P, Diaz A, Stuckey J, Shelden E, Wadhwa A, Gumucio DL. Interaction between pyrin and the apoptotic speck protein (ASC) modulates ASC-induced apoptosis. *J Biol Chem* 2001; 276: 39320.

29. Shiohara M, Taniguchi S, Masumoto J, Yasui K, Koike K, Komiyama A, Sagara J. ASC, which is composed of a PYD and a CARD, is up-regulated by inflammation and apoptosis in human neutrophils. *Biochem Biophys Res Commun* 2002; 293: 1314.

30. Martinon F, Petrilli V, Mayor A, Tardivel A, Tschopp J. Gout-associated uric acid crystals activate the NALP3 inflammasome. *Nature* 2006; 440: 237.

31. Mariathasan S, Newton K, Monack DM, Vucic D, French DM, Lee WP, Roose-Girma M, Erickson S, Dixit VM. Differential activation of the inflammasome by caspase-1 adaptors ASC and Ipaf. *Nature* 2004; 430: 213.

32. Evans HG, Lewis MJ, Shah AM. Interleukin-1β modulates myocardial contraction via dexamethasone sensitive production of nitric oxide. *Cardiovasc Res* 1993; 27: 1486.

33. Beasley D, Cohen RA, Levinsky NG. Interleukin-1 inhibits contraction of vascular smooth muscle. *J Clin Invest* 1989; 83: 331.

34. Geng Y-J, Wu Q, Muszinski M, Hansson G, Libby P. Apoptosis of vascular smooth muscle cells induced by *in vitro* stimulation with interferon-γ, tumor necrosis factor-α, and interleukin-1β. *Arterioscler Thromb Vasc Biol* 1996; 16: 19.

35. Englesbe MJ, Deou J, Bourns BD, Clowes AW, Daum G. Interleukin-1β inhibits PDGF-BB-induced migration by cooperating with PDGF-BB to induce cyclooxygenase-2 expression in baboon aortic smooth muscle cells. *J Vasc Surg* 2004; 39: 1091.

36. Li Z, Froehlich J, Galis ZS, Lakatta EG. Increased expression of matrix metalloproteinase-2 in the thickened intima of aged rats. *Hypertension* 1999; 33: 116.

37. Knipp BS, Ailawadi G, Ford JW, Peterson DA, Eagleton MJ, Roelofs KJ, Hannawa KK, Deogracias MP, Ji B, Logsdon C, Graziano KD, Simeone DM, Thompson RW, Henke PK, Stanley JC, Upchurch Jr. GR. Increased MMP-9 expression and activity by aortic smooth muscle cells after nitric oxide synthase inhibition is associated with increased nuclear factor-kappaB and activator protein-1 activity. *J Surg Res* 2004; 116: 70.

38. Hattori Y, Nakanishi N, Kasai K. Statin enhances cytokine-mediated induction of nitric oxide synthesis in vascular smooth muscle cells. *Cardiovasc Res* 2002; 54: 649.

39. Loppnow H, Westphal E, Buchhorn R, Wessel A, Werdan K. Interleukin-1 and related proteins in cardiovascular disease in adults and children. *Shock* 2001; 16(Supp 1): 3.

40. Loppnow H, Werdan K, Reuter G, Flad H-D. The interleukin-1 and interleukin-1-converting enzyme families in the cardiovascular system. *Eur Cytokine Netw* 1998; 9: 675.

41. Schönbeck U, Herzberg M, Petersen A, Wohlenberg C, Gerdes J, Flad H-D, Loppnow H. Human vascular smooth muscle cells express interleukin-1β-converting enzyme (ICE), but inhibit processing of the interleukin-1β precursor by ICE. *J Exp Med* 1997; 185: 1287.

42. Funayama H, Ikeda U, Takahashi M, Sakata Y, Kitagawa S, Takahashi Y, Masuyama J, Furukawa Y, Miura Y, Kano S, Matsuda M. K. S. Human monocyte-endothelial cell interaction induces platelet-derived growth factor expression. *Cardiovasc Res* 1998; 37: 216.

43. Nicklin MJH, Hughes DE, Barton JL, Ure JM, Duff GW. Arterial inflammation in mice lacking the interleukin-1 receptor antagonist gene. *J Exp Med* 2000; 191: 303.

44. Lord PC, Wilmoth LM, Mizel SB, McCall CE. Expression of interleukin-1α and β genes by human blood polymorphonuclear leukocytes. *J Clin Invest* 1991; 87: 1312.

45. Herzbeck H, Blum B, Rönspeck W, Frenzel B, Brandt E, Ulmer AJ, Flad H-D. Functional and molecular characterization of a monoclonal antibody against the 165-186 peptide of human IL-1β. *Scand J Immunol* 1989; 30: 549.

46. Loppnow H, Libby P. Proliferating or interleukin-1-activated human vascular smooth muscle cells secrete copious interleukin-6. *J Clin Invest* 1990; 85: 731.

47. Böyum A. Isolation of mononuclear cells and granulocytes from human blood. *Scand J Clin Lab Invest* 1968; 21(Suppl. 97): 77.

48. Westphal E, Friedrich I, Silber RE, Werdan K, Loppnow H. Processing of interleukin-18 by human vascular smooth muscle cells. *Adv Exp Med Biol* 2000; 477: 343.

49. Schmidt JA, Mizel SB, Cohen D, Green I. Interleukin-1, a potential regulator of fibroblast proliferation. *J Immunol* 1982; 128: 2177.

50. Loppnow H, Flad H-D, Dürrbaum I, Musehold J, Fetting R, Ulmer AJ, Herzbeck H, Brandt E. Detection of interleukin-1 with human dermal fibroblasts. *Immunobiology* 1989; 179: 283.

51. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987; 162: 156.

52. Marucha PT, Zeff RA, Kreutzer DL. Cytokine regulation of IL-1β gene expression in the human polymorphonuclear leukocyte. *J Immunol* 1990; 145: 2932.

53. Nakahara H, Sato EF, Ishisaka R, Kanno T, Yoshioka T, Yasuda T, Inoue M, Utsumi K. Biochemical properties of human oral polymorphonuclear leukocytes. *Free Rad Res* 1998; 28: 485.

54. Black RA, Kronheim SR, Cantrell M, Deeley MC, March CJ, Prickett KS, Wignall J, Conlon PJ, Cosman D, Hopp TP, Mochizuki DY. Generation of biologically active interleukin-1β by proteolytic cleavage of the inactive precursor. *J Biol Chem* 1988; 263: 9437.

55. Hazuda DJ, Strickler J, Kueppers F, Simon PL, Young PR. Processing of precursor interleukin-1ß and inflammatory disease. *J Biol Chem* 1990; 265: 6318.

56. Mizutani H, Black R, Kupper TS. Human keratinocytes produce but do not process pro-interleukin-1ß. Different strategies of IL-1 production and processing in monocytes and keratinocytes. *J Clin Invest* 1991; 87: 1066.

57. Mosley BD, Dower SK, Gillis S, Cosman D. Determination of the minimum polypeptide lenghts of the functionally active sites of human interleukins-1α and -1β. *Proc Natl Acad Sci USA* 1987; 84: 4572.

58. Garcia-Calvo M, Peterson EP, Leiting B, Ruel R, Nicholson DW, Thornberry NA. Inhibition of human caspases by peptide-based and macromolecular inhibitors. *J Biol Chem* 1998; 273: 32608.

59. Tiku K, Tiku ML, Skosey JL. Interleukin-1 production by human polymorphonuclear neutrophils. *J Immunol* 1986; 136: 3677.

60. Bossu P, del Grosso E, Cesaroni MP, Maurizi G, Balestro N, Stoppacciaro A, del Giudice E, Ruggiero P, Boraschi D. Balance between autocrine interleukin-1β and caspases defines life versus death of polymorphonuclear cells. *Eur Cytokine Netw* 2001; 12: 177.

61. Rowe SJ, Allen L, Ridger VC, Hellewell PG, Whyte MK. Caspase-1-deficient mice have delayed neutrophil apoptosis and a prolonged inflammatory response to lipopolysaccharide-induced acute lung injury. *J Immunol* 2002; 169: 6401.

62. Re F, Mengozzi M, Muzio M, Dinarello CA, Mantovani A, Colotta F. Expression of interleukin-1 receptor antagonist (IL-1ra) by human circulating polymorphonuclear cells. *Eur J Immunol* 1993; 23: 570.

63. Suh B-C, Kim J-S, Namgung U, Ha H, Kim K-T. P2X7 nucleotide receptor mediation of membrane pore formation and superoxide generation in human promyelocytes and neutrophils. *J Immunol* 2001; 166: 6754.

64. Perregaux D, Gabel CA. Interleukin-1β maturation and release in response to ATP and nigericin. Evidence that potassium depletion mediated by these agents is a necessary and common feature of their activity. *J Biol Chem* 1994; 269: 15195.

65. Andrei C, Margiocco P, Poggi A, Lotti LV, Torrisi MR, Rubartelli A. Phospholipases C and A2 control lysosome-mediated IL-1β secretion: Implications for inflammatory processes. *Proc Natl Acad Sci USA* 2004; 101: 9745.

66. Elssner A, Duncan M, Gavrilin M, Wewers MD. A novel P2X7 receptor activator, the human cathelicidin-derived peptide LL37, induces IL-1 beta processing and release. *J Immunol* 2004; 172: 4987.

67. Kahlenberg JM, Lundberg KC, Kertesy SB, Qu Y, Dubyak GR. Potentiation of caspase-1 activation by the P2X7 receptor is dependent on TLR signals and requires NF-{kappa}B-driven protein synthesis. *J Immunol* 2005; 175: 7611.

68. Humke EW, Shriver SK, Starovasnik MA, Fairbrother WJ, Dixit VM. ICEBERG: A novel inhibitor of interleukin-1β generation. *Cell* 2000; 103: 99.

69. Annand RR, Dahlen JR, Sprecher CA, De Dreu P, Foster DC, Mankovich JA, Talanian RV, Kisiel W, Giegel DA. Caspase-1 (IL-1β–converting enzyme) is inhibited by the human serpin analogue proteinase inhibitor 9. *Biochem J* 1999; 342: 655.

70. Young JL, Sukhova GK, Foster D, Kisiel W, Libby P, Schönbeck U. The serpin proteinase inhibitor 9 is an endogenous inhibitor of IL-1β-converting enzyme (caspase-1) activity in human vascular smooth muscle cells. *J Exp Med* 2000; 191: 1535.

71. Chamaillard M, Girardin SE, Viala J, Philpott DJ. Nods, Nalps and Naip: intracellular regulators of bacterial-induced inflammation. *Cell Microbiol* 2003; 5: 581.

72. Shi Y. Caspase activation: revisiting the induced proximity model. *Cell* 2004; 117: 855.

73. Kollum M, Kaiser S, Kinscherf R, Metz J, Kubler W, Hehrlein C. Apoptosis after stent implantation compared with balloon angioplasty in rabbits. Role of macrophages. *Arterioscler Thromb Vasc Biol* 1997; 17: 2383.

74. Kockx MM, De Meyer GR, Bortier H, de Meyere N, Muhring J, Bakker A, Jacob W, Van Vaeck L, Herman A. Luminal foam cell accumulation is associated with smooth muscle cell death in the intimal thickening of human saphenous vein grafts. *Circulation* 1996; 94: 1255.

75. Geng Y-J, Libby P. Evidence for apoptosis in advanced human atheroma. Colocalization with ICE. *Am J Path* 1995; 147: 251.

76. Slim RM, Song Y, Albassam M, Dethloff LA. Apoptosis and nitrative stress associated with phosphodiesterase inhibitor-induced mesenteric vasculitis in rats. *Toxicol Pathol* 2003; 31: 638.

77. Donetti E, Baetta R, Comparato C, Altana C, Sartore S, Paoletti R, Castano P, Gabbiani G, Corsini A. Polymorphonuclear leukocyte-myocyte interaction: an early event in collar-induced rabbit carotid intimal thickening. *Exp Cell Res* 2002; 274: 197.

78. Welt FG, Edelman ER, Simon DI, Rogers C. Neutrophil, not macrophage, infiltration precedes neointimal thickening in balloon-injured arteries. *Arterioscler Thromb Vasc Biol* 2000; 20: 2553.

79. Gonschior P, Gerheuser F, Gonschior GM, Maier GR, Mack B, Nerlich A, Lehr HA, Hofling B. Experimental directional atherectomy injury in arterial vessels: impact of trauma depth on cellular response. *Am Heart J* 1995; 129: 1067.