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Caspase-1 Independent IL-1 β Activation in Neutrophil Dependent Inflammation

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Abstract

Objective—IL-1 β is a key cytokine linked to the pathogenesis of acute arthritis. Caspase-1, neutrophil elastase, and chymase all process pro-IL-1 β to its biologically active form. The potential contributions of these proteases were examined.

Methods—Caspase-1 (*Casp1*^{−/−}) deficient and wild type (WT) mice were tested for their response to K/BxN arthritogenic serum transfer and monosodium urate (MSU) crystal induced peritonitis while prophylactically treated with elastase or chymase inhibitors. Arthritic paws were tested for presence of IL-1 β protein by ELISA and Western blot. Neutrophils and mast cells from WT and mutant mice were tested for their ability to secrete IL-1 β after *in vitro* stimulation in the presence of protease inhibitors.

Results—*Casp1*^{−/−} and WT mice developed paw swelling to the same extent in the K/BxN serum transfer arthritis model. MSU crystal injection into *Casp1*^{−/−} mice also resulted in neutrophil influx and measurable peritoneal IL-1 β protein. Both of these responses were attenuated with neutrophil elastase inhibitors. K/BxN serum induced arthritis was also reduced by treatment with a chymase inhibitor. *Casp1*^{−/−} neutrophils and mast cells secreted similar amounts of IL-1 β protein upon *in vitro* stimulation with LPS, albeit at lower levels than WT cells when *Casp1*^{−/−} neutrophils were exposed to MSU crystals. Elastase and chymase inhibitors reduced IL-1 β released by these cells.

Conclusion—The production of IL-1 β by neutrophils and mast cells is not exclusively dependent on caspase-1 and other proteases can compensate for loss of caspase-1 *in vivo*. These pathways might therefore compromise the caspase-1 targeted therapies in neutrophil-predominant arthritis.

INTRODUCTION

Inflammatory arthritis is perpetuated by the innate immune system in addition to the driving force of the adaptive immune system. Key cells involved in barrier protection and innate immunity are recruited to the joint under inflammatory conditions. Amongst these cells, macrophages, neutrophils and mast cells have been implicated in the pathogenesis of both acute and chronic joint diseases. IL-1 is a cytokine secreted by innate immune cells, that has been implicated in promoting self-perpetuating inflammatory cascades (1).

The two forms of IL-1, IL-1 α and IL-1 β are subject to distinct regulation although they can both activate the same receptor, IL-1R, that transduces its signal via the Myeloid differentiation factor 88 (MyD88) adapter protein (2). Of these two IL-1 forms, IL-1 β is more frequently involved in chronic inflammatory disease and is subject to intricate controls at many different levels that limit its production and thereby prevent inadvertent of activation of inflammation (2). IL-1 β is produced in a proform without a leader sequence whose production and release depend on transcriptional and post-transcriptional processes (3–5). To be released in its active form pro-IL-1 β requires proteolytic cleavage (5,6). In macrophages the activation of caspase-1, also known as IL-1 converting enzyme (ICE), by inflammasome complexes is required for the pro-IL-1 β processing and IL-1 β secretion (4,6–8). Caspase-1 is a member of the caspase family of cysteine proteases and specifically cleaves the 31 kD pro-IL-1 β precursor to generate the mature, 17kD biologically active IL-1 β (4). Alternatively, neutrophil-derived serine proteases [cathepsin G (CG), neutrophil elastase (NE), and proteinase 3 (PR3)] as well as mast cell derived serine proteases (granzyme A and chymase) cleave the IL-1 β precursor at distinct sites into a secreted biologically active form (5,9–12).

In innate immune inflammation, the release of mature IL-1 β has been attributed primarily to activation of the inflammasome and consequently caspase-1 activation in monocytic cells (13). However, in the inflamed joint there are a variety of contributing cell types. Although macrophages are present in the inflamed synovium, we hypothesized that there are redundant mechanisms in IL-1 β processing and activation used by neutrophils and mast cells. We examined two murine model systems previously described to be neutrophil, IL-1 receptor (IL-1R)- and MyD88-dependent: K/BxN serum transferred arthritis and monosodium urate (MSU) crystal-induced peritonitis (14–19). In both systems, inflammation was not entirely caspase-1 dependent and exhibited redundancy in the mechanisms for IL-1 β processing. Potential therapies targeting the inflammasome and caspase-1 might therefore be of limited benefit compared to IL-1R antagonism, as other proteases can continue to produce active IL-1 β in the absence of caspase-1 activity.

METHODS

Reagents

Lipopolysaccharide (LPS; *Escherichia coli* 0111:B4) and MSU was purchased from Sigma (St. Louis, MO). Calcium ionophore A23187, elastase inhibitor III (Methoxysuccinyl-alanyl-alanyl-prolyl-valine-chloromethylketone, MeOSuc-Ala-Ala-Pro-Val-cmk) and elastase inhibitor IV (N-(2-(4-(2, 2-Dimethylpropionyloxy)phenylsulfonylamino)benzoyl)aminoacetic acid N-(o-(p-Pivaloyloxybenzene)sulfonylamino)benzoyl)glycine) (previously published as ONO-5046) (20) were purchased from Calbiochem (La Jolla, CA). The chymase inhibitor Suc-Val-Pro-Phe^p(OPh)₂ was synthesized by Peptide Institute Inc. (Minoh, Japan) (21–23). The inhibitors were resuspended in DMSO and then diluted in normal saline not to exceed 10% DMSO. Control injections matched the vehicle. The inhibitors were added to cell cultures 1 hr before stimulation or injected intraperitoneally (i.p.) 1 hr before KxB/N sera or MSU crystals

injection. MSU crystals were prepared under pyrogen-free conditions, using uric acid pretreated for 2 hrs at 200°C prior to crystallization as described (17). The crystals were suspended at 25 mg/ml in sterile, endotoxin-free phosphate buffered saline (PBS), and verified to be free of detectable LPS contamination (<0.025 endotoxin units/ml) by the Limulus amebocyte cell lysate assay (BioWhittaker, Walkersville, MD).

Mice

KRN T cell receptor (TCR) transgenic mice were a gift from Drs. D. Mathis and C. Benoist (Harvard Medical School, Boston, MA) and Institut de Génétique et de Biologie Moléculaire et Cellulaire (Strasbourg, France), and were maintained on a C57BL/6 background (K/B) (24). Arthritic mice were obtained by crossing K/B with NOD/Lt (N) animals (K/BxN). C57BL/6, *Il1r1*^{-/-}, NOD/Lt mice were purchased from The Jackson Laboratory (Bar Harbor, ME). *Casp1*^{-/-} mice were generously provided by Dr. Richard Flavell (Howard Hughes Medicine Institute Investigator at Yale University, New Haven CT) (6). *Casp1*^{-/-} and *Il1r1*^{-/-} mice were also maintained on a C57BL/6 background. The mice were bred and maintained under standard conditions at a University of California, San Diego Animal Facility that is accredited by the American Association for Accreditation of Laboratory Animal Care. All animal protocols received prior approval by the institutional review board.

Serum transfer and arthritis scoring

Arthritic adult K/BxN mice were bled and their sera were pooled. Recipient mice were injected with 150 µl i.p. on day 0. Some groups of mice also received 30 mg/kg/day ONO-5046 modified from a previous report (25) or 3 mg/kg/day chymase inhibitor. Clinical arthritis scores were evaluated using a scale of 0–4 for each paw (0, normal; 1, minimal erythema and mild swelling; 2, moderate erythema and mild swelling; 3, marked erythema and severe swelling, digits not yet involved; 4, maximal and swelling, digits involved). Ankle thickness in mm was measured with a caliper (Manostat, Switzerland).

MSU crystal induced inflammation

Peritonitis was induced by i.p. injection of 3 mg MSU crystals in 1 ml sterile PBS. Some mice received 1 mg MeOSuc-Ala-Ala-Pro-Val-cmk i.p. prior to MSU administration. Mice were sacrificed after 6 hrs and peritoneal cells were removed by lavage with 5 ml of 3 mM EDTA in PBS. Lavage fluids were separated by centrifugation and supernatants were used for cytokine analysis. The cell yield was obtained by counting and the relative percent of neutrophils was determined by flow cytometry after staining with anti-CD11b (BD Biosciences, San Jose, CA) and anti-Gr-1 (BD Biosciences) antibodies.

Histology

Whole hind paws were fixed in 10% formalin, decalcified, trimmed and embedded. Sections were prepared from the tissue blocks and stained with hematoxylin and eosin (H&E) and Safranin O (HistoTox, Boulder CO). Histopathological scoring was performed as described below. Joints of arthritic mice were given scores of 0–4 for inflammation, according to the following criteria: 0 = normal; 1 = minimal infiltration of inflammatory cells in periarticular area; 2 = mild infiltration; 3 = moderate infiltration; and 4 = marked infiltration. Joints of arthritic mice were given scores of 0–4 for bone resorption, according to the following criteria: 0 = normal; 1 = minimal (small areas of resorption, not readily apparent on low magnification); 2 = mild (more numerous areas of resorption, not readily apparent on low magnification, in trabecular or cortical bone); 3 = moderate (obvious resorption of trabecular and cortical bone, without full thickness defects in the cortex; loss of some trabeculae; lesions apparent on low magnification); and 4 = marked (full-thickness defects in the

cortical bone and marked trabecular bone loss). Cartilage depletion was identified by presence of diminished Safranin O staining of the matrix and was scored on a scale of 0–4, where 0 = no cartilage destruction (full staining with Safranin O), 1 = localized cartilage erosions, 2 = more extended cartilage erosions, 3 = severe cartilage erosions and 4 = depletion of entire cartilage. Histologic analyses were performed in a blinded manner.

Mast cell culture

Bone marrow cells were harvested from femurs and tibia of donors and cultured in RPMI with 10% FCS (Omega Scientific, Tarzana, CA), 1% penicillin/streptomycin, 0.1 mM nonessential amino acids, 5×10^{-5} M 2-mercaptoethanol and 3 ng/ml IL-3 (Peprotech) for 4 weeks, serially monitoring the cultures until they were >95% pure by toluidine blue staining (26). Cells were then dispersed into 96 well plates at a density of 500,000/well. Antibodies used for cross linking included 10 µg/ml anti-FcγRII/III clone 2.4G2, isotype control (BD Biosciences), and 10 µg/ml goat anti-rat F(ab')₂ (Jackson ImmunoResearch Laboratories, Inc, West Grove, PA) as described (27).

Neutrophil isolation

Mice were i.p. injected with 1 ml of 3% thioglycolate (DIFCO, Franklin Lakes, NJ). After 3–5 hrs the mice were euthanized and peritoneal cells were removed by lavage with 5 ml of 3 mM EDTA in PBS. The cells were incubated with anti-CD16/32 (BD Biosciences) and then with PE-labelled anti-Gr-1 (BD Biosciences). The cells were magnetically separated using anti-PE coated beads per manufacturer's instructions (Miltenyi Biotec, Auburn, CA). Cell purity was verified to be >95% by flow cytometry. Cells were seeded into 96 well plates at a density of 250,000/well.

Quantitative PCR

Mast cells and neutrophils were lysed in Trizol (Invitrogen, Carlsbad, CA). cDNA was generated using a Superscript II kit (Invitrogen) and amplicons were generated using SYBR green quantitative polymerase chain reaction (qPCR) master mix (Applied Biosystems, Foster City, CA). The primers used included IL-1β-forward 5'-CAACCAACAAGTGATATTCTCCATG-3' and reverse 5'-GATCCACACTCTCCAGCTGCA-3', and 18 S rRNA: forward 5'-CGCCGCTAGAGGTGAAAT TCT-3' and reverse 5'-CGAACCTCCGACTTTTCGTTCT-3'. The relative amounts of IL-1β transcripts were compared to those of 18S mRNA and normalized to untreated samples by the $\Delta\Delta C_t$ method.

Immunoblot

After snap freezing in liquid nitrogen and mechanical pulverization, paw tissues were disrupted in lysis buffer (PhosphoSafe™, Novagen, Gibbstown, NJ) containing a protease inhibitor cocktail. Proteins were separated by SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane. Blots were probed with IL-1β (Cell Signaling Technology, Danvers, MA) and actin (Chemicon International Inc) antibodies. Horseradish peroxidase-conjugated anti-IgG (Santa Cruz Biotechnology Inc, Santa Cruz, CA) was used as the secondary antibody. The membranes were developed using a chemiluminescence system (ECL detection reagent: Amersham Life Science, Aylesbury, UK).

ELISA

IL-1β and IL-18 amounts were measured by enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN and MBL International, Woburn, MA), following manufacturers' protocols.

Statistical analysis

Data are expressed as mean $\pm$ standard error of the mean (SEM). The analysis used unpaired Students' *t* test for comparing two groups and ANOVA for multiple group comparisons. Dunnett's *post hoc* tests were used for multiple comparisons to a control group and Bonferroni *post hoc* tests for multiple pair-wise comparisons. All statistical analyses were performed using PRISM version 4.0b (GraphPad Software, San Diego, California).

RESULTS

Caspase-1 is dispensable for K/BxN induced arthritis

The K/BxN serum transfer model is IL-1R- and MyD88-dependent (14,15). Pro-IL-1 β requires proteolytic cleavage for secretion of biologically active IL-1 β (1). Recently, inflammasomes were implicated as primarily responsible for activating caspase-1, and therefore IL-1 β secretion (13). Surprisingly, *Casp1*^{-/-} mice developed arthritis in the K/BxN serum transfer model with the same robustness as WT mice (Figure 1A). Clinical swelling, joint inflammation and damage were comparable between WT and *Casp1*^{-/-} mice (Figure 1A and B). There were no significant differences in the histology scores between WT and *Casp1*^{-/-} mice (n=8/group) for inflammation (3.5 $\pm$ 0.3 vs 3.3 $\pm$ 0.3), bone erosion (2.6 $\pm$ 0.4 vs 2.7 $\pm$ 0.3) and cartilage damage (2.4 $\pm$ 0.3 vs 2.1 $\pm$ 0.3) respectively. Consistent with their arthritic phenotype, the joint tissues of *Casp1*^{-/-} mice had elevated levels of IL-1 β and cleaved IL-1 β was locally detectable in the affected paws (Figure 1C). In addition the joint tissue had elevated levels of IL-18, another interleukin that can be processed by caspase-1, as well as other enzymes (28).

Mast cells and neutrophil exploit alternative enzymes in IL-1 β processing

Previously, mast cells and neutrophils were reported to play prominent roles in the pathogenesis of paw swelling in the K/BxN serum transfer model (18,26,29). Hence, these cell types were examined for their ability to express and secrete IL-1 β in the absence of caspase-1. First, bone marrow was enriched for mast cells by culturing in the presence of IL-3. Mast cells from both WT and *Casp1*^{-/-} mice generated comparable levels of IL-1 β mRNA transcripts (Figure 2A) and secreted similar amounts of IL-1 β protein when stimulated with LPS (Figure 2B).

In a previous report *Casp1*^{-/-} neutrophils competently released mature IL-1 β in response to LPS stimulation and were shown to utilize PR3 and NE as alternative proteases to process IL-1 β (30). Correspondingly, release of IL-1 β into the supernatant by WT neutrophils was limited by a potent elastase inhibitor, MeOSuc-Ala-Ala-Pro-Val-cmk, as previously published (Figure 2C)(30). As elastase is not typically found in the granules of mast cells, chymase was tested as a potential IL-1 β secretory protease, as it was reported to cleave pro-IL-1 β into its active form (9). After 16 hrs of stimulation with LPS, WT mast cells demonstrated a reduced ability to secrete IL-1 β in the presence of a chymase inhibitor, Suc-Val-Pro-Phe^P(OPh)₂, compared to vehicle treated control wells (Figure 2D). Suc-Val-Pro-Phe^P(OPh)₂ treatment also reduced the level of IL-1 β produced with synergistic stimulation with LPS and the calcium ionophore A23187. This inhibitor potently affects chymase, but has also been reported to influence the activity of chymotrypsin and cathepsin G (21). Mast cells contribute to the initiation of inflammation within the joint by elaboration of IL-1 β in response to activation of the IgG immune complex receptor Fc γ RIII (27). Suc-Val-Pro-Phe^P(OPh)₂ was also able to reduce IL-1 β secretion by mast cells after ligation of Fc γ RII/III (Figure 2D).

Arthritis is attenuated *in vivo* by chymase and elastase inhibitors

To extend the findings described above into the K/BxN arthritis model, WT mice were treated with the chymase inhibitor, Suc-Val-Pro-Phe^P(OPh)₂, without demonstrable reduction in joint swelling (data not shown). As there are multiple cell types and enzymatic pathways that could be involved in IL-1 β processing and secretion *in vivo*, we used caspase-1 deficient mice to eliminate at least one pro-IL-1 β processing protease. After K/BxN serum transfer, *Casp1*^{-/-} mice had attenuated paw swelling when treated with 3 mg/kg/day of a chymase inhibitor, Suc-Val-Pro-Phe^P(OPh)₂ (Figure 3A). In other experiments, the areas under the curve (AUC) for joint swelling for doses of 1mg/kg/day, 3mg/kg/day and 10mg/kg/day were 83%, 20% and 14% of vehicle injected *Casp1*^{-/-} mice respectively. We tested additional protease inhibitors in these mice. The elastase inhibitor, MeOSuc-Ala-Ala-Pro-Val-cmk, had no effect on K/BxN serum-induced paw swelling in *Casp1*^{-/-} mice (data not shown), despite its *in vitro* activity in cultured neutrophils (Figure 2C). However administration of the ONO-5046 inhibitor significantly reduced paw swelling (Figure 3A). The areas under the curve (AUC) for joint swelling in mice that received doses of 10mg/kg/day, and 30mg/kg/day of ONO-5046 were 83%, and 21% of vehicle injected *Casp1*^{-/-} mice respectively. The reduction in paw swelling seen with Suc-Val-Pro-Phe^P(OPh)₂ and ONO-5046 was associated with decreased amounts of mature IL-1 β and IL-18 in wrist tissues (Figure 3B). Concordantly, the cleaved form of IL-1 β was detectable in paw lysates from vehicle treated mice, but not from mice that received active inhibitors (Figure 3C).

Neutrophils employ alternative proteases to generate soluble IL-1 β after MSU crystal stimulation

Caspase-1 has been reported to be the predominant activator of IL-1 β in macrophages after MSU crystals stimulation (16). However in gout, neutrophils are heavily recruited to the joint (31). To assess whether the ability to secrete IL-1 β in response to MSU stimulation is dependent on caspase-1 or other proteases in neutrophils, neutrophils from caspase-1 deficient and WT mice were tested *in vitro*. Although IL-1 β mRNA was similarly induced in WT and *Casp1*^{-/-} neutrophils, the latter release less mature IL-1 β protein than MSU stimulated WT cells (Figure 4A and B). However, some IL-1 β secretion was detected from MSU stimulated *Casp1*^{-/-} neutrophils, suggesting the involvement of other proteases. Indeed, the elastase inhibitor, MeOSuc-Ala-Ala-Pro-Val-cmk, reduced the level of IL-1 β secretion by WT neutrophils by approximately 40% (Figure 4C). MSU crystal stimulated caspase-1 deficient neutrophils secreted less IL-1 β than the WT neutrophils; however IL-1 β secretion was abrogated in the presence of the MeOSuc-Ala-Ala-Pro-Val-cmk (Figure 4D).

Redundant proteases are activated in MSU crystal induced peritonitis

To assess the response of caspase-1 deficient mice to MSU crystals, MSU induced peritonitis was evaluated. Neutrophil recruitment was assessed by injecting MSU crystals into the peritoneum of WT, *Il1r1*^{-/-} and *Casp1*^{-/-} mice. After 6 hrs the peritoneal cavities were lavaged and the number of neutrophils were quantified. The IL-1R deficient mice did not significantly recruit neutrophils into the peritoneum after MSU injection, consistent with a previous report (16). Interestingly the caspase-1 deficient mice had significant influx of neutrophils, albeit at reduced levels compared to the WT mice. When the mice were pretreated with the elastase inhibitor MeOSuc-Ala-Ala-Pro-Val-cmk the number of neutrophils in the WT mice was reduced to similar to those seen in untreated caspase-1 deficient mice, which exhibited only a small reduction in neutrophil influx in response to the elastase inhibitor (Figure 5A). The numbers of recruited neutrophils correlated with the level of IL-1 β measured in the lavage fluids (Figure 5B). Despite the potential role of mast cells in the MSU peritonitis model (32), the chymase inhibitor did not reduce the number of neutrophils or the levels of IL-1 β measured in the lavage fluids of either WT or *Casp1*^{-/-} mice (data not shown).

DISCUSSION

In most arthritic conditions, sterile inflammation driven by the innate immune system is involved in both the initiation and chronic phases of the disease (33). In the K/BxN serum transfer model the effector phase is marked by several innate immune pathways and is absolutely dependent upon IL-1R and its adaptor protein, MyD88, for disease manifestation (14,15). Similarly the MSU crystal-induced peritonitis model is also absolutely dependent upon the IL-1R and MyD88 (17,19). IL-1 β has been implicated in promoting a self-perpetuating inflammatory cycle, whereby the initial IL-1 β release results in secondary production of chemokines and in the recruitment of neutrophils that in turn release more cytokines including IL-1 β family members (2,34,35). In the K/BxN serum transfer model, mast cells may be the cells that provide the initial release of IL-1 β (27). In the MSU crystal induced peritonitis model mast cells are also recruited (32), but the first cells to produce IL-1 β remain to be identified. In both models, however, neutrophil recruitment is rapid and is required for most of the pathological response (16,18). Surprisingly, caspase-1 deficient mice developed paw swelling response to K/BxN serum transfer and MSU crystal induced peritonitis. Seeking to resolve this discrepancy we examined whether other proteases could be involved in IL-1 β processing and therefore compensate for the loss of caspase-1. Review of the literature suggested neutrophil and mast cell-derived serine proteases such as NE, PR3 and chymase as potential candidates (5,9–12). All of them cleave the IL-1 β precursor into a secreted biologically active form and these alternatively cleaved forms of functional IL-1 β have been detected in synovial fluid samples from patients with polyarticular inflammatory arthritis and gout (11).

Although neutrophil and mast cell-derived serine proteases have a pathogenic role in several arthritis models, the ability to produce bioactive IL-1 β was not directly assessed (25,36,37). The elastase inhibitor ONO-5046 has previously been demonstrated to alleviate arthritis in a rat collagen induced arthritis (CIA) model, suggesting that NE may play a key role in the pathogenesis of this model (25). The role of mast cell-derived proteases have been more recently examined. A recent study showed that mice lacking murine mast cell proteases (mMCP)-6 and -7 (tryptases) developed less severe arthritis than did WT mice, suggesting that MC tryptase may have a pathogenic role in arthritis (38). However we are not aware that tryptase has been reported to cleave pro-IL-1 β into a biologically mature active form. An additional report utilizing mMCP4 deficient mice demonstrated attenuated response in both active CIA and passive anti-collagen transfer models (37). mMCP4 is the closest ortholog of the human chymase and is expressed by synovial mast cells (39). However, the ability of the mMCP4 deficient mice to produce IL-1 β was also not directly addressed.

In our studies both Suc-Val-Pro-Phe^P(OPh)₂ and ONO-5046 reduced the release of mature IL-1 β and attenuated the induction of K/BxN serum transferred arthritis in *Casp1*^{-/-} mice, suggesting that other proteases can continue to produce active IL-1 β in the absence of caspase-1 activity. Presumably, the chymase inhibitor may have influenced the release of IL-1 β by mast cells as implicated by the *in vitro* experiment (Figure 2D), whereas ONO 5046 may have reduced neutrophil migration and IL-1 β secretion by neutrophils. Although the implication was that all of these inhibitors abrogated functional IL-1 β secretion, protease inhibitor might have had other functional consequences. Serine-proteases (CG, NE and PR3) have also been reported to catalyze the release of active forms of CXC chemokines (40, 41). In addition, there are inhibitors of inflammatory pathways such as progranulin that are also inactivated by PR3 and NE (42). Also, impairing NE and PR3 activity on the cell surface of neutrophils might impact their ability to access inflamed spaces and activate adhesion dependent oxidative burst (42–45).

Although Suc-Val-Pro-Phe^P(OPh)₂ was originally characterized as a potent chymase inhibitor, this peptidomimic was also noted to have effects on other proteases such as cathepsin G and chymotrypsin (21). Likewise, MeOSuc-Ala-Ala-Pro-Val-cmk was previously noted to also inhibit PR3, in addition to its effect on NE (30). However, ONO-5046 specifically inhibits leukocyte elastase and was not found to inhibit trypsin, thrombin, plasmin, plasma kallikrein, pancreas kallikrein, chymotrypsin or cathepsin G even at a high dose of 100 μ M (20). In the K/BxN serum transfer model the elastase inhibitor, MeOSuc-Ala-Ala-Pro-Val-cmk, did not reduce arthritis at the same dose that was effective in the MSU peritonitis model, yet ONO-5046 was therapeutically beneficial. The potentially broader off-target effects of MeOSuc-Ala-Ala-Pro-Val-cmk, including its effect on PR3, may have negated the beneficial effects of inhibiting IL-1 β processing with concomitantly reducing the processing or shedding on unknown anti-inflammatory mediators.

The MSU crystal induced peritonitis model is also absolutely dependent upon the IL-1R and MyD88 for disease manifestation (17,19). Macrophages from mice deficient in various inflammasome components such as caspase-1, apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) and NALP3 are defective in MSU-induced IL-1 β secretion (16). In our studies and in prior reports (16,19) the influx of neutrophils in sterile peritonitis from MSU crystals was reduced six fold in *Il1r1*^{-/-} mice. However, there was only a 2-fold reduction in neutrophil influx in *Casp1*^{-/-} mice. In our study the inhibitor MeOSuc-Ala-Ala-Pro-Val-cmk reduced the influx of neutrophils in the WT mice by two-fold; yet the same inhibitor did not diminish the numbers of peritoneal neutrophils in the *Casp1*^{-/-} mice to the same extent as the ablation of IL-1R. This suggests that there are other proteases, such as chymase or cathepsin G that can further compensate for the loss of caspase-1 deficiency in this model.

Collectively, our results illustrate that there were redundant proteolytic activities produced by innate immune cells that can process pro-IL-1 β , and promote secretion of mature IL-1 β and induction of IL-1 β dependent inflammation. Indeed, it has been suspected for some time that caspase-1 is not the only protease involved in IL-1 β and IL-18 processing. In neutrophils, production of IL-1 β is not entirely dependent on caspase-1 and several serine proteases including CG, NE and PR3 can also process pro-IL-1 β and pro-IL18 (28,30,36,46). The important innate immune function of IL-1 β in host defense might have to rely on several alternative proteases to induce IL-1 β secretion and thereby trigger neutrophil mobilization. Given this complexity and redundancy, targeting of inflammasome and caspase-1 in gout and other neutrophil dependent arthritis may not be as successful as the use of IL-1 receptor antagonism (47). On the other hand, caspase-1 and inflammasome inhibitors may not compromise host defense to the same extent as IL-1 receptor inhibition.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. IL-1 β is processed and secreted in K/BxN serum transferred arthritis even in the absence of caspase-1

(A) Clinical scores and joint swelling were similar in *Casp1*^{-/-} and WT mice. *Casp1*^{-/-} (squares, N=19) and C57BL/6 (circles, n=20) mice were injected with 150 μ l of pooled K/BxN sera on day 0. Swelling and clinical scores were serially monitored, and averages $\pm$ SEM are shown. No significant differences were observed ($p > 0.05$ by ANOVA). Data are pooled from five independent experiments. (B) Joint inflammation and destruction are similar in C57BL/6 and *Casp1*^{-/-} mice. Mice were sacrificed on day 7 at the peak of arthritis and rear paws were removed, and prepared for histology. Representative ankles joints stained with H&E and safranin O are shown (original magnification x200). (C) IL-1 β and IL-18 were assayed by ELISA in disrupted joint tissue from C57BL/6 (solid) and *Casp1*^{-/-} (striped) mice on day 0 and day 7. Shown are means $\pm$ SEM. Processed IL-1 β was detectable by immunoblotting lysates of paws from C57BL/6 and *Casp1*^{-/-} mice on day 7.

Figure 2. Mast cells and neutrophils use several proteases to process IL-1 β

(A) C57BL/6 and *Casp1*^{-/-} mast cells were stimulated for 4 hr with LPS (1 μ g/ml) and IL-1 β mRNA transcript levels were analyzed by qPCR and normalized to 18S mRNA. (B) Mast cells from C57BL/6 (B6, dots) and *Casp1*^{-/-} (C1, stripes) mice were stimulated for 16 hrs with LPS (1 μ g/ml). The levels of secreted IL-1 β were assayed by capture ELISA and cell lysates were probed for the presence of IL-1 β . (C) Thioglycolate induced neutrophils from C57BL/6 micewere stimulated with LPS (100 ng/ml) in the presence (dark) or absence (light) of the MeOSuc-Ala-Ala-Pro-Val-cmk (500 μ M) inhibitor. After 16 hrs levels of IL-1 β secretion were measured by ELISA. (D) WT mast cells were stimulated for 16 hrs with LPS (1 μ g/ml) in the presence of the Suc- Val-Pro-Phe^P(OPh)₂ inhibitor (dark) or vehicle (light). Additional wells received a calcium ionophore (A23187, 0.5 μ g/ml) for the last hr of incubation with LPS to enhance IL-1 β secretion. Mast cells were also stimulated for 16 hrs with anti-Fc γ RII/III crosslinking in the presence of Suc-Val-Pro-Phe^P(OPh)₂ (dark) or vehicle (light). IL-1 β secretion was measured by ELISA. Results are averages of three independent experiments $\pm$ SEM. * denotes $p < 0.05$ versus control.

Figure 3. Serum transferred arthritis is attenuated by chymase and elastase inhibitors
Caspr^{-/-} mice were injected with 150 μ l pooled K/BxN sera on day 0. Groups of mice were treated with vehicle (square, n=7), chymase inhibitor (Suc-Val- Pro-Phe^p(OPh)₂)(circle, n=7) or elastase inhibitor ONO-5046 (triangle, n=8) daily i.p. (A) Ankle thickness was assessed daily. Data shown were pooled from two independent experiments. * designates p<0.05 by ANOVA and post hoc Bonferonni comparisons. (B) On day 5 after serum transfer, mice were sacrificed and their paws were snap frozen. Pulverized paws were lysed and assayed for IL-1 β and IL-18 y ELISA (n=4/group). * designates p<0.05 by ANOVA and significantly different to the WT vehicle treated mice by Dunnet's test. (C) Lysates of pooled paws were immunoblotted for the presence of IL-1 β and actin.

Figure 4. MSU stimulated IL-1 β secretion by neutrophils depends on caspase-1 and other proteases

(A) Neutrophils from WT (solid) and *Casp1*^{-/-} (striped) were stimulated in culture for 4 hrs with MSU crystals (0.5 mg/ml), lysed and IL-1 β mRNA transcripts were assessed by qPCR. The fold induction is relative to PBS treated control cultures. (B) Thioglycolate elicited neutrophils from WT (solid) and *Casp1*^{-/-} (striped) mice were harvested and stimulated with the indicated doses of MSU crystals. The levels of IL-1 β released into the supernatant were measured after 16 hrs by ELISA. Thioglycolate stimulated peritoneal neutrophils from C57BL/6 (C) and *Casp1*^{-/-} (D) mice were stimulated *in vitro* with the indicated doses of MSU crystals in the presence of the MeOSuc-Ala-Ala-Pro-Val-cmk (500 μ M, dark) inhibitor or vehicle (light). After 16 hrs IL-1 β secretion to the supernatant was measured by ELISA. Results are averages of three independent experiments $\pm$ SEM.

Figure 5. Redundant roles for caspase-1 and elastase in murine MSU induced peritonitis WT, *Casp1*^{-/-} or *Il1r1*^{-/-} mice (n=4/group) were i.p. injected with 1ml PBS or 3 mg/ml MSU crystals as indicated. Some groups of mice were i.p. injected with 1 mg MeOSuc-Ala-Ala-Pro-Val-cmk (AAPV) 1 hr before MSU crystal injection. The peritoneum was lavaged after 6 hrs. (A) Neutrophil influx was determined by cell count and flow cytometry. (B) IL-1β levels in lavage fluids were assayed by ELISA. Shown were means ± SEM. Significance was assessed by ANOVA with *post hoc* Bonferroni pair wise comparisons.