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Inflammatory arthritis in caspase-1 gene deficient mice: Contribution of proteinase 3 for caspase-1-independent production of bioactive IL-1β

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Abstract

Objective—Caspase-1 is a known cysteine proteases and is a critical component of the inflammasome. Caspase-1 and neutrophil serine proteases, such as proteinase 3 (PR3) can process pro-IL-1β a crucial cytokine linked to the pathogenesis of rheumatoid arthritis, but their relative importance is unknown.

Methods—To this end we induced acute and chronic arthritis in caspase-1−/− mice and investigated the lack of caspase-1 on joint swelling, cartilage metabolism and joint pathology. In addition, caspase-1 activity was inhibited in mice lacking active cysteine proteases and evaluated the effect of dual blockade of caspase-1 and serine proteinase on arthritis severity and joint pathology.

Results—Surprisingly, caspase-1−/− mice developed joint swelling similar to wild-type mice in models of neutrophil-dominated arthritis. Joint fluid concentrations of bioactive IL-1β were comparable in caspase-1−/− mice and controls. In contrast, induction of chronic arthritis with minimal numbers of neutrophils in caspase-1−/− mice lead to reduced joint inflammation and cartilage damage, implying caspase-1 dependence. In mice lacking neutrophil serine PR3, inhibition caspase-1 activity results in decreased bioactive IL-1β concentrations in synovial tissue and less suppression of chondrocyte anabolic function. In addition, dual blockade of both PR-3 and caspase-1 lead to protection against cartilage and bone destruction.

Conclusions—We conclude that caspase-1 deficiency does not affect neutrophil-dominated joint inflammation, whereas in chronic arthritis the lack of caspase-1 results in reduced joint pathology. This study implies that caspase-1 inhibitors are not able to interfere with the whole spectrum of IL-1β production and hence may be of therapeutic value only in inflammatory conditions where limited numbers of neutrophils are present.

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Cytokines such as interleukin-1β (IL-1β) produced by cells of the innate immune system are induced in response to a variety of pathogen- or damage-associated molecular patterns. Due to its potent inflammatory properties, IL-1β can be deleterious if released in high amounts in various sites of the body (1). Both production and activity of IL-1β are tightly regulated at several levels: transcription and translation (2), conversions of the inactive pro-IL-1β form into the bioactive IL-1β (3), excretion in microvesicles through K^+-dependent mechanisms (4), as well as at the level of its receptors by natural antagonists (IL-1 receptor antagonist, IL-1Ra), decoy receptors (IL-1R type II) and IL-1RI receptor shedding (5,6).

Much interest has been generated in recent years in the regulation of IL-1β, especially since the discovery that many of the manifestations of the so called autoinflammatory disorders that include familial Mediterranean fever, Muckle-Wells syndrome, hyperimmunoglobulin D syndrome, familial cold urticaria, juvenile rheumatoid arthritis, adult-onset Still's disease, relapsing polychondritis, and Schnitzler syndrome are due to a dysregulated IL-1β production (7). Consequently, treatments that block IL-1 activity, either IL-1Ra or anti-IL-1β antibodies, are highly-effective in these disorders (8). Even β–cell destruction in patients with type 2 diabetes is related to IL-1β-mediated toxicity, and can be prevented by IL-1Ra (anakinra) treatment (9). Blockade of IL-1β activity has also been proved to be beneficial in rheumatoid arthritis (10). The relative short half life of IL-1Ra and the necessity for injections make it a suboptimal drug. Further elucidation of the molecular mechanisms behind production of bioactive IL-1β is needed for designing more effective treatment interfering with IL-1β production and action.

In contrast to many other proinflammatory cytokines, IL-1β lacks a signal peptide, and its processing and secretion depend on cleavage by proteolytic enzymes such as caspase-1. Caspase-1 activation in turn has been proposed to be mediated by protein platforms called the inflammasomes (11). Several of such inflammasomes are able to activate caspase-1, all of them including members of the NOD-like receptor (NLR) family of proteins, such as NALP3, NALP1, and IPAF (12). Mutations in NALP3 (cryopyrin) cause Muckle-Wells syndrome, NOMID and CINCA syndromes (13,14), whereas NALP-1 polymorphisms are associated with vitiligo and autoimmune diseases (15). Not only caspase-1, but also serine proteinases such as PR3, elastase or cathepsin-G, can process pro-IL-1β (16,17), but the role of joint inflammation and cartilage destruction is limited (18). In addition, mast cell proteases granzyme A and chymase have been also implicated in the activation pro-IL-1β (19,20).

Although the role of the NLR inflammasomes for IL-1β activation is supported by in-vitro studies and clinical data in patients with autoinflammatory disorders, it is unclear whether activation of the inflammasome is also involved in other inflammatory disorders such as arthritis. Moreover, the relative role of caspase-1 and serine proteases for activation of pro-IL-1β is not known in arthritis. As both caspase-1 and proteinase 3 (PR3) are considered to be potential targets in inflammation, discerning their roles in arthritis is important for the design of novel anti-IL-1β therapies. In the present study we investigated the specific contribution of caspase-1 and serine proteinases to acute and chronic inflammation in experimental models of arthritis. To this end we used caspase-1 and - DPPI deficient mice, which lack neutrophil proteases in combination with a potent caspase-1 inhibitor.

Materials and Methods

Animals

Male C57Bl/6 and Balb/c mice were obtained from Charles River Wiga (Sulzfeld, Germany). IL-1β gene deficient mice were kindly provided by J. Mudgett, Merck, Rahway, NJ, USA. Caspase-1 deficient mice were originally obtained from R.A. Flavell, New Haven,

CT, USA (21). Dipeptidyl peptidase I (DPPI) gene deficient mice (Balb/C background) were obtained from Christine T. Pham. The homozygous MMP-9 deficient mice were obtained from Robert Thompson, Washington University, St. Louis, Missouri, USA. C57/Bl6-Beige/Beige mice (lacking neutrophil elastase and cathepsin G) were obtained from Harlan/Olac, UK. All mice were bred at the Central Animal Laboratory, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands. All mice were housed in filter top cages and water and food were supplied *ad libitum*. The mice were used at the age of 10–14 weeks. All animal experiments were approved by the university animal ethic committee.

Materials

All PCR primers were purchased from Biolegio, Malden, The Netherlands. Bovine serum albumin was purchased from Sigma-Aldrich (St Louis, MO). TRIzol reagent, TaqDNA polymerase, and RPMI 1640 medium were obtained from Life Technologies (Breda, The Netherlands). SyberGreen was purchased from Applied Biosystems (Foster City, CA, USA). Cytokine kits for the Luminex x-MAP technology were purchased from Bio-Rad (Hercules, CA, USA). TNFα bead kit was obtained from R&D systems (Abingdon,UK). Caspase-1 inhibitor was provided by Novartis, Basel, Switzerland.

K/BxN arthritis model

The K/BxN serum transfer model is based on antibodies that recognize glucose-6-phosphate isomerase (GPI) (22). Transfer of serum or purified immunoglobulin (Ig)G from K/BxN mice leads to induction of a robust and reproducible acute disease in several mouse strains, including C57black/6 mice. Caspase-1−/− and wild type mice were injected with 200μl of arthritic serum and the expression of arthritis was scored macroscopically on a scale ranging from 0–2 per hind paw.

SCW preparation and induction of acute or chronic SCW arthritis

Streptococcus Pyogenes T12 organisms were cultured overnight in Todd-Hewitt broth. Cell walls were prepared as described previously (23). The resulting 10.000×g supernatant was used throughout the experiments. These preparations contained 11% muramic acid. Unilateral arthritis was induced by intraarticular injection of 25 μg SCW (rhamnose content) in 6 μl of PBS into the right knee joint of naïve mice. To create a chronic streptococcal cell wall (SCW)-induced arthritis, multiple i.a. injections into the right knee joint were performed at days 0, 7, 14 and 21. These repeated injections resulted in a chronic inflammation at day 28.

Treatment of SCW-induced arthritis with caspase-1 inhibitor

Mice were daily given orally a potent caspase-1 inhibitor Pralnacasan (24) at a dose of 100 mg/kg in 0.5% Tylose. For the acute SCW-induced arthritis model mice were treated from day 0 (−2h) until day 2 with vehicle or caspase-1 inhibitor. To block caspase-1 during chronic SCW-induced model, mice were treated for 14 days with the caspase-1 inhibitor starting at day 14 (−2h) up to day 28 of arthritis. As control mice were treated with vehicle for 14 days.

Joint swelling measurement

Joint inflammation was measured by the ^{99m}Tc uptake method as described previously (25,26). In brief, 20μCi of the radioisotope ^{99m}Technetium in 200μl saline was injected subcutaneously and thereafter the ^{99m}Tc distributed over the whole body in a few minutes. Due to increased blood flow and edema formation, accumulation of ^{99m}Tc in the right (inflamed) joint can be measured with external gamma counting. Joint swelling is expressed

as the ratio of the ^{99m}Tc uptake in the inflamed over the control joint (left knee joint). All values exceeding 1.10 are assigned as inflammation.

Chondrocyte proteoglycan (PG) synthesis

Patellae were isolated with minimal surrounding synovial tissue and chondrocyte PG synthesis was determined by ex-vivo 35S-sulphate incorporation. Briefly, patellae were incubated in RPMI 1640 medium complemented with 740 μBq/ml ^{35}S-sulphate for 2h at 37°C. Thereafter patellae were washed for 3 times in saline and fixed in 4% buffered formaldehyde. After decalcification in 5% formic acid, the patellae were separated from the surrounding tissue and dissolved in Lumasolve (25). The radioactivity was determined by liquid scintillation counting.

RNA isolation and PCR amplification

Immediately after cervical dislocation synovial tissue was isolated from the inflamed knee joints. The synovium samples were immediately stored in N_2 until total RNA isolation. Using Magnalyzer system (Roche, Basel, Switzerland) the synovium samples were grinded and total RNA was extracted in 1 ml TRIzol reagent (Life technologies, Breda, The Netherlands). Subsequently 200μl chloroform and 500μl 2-propanol (Merck, Darmstadt, Germany) were used to separate the RNA from DNA and proteins. Finally, after a wash step with 75% ethanol (Merck, Darmstadt, Germany) the dry RNA was dissolved in 30 μl of water. To obtain ds cDNA, standard RT-PCR was performed using oligo dT primers. Subsequently quantitative PCR was performed using ABI/PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). PCR's of GAPDH, F4/80 and MPO were performed with Sybr Green PCR Master Mix (Applied Biosystems). Quantification of the PCR signals of each sample was performed by comparing the cycle threshold values (C_t), in duplicate, of the gene of interest with the C_t values of the GAPDH housekeeping gene. We validated all primers according to the protocol and the standard curves were all within the tolerable range.

Cytokine determination

Protein levels of IL-1β or TNFα were measured in patellae washouts. At several time points after induction of acute or chronic SCW arthritis patellae were isolated from inflamed knee joints and cultured 1 hour at RT in RPMI 1640 medium containing 0.1% bovine serum albumin (200μl/patella). Thereafter supernatant was harvested and centrifugated for 5 minutes at 1000 × *g*. IL-1β and TNFα were measured in 50 μl patella washouts, by using Luminex xMAP technology. Bioactive IL-1 was measured using the NOB-1 bioassay as previously described (27), with a modification of the IL-2 measurement. This was performed by Luminex xMAP using Bio-Plex IL-2 kit (Bio-Rad, Hercules, CA, USA).

Processing of pro-IL-1 β by PR3 or caspase-1

Human recombinant pro-IL-1β (R&D systems, Minneapolis, MN, USA) was incubated for 120 minutes with purified 10 μg/ml human PR3 or caspase-1 (Athens Research & Technology, Athens GA, USA). Thereafter, cleaved IL-1β was tested for bioactivity using A549 cells for IL-1β-induced IL-8 production. Cells were incubated for 24h with pro-IL-1β, cleaved product or mature IL-1β. As comparison, pro-IL-1β was processed by caspase-1. Specific ELISA's were used to detect pro-IL-1β or total IL-1β (DLBP00, R&D systems, Minneapolis, MN, USA).

Histological analysis

Mice were sacrificed by cervical dislocation. Whole knee joints were removed and fixed in 4% formaldehyde for 7 days before decalcification in 5% formic acid and processing for

paraffin embedding. Tissue sections (7μm) were stained with Haematoxylin/Eosin or Safranin O/Fast Green. Histopathological changes in the knee joints were scored in the patella/femur region on 5 semi-serial sections, spaced 140 μm apart. Scoring was performed on decoded slides by two separate observers, using the following parameters: in the haematoxylin/eosin stained slides the amount of cells infiltrating the synovial lining and the joint cavity was scored from 0–3. Proteoglycan (PG) depletion was scored in the safranin O stained slides on a scale from 0–3 (ranging from stained cartilage to fully destained cartilage). Cartilage damage was scored from 0–3, ranging from non-effected cartilage to maximum of chondrocyte death and cartilage destruction (28).

Statistical analysis

Differences between experimental groups were tested using the Mann-Whitney U-test. Data are expressed as mean±SEM, unless stated elsewhere. *P<0.05, compared to wild control mice.

Results

Deficiency of caspase-1 during acute arthritis does not lead to reduced joint inflammation or cartilage damage

Although there are previous reports using caspase-1 inhibitors in models of (osteo)arthritis, the precise role of caspase-1 in the production of active IL-1β in models of arthritis is not known. To investigate the function of caspase-1 in a model of acute arthritis, we examined the passive K/BxN model, an IL-1-dependent model of acute arthritis in caspase-1−/− mice (22). Figure 1A shows that caspase-1 activity is not needed for induction of arthritis induced by injection of arthritic K/BxN serum. To corroborate these findings, we explored another model of arthritis in which IL-1β is produced and IL-1-mediated cartilage damage can be analyzed. As reported previously, intraarticular injection of cell wall fragments of *Streptococcus pyogenes* (SCW) results in joint inflammation and concomitant cartilage damage, the latter being highly IL-1 dependent (25,26,29). When we induced acute SCW arthritis in caspase-1−/− mice we found no evidence for reduced joint swelling, compared to wild-type mice (Figure 1B). Since active caspase-1 converts pro-IL-1β to active IL-1β, we compared caspase-1−/− mice with IL-1β−/− mice (Figure 1). As shown previously, IL-1β−/− mice respond similar as wild-type mice to induction of acute SCW arthritis (26). Interestingly, caspase-1−/− mice produced more local IL-1β (pro- and mature-IL-1β) than the wild-type mice (Figure 1C). This might be explained by excessive storage of pro-IL-1β due to the lack of caspase-1.

Inhibition of chondrocyte proteoglycan (PG) synthesis is a hallmark of joint inflammation that is almost exclusively IL-1-dependent (25,29). This is illustrated in figure 1D, in which chondrocyte PG synthesis is strongly inhibited at days 1, 2 and 4 after induction of SCW arthritis in wild-type mice, while the chondrocyte PG synthesis in cartilage from IL-1β−/− mice is fully protected. Lack of caspase-1 on the other hand did not result in protection against inhibition of chondrocyte PG synthesis, which is in line with the local concentration of IL-1β shown in Figure 1C. However, on day 4 of SCW arthritis a partial recovery of the chondrocyte PG synthesis was noted in the caspase-1−/− mice (Figure 1D). In addition, histology at day 7 of SCW arthritis revealed that the loss of matrix PG, due to the arthritic process, was ameliorated in the caspase-1−/− mice compared to wild-type mice (Table 1 and Figure 1E/F). As reported previously (23), IL-1β−/− mice were completely protected against inflammation-induced cartilage PG loss (Table 1).

Caspase-1 deficiency during chronic arthritis partially protects against severe cartilage damage

To investigate the role of caspase-1 in chronic destructive arthritis we induced a model of chronic SCW-induced arthritis in caspase-1−/− mice. It was recently demonstrated that this model is highly IL-1β-dependent (26) and we compared the role of caspase-1 with that of IL-1β. Figure 2A displays the protocol for induction of chronic SCW-induced arthritis. After the fourth local injection of bacterial fragments (SCW) chronic inflammation develops with concomitant joint destruction at day 28 (Figure 2A, Table 1). Microscopic analysis of the cellular infiltrate of both joint cavity and synovial lining showed that the percentage of neutrophils (PMNs) was notably different between acute and chronic SCW arthritis (Figure 2B). The neutrophil content in the joint cavity was >90% at day 1 after intraarticular injection of SCW fragments, whereas less than 20% of the cells in the joint cavity were PMNs at 24h after the last reactivation (day 23) of chronic SCW arthritis (Figure 2B). Figures 2C/D show the PMNs content of the synovial cavity at day 1 and day 23 in wild type mice. To demonstrate the distinct phases in this particular arthritis model we investigated cellular content of the inflamed synovium by using cell markers. Both microarray and real-time PCR technology were used. Figure 2E showed that PMN-influx (determined as MPO gene expression) is clearly higher in the acute phase than in the late phase. In contrast to MPO, we found that F4/80 (macrophage marker) was strongly upregulated in the late chronic stage.

Mice deficient for caspase-1 tended to have less joint swelling, although this was not significantly different from wild-type mice (Figure 3A). In contrast, IL-1βko mice did not develop the chronic stage of this arthritis model, as previously described (26). Histology taken at day 28 demonstrated that caspase-1−/− mice expressed significantly less synovial inflammation, cartilage damage and bone erosion than wild-type controls (Table 1). Of interest, enhanced cartilage matrix PG production was noted in caspase-1−/− mice indicating less IL-1 activity since IL-1β−/− mice were completely protected against cartilage PG loss (see also Figure 3E/F, 25). Moreover, synovial mRNA expression at day 28 for IL-1β, iNOS and COX-2 were strongly reduced, all three mediators are known to be involved in suppression of chondrocyte PG synthesis (data not shown).

Enhanced synovial concentrations of IL-1β in caspase-1 deficient mice in both acute and chronic SCW arthritis

Several studies indicated that IL-1β is the pivotal cytokine with respect to cartilage catabolism during experimental arthritis (26,29,30). Since we observed striking differences in the way caspase-1−/− mice responded during acute and chronic experimental arthritis, in terms of chrondrocyte metabolism and cartilage damage, we measured immune-reactive and bioactive IL-1β in synovial tissue washouts. Figure 3B shows that shortly after injection of SCW fragments, both in acute and chronic SCW arthritis, IL-1β protein concentrations were 2–3 fold higher ($p<0.01$) in caspase-1−/− mice than in wild type mice. Of interest, IL-1β protein concentrations declined rapidly in caspase-1−/− in chronic SCW arthritis but not in acute SCW arthritis (Figure 3B). Analysis of bioactive IL-1β concentrations, using a cell-based assay, showed similar results as total protein measurement of IL-1β, although the synovial concentrations of bioactive IL-1β were 2-fold lower than total IL-1β concentrations (Figure 3C). The metabolic function of chondrocytes was rapidly restored in mice deficient for caspase-1 in chronic SCW arthritis (Figure 3D). In contrast, during acute SCW arthritis chondrocyte anabolic function PG was strongly affected in both caspase-1 and wild-type mice (Figure 3D).

Proteinase 3 can process precursor IL-1β to active IL-1β

Enzymes other than caspase-1 can process IL-1β in vitro and these enzymes are predominantly neutrophil-derived serine proteases (16,31). Since we noted a strong influx of neutrophils into the joint cavity after induction of SCW arthritis, we investigated whether one of these serine proteinases, namely proteinase 3 (PR3) can process pro-IL-1β into bioactive IL-1β. Figure 4A shows that PR3 cleaves inactive pro-IL-1β into bioactive IL-1β, using A549 cells for IL-1β-induced IL-8 production. Processing of pro-IL-1β by PR3 was further confirmed by the use of a specific ELISA. The concentration of pro-IL-1β clearly decreased after incubation with PR3 whereas mature IL-1β concentrations were increased (Figure 4B/C).

Blockade of both caspase-1 and PR3 results in reduction of bioactive IL-1β

To investigate whether blockade of PR3 and caspase-1 leads to less active IL-1β in SCW-induced acute as well as chronic arthritis, we performed studies in mice lacking neutrophil proteases, including active PR3 (DPPI−/− mice, 32) treated with a potent caspase-1 inhibitor (24). Deficiency of PR3 in combination with caspase-1 inhibition did not reduce joint swelling in acute SCW arthritis but showed a moderate reduction of joint swelling in chronic SCW arthritis (data not shown). However, chondrocyte PG synthesis was completely protected in DPPI−/− mice exposed to the caspase-1 inhibitor compared to the wild type control mice (Figure 5A). These results are comparable to those found in IL-1β−/− mice (Figure 1B). Analysis of total IL-1β protein concentrations revealed that in wild type and DPPI−/− mice, total IL-1β protein content was roughly similar with or without caspase-1 inhibitor (data not shown). As indicated by the chondrocyte metabolic function, the amount of bioactive IL-1β was strongly reduced in the DPPI−/− mice treated with caspase-1 inhibitor (Figure 5B). Histological analysis at day 2 showed that the PG content of the articular cartilage displayed intense loss of matrix PG in wild-type, wild-type/caspase-1 inhibitor, and DPPI−/− mice (Figure 5C/D/E). Of great interest, DPPI−/− mice injected with caspase-1 inhibitor showed no loss of cartilage PG (Figure 5F/Table 1). Furthermore, there was protection against severe cartilage damage at day 28 in DPPI−/− mice treated with caspase-1 inhibitor when compared with DPPI−/−, wild-type/caspase-1 inhibitor or wild-type mice (Table 1). In addition, bone erosion noted in chronic SCW arthritis at day 28 was reduced when caspase-1 was inhibited in DPPI−/− mice when compared to control DPPI−/− mice (Table 1). This latter is highly IL-1β dependent since IL-1β−/− mice were fully protected against bone erosion (Table 1). Induction of acute or chronic SCW arthritis in mice deficient for other classes of neutrophil serine proteinases revealed that matrixmetalloproteinase (MMP)-9, cathepsin G or elastase (Beige/Beige mice) were not involved in joint inflammation or cartilage damage (Table 1).

Discussion

In the present study we dissected the mechanisms of IL-1β activation during an experimental model of arthritis, and the differential role played in this process by caspase-1 on the one hand and serine proteinases, especially PR3, on the other. In line with previous studies, we demonstrated that while joint swelling is mainly mediated by TNF, production of IL-1β is crucial for proteoglycan loss. Caspase-1 and serine proteinases like PR3 are redundant for the release of bioactive IL-1β during joint inflammation, but blocking both processing systems results in an almost complete inhibition of IL-1β activation and protection against severe articular cartilage damage.

IL-1β is a proinflammatory cytokine that lacks a signal peptide and needs cleavage in order to be activated and released (33). The cysteine protease caspase-1 as well as the neutrophil serine proteinases cathepsin G, elastase and in particular PR3 are known to cleave pro-IL-1β

(16,34,35). We demonstrate that cleavage of pro-IL-1β by caspase-1 and by PR3 results in the release of bioactive IL-1β. Although IL-1β seems to be dispensable for induction of edema and swelling during the acute phase of the inflammatory reaction, as shown in the IL-1β−/− mice, its role is crucial for chondrocyte proteoglycan synthesis and tissue destruction (36). In contrast to the acute phase, IL-1β strongly contributes to joint swelling and inflammation during the chronic phase of arthritis.

During inflammation, IL-1β can be released by a variety of leukocytes such as monocytes, macrophages and neutrophils (2,8). The acute inflammation of arthritis is characterized by a rich infiltrate consisting of both neutrophils and monocytes/macrophages. When caspase-1 is absent in the knock-out mice, little effect is observed on IL-1β production and proteoglycan loss during acute arthritis, demonstrating that caspase-1 activation of IL-1β is not necessary in that stage. Most probably, the abundance of neutrophils is responsible for the release of serine proteinases during acute inflammation. These enzymes are produced as *zymogens* that require enzymatic processing to become active. The signal-peptide of the serine proteases is cleaved by a signal peptidase called dipeptidyl peptidase I (DPPI). The latter activation occurs just before the serine proteases are stored in the granules of neutrophils (32). It has been shown in the past that DPPI−/− mice do not develop anti-collagen antibody- and type II collagen-induced arthritis, two experimental models on which initiation is based on immune complex formation in the joint (37,38). One of the mechanisms why DPPI−/− mice do not develop immune complex induced arthritis may be that fact that mast cells of DPPI−/− mice lacking active tryptase. Tryptase needs to be activated by DPPI for the production of chemokines, such as MCP-1 and IL-8, by mast cells. It is known that mast cells are crucial for the development of immune complex induced arthritis models (39,40). However, we demonstrated here that DPPI−/− mice develop aggravated joint inflammation when arthritis is induced by intraarticular injection of arthritic stimuli, such as SCW fragments or zymosan (Table 1, CTP unpublished data). Due to the lack of active serine proteinase in DPPIko mice the clearance of either SCW fragments or zymosan particles from the joint may be delayed.

The mere absence of PR3 alone in the DPPI−/− mice did not lead to decreased IL-1β production, likely due to presence of caspase-1. However, when the activity of both enzymes was inhibited due to administration of a potent caspase-1 inhibitor to DPPI−/− mice, IL-1β bioactivity was completely blocked and proteoglycan loss reversed. During the chronic phase of arthritis, the relative importance of caspase-1 increases as suggested by the partial protection of caspase-1−/− mice, although a clear role of PR3 is still present as shown in the DPPI−/− mice. The importance of caspase-1 in chronic inflammation is most likely a consequence of the predominance of macrophages and the presence of few neutrophils, in the infiltrate in this phase of arthritis. The dichotomy between the role of caspase-1 in acute versus chronic inflammation is supported by experimental colitis models in which disruption of the caspase-1 gene led to protection in chronic models (41). These colitis models are also characterized by a minor role of neutrophils and a crucial role for monocytes and T-cells (42). However, in LPS-induced endotoxic shock models it was shown that caspase-1 was essential. LPS shock models are hyper acute models of inflammation in which the IL-1β production peaked at 90 minutes. Monocytes are crucial for the LPS-induced IL-1β production due to that fact that monocytes express already active caspase-1 they can rapidly produce mature IL-1β (43). In the most arthritis models the local production and activation of IL-1β is macrophage- or granulocyte (PR3) - dependent.

Our studies point towards PR3 as the pivotal serine protease responsible for the effects observed in the DPPI−/− mice, since the two other major neutrophil serine proteinases elastase (NE) and cathepsin G were not able to process proIL-1β in vitro (16,35). In addition, it was shown that mice lacking elastase and cathepsin G were not protected against severe cartilage destruction in a model of experimental arthritis (18). The present studies

have shown that lack of MMP-9, neutrophil elastase and cathepsin G (Beige/Beige mice) does not impact the development of SCW arthritis, and this suggests that PR3 plays the most important role (Table 1). It has been demonstrated that PR3 is the most important serine protease released by neutrophils (44) that process IL-1β, as well as IL-32 (45). From this perspective, PR3 is important in inflammation. The final proof for the role of PR3 in arthritis should come from PR3−/− mice, which are not available at this moment. However, we cannot exclude that potentially DPPI has a PR3-unrelated effect on IL-1β release.

The findings of this study have clear clinical relevance. They provide an explanation for the clinical effect of anti-IL-1-based therapy such as anakinra (recombinant IL-1 receptor antagonist) and the fully human anti-IL-1β antibody ACZ885 (46,47), but the possible failure in early clinical trials of caspase-1 inhibitors such as VX-765. In the present study it is shown that under inflammatory conditions caspase-1 activity and neutrophil serine proteases can process pro-IL-1β to mature IL-1β. Moreover, our data clearly demonstrate that processing of pro-IL-1β by serine proteases can compensate for the inhibition of caspase-1, especially in inflammatory foci in which neutrophils are abundant. From this perspective one should probably apply therapies directed towards caspase-1 only in inflammatory conditions in which neutrophils do not play a major role. A therapy which should be probably favored is one in which a combination of caspase-1 and serine protease inhibitors is used.

In conclusion, we show here that caspase-1-independent processing of IL-1β occurs in arthritis by serine proteinases such as PR3. Activation of IL-1β independent from caspase-1 is especially apparent in the acute phase of inflammation characterized by a predominantly neutrophilic infiltrate that serves as a source for PR3. It is therefore likely that only therapies based on the dual inhibition of caspase-1 and serine proteinases will be successful in the complex inflammatory diseases of humans.

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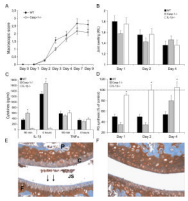

Figure 1. Acute arthritis in caspase-1−/− mice

A. K/BxN arthritis in caspase-1−/− mice. Arthritis was induced by i.p injection of 200μl arthritic serum at day 0. Data are expressed as mean±SEM of 7 mice per group. B. Acute SCW-induced arthritis in caspase-1−/− mice. Joint swelling at days 1, 2 and 4 after intraarticular injection of 25μg SCW fragments, measured by radioactive ^{99m}Tc-uptake method. The swelling is expressed as a right-left ratio and a ratio > 1.15 is indicated as inflammation. Data are expressed as mean±sem of 7 mice per group. C. Local IL-1β (both IL-1β and proIL-1β) and TNFα concentrations in synovial tissue explants, 90 minutes and 4 hours after induction of arthritis. Data represents the mean±SEM of 5 explants per group. D. Chondrocyte metabolic function determined in patellar cartilage explants by 35S-sulphate incorporation. Note almost complete protection against inhibition of chondrocyte PG synthesis in IL-1β−/− mice at day 1 and 2. Data represents the mean±SEM of 5 explants per group. E. Cartilage proteoglycan (PG) depletion visualized by Safranin O staining (arrows indicated PG depletion) at day 7 of SCW arthritis, 200 × magnifications. P= patella, F= femur, C = cartilage, JS = joint space. F. Cartilage PG depletion in caspase-1−/−mice, noted the reduction of PG loss. *P<0.01 compared to wild-type control mice, Mann-Whitney U-test.

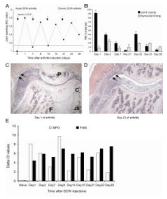

Figure 2. Induction of chronic SCW arthritis in C57Bl/6 mice

A: Arthritis, induced by intraarticular (i.a.) injections of 25 μg SCW fragments at days 0, 7, 14 and 21, leads to a chronic inflamed joint at day 28. Note that the joint swelling remains detectable after the second i.a. injection (dotted line indicates detectable joint swelling). B. Cellular influx during the course of chronic SCW-induced arthritis. Predominantly PMN influx in acute (1st injection) SCW- and shortly after the 4th reactivation (day 22) of chronic SCW-induced arthritis. C. PMN influx in the joint cavity at day 1 of SCW arthritis (wild-type mouse). H&E staining, 40×. D, Cellular influx in synovial lining and joint cavity at day 23 of chronic SCW arthritis. H&E staining, 40×. Note the difference in synovial lining between day1 and day 23 (arrows). E, Cell marker expression in inflamed synovium. MPO (PMN) and F4/80 (Macrophage) mRNA expression was determine during the course of SCW-induced arthritis. Note that MPO is predominantly expressed in the acute stages whereas F4/80 is seen at the more chronic stages. For details, see Figure 1.

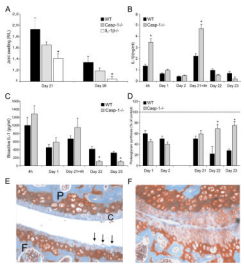

Figure 3. Chronic SCW-induced arthritis in caspase-1−/− mice

A. Joint swelling at days 21 and 28 in caspase-1−/− mice, compared to wild-type and IL-1β −/− mice. Data are expressed as mean±SEM of 7 mice per group. *P<0.01 compared to wild-type control mice, Mann-Whitney U-test. B. Total (pro-IL-1β and mature) IL-1β protein concentrations were measured at several time points after induction of SCW arthritis in wild-type and caspase-1−/− mice. Significantly higher IL-1β concentrations were found in synovial tissue explants isolated from casapase-1−/− mice at 4h and day 21, shortly after SCW exposure. Data represents the mean±SEM of 5 explants per group. C. Bioactive IL-1 was determined by using modified NOB-1 bioassay. Significant reduction of bioactive IL-1 in synovial explants from caspase-1−/− mice at days 22 and 23. Data represents the mean ±SEM of 5 explants per group. D. Chondrocyte metabolic function determined in patellar cartilage explants. Less suppression of chondrocyte PG-synthesis in caspase-1−/− mice was seen at days 22 and 23, when compared to wild-type mice. *P<0.01 compared to wild-type control mice, Mann-Whitney U-test. Data represents the mean±SEM of 5 explants per group. E. Cartilage proteoglycan (PG) depletion (indicated by arrows) at day 28 of chronic SCW arthritis, 200× magnification. F. Cartilage PG loss in a caspase-1−/− mouse. Note the intense staining around the chondrocytes, indicating enhanced PG synthesis (200×). *P<0.01 compared to wild-type control mice, Mann-Whitney U-test.

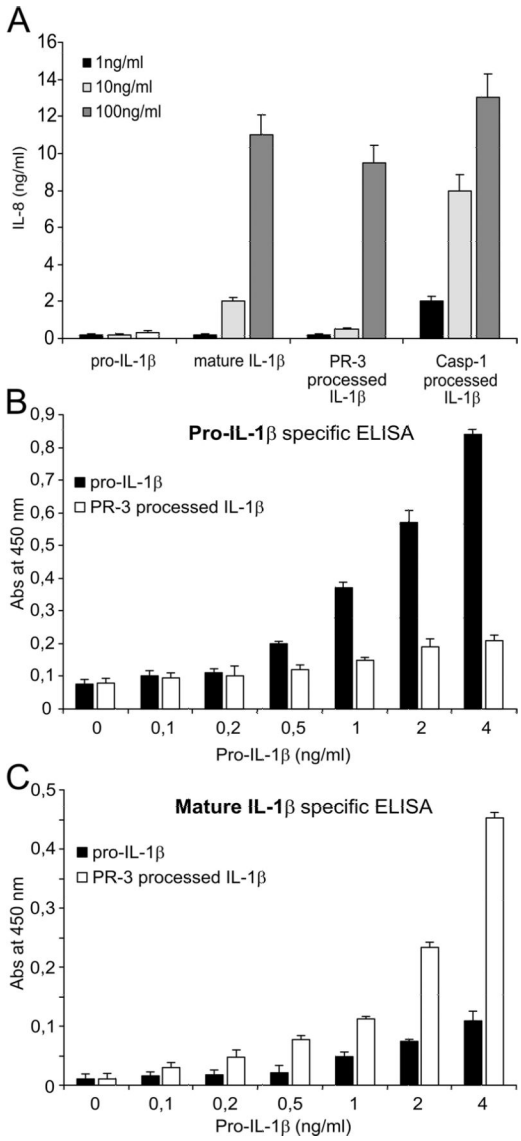

Figure 4. PR3 activates pro-IL-1β

A. PR3-cleaved IL-1β induced IL-8 production. Human recombinant pro-IL-1β (1–100 ng/ml) was incubated for 120 minutes with either PR3 or caspase-1 (10 μg/ml). The resulting products were used to stimulate A549 cells. After 24h, supernatants were collected and IL-8 was measured. B/C. Cleavage of pro-IL-1β. Human recombinant pro-IL-1β (10ug/ml) was incubated for 1h with PR3 (100ng/ml). The resulting products as were assayed using an ELISA specific for pro-IL-1β (B) or mature IL-1β (C). Incubation of pro-IL-1β with PR3 lead to reduced recognition of molecule in the pro-IL-1β assay and increased recognition in the mature assay. The cleavage experiments were performed in triplo. Data are expressed as mean±SEM.

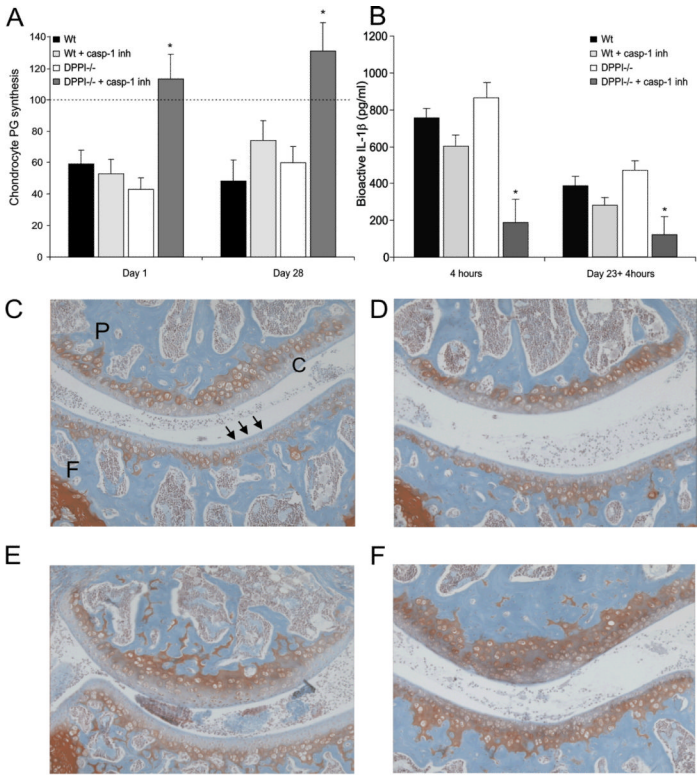

Figure 5. Blocking of caspase-1 and PR3 in acute and chronic SCW arthritis

Chondrocyte PG-synthesis at days 1 and 28 (A). Mice were treated with 100mg/kg caspase-1 inhibitor daily. Acute arthritis from days 0 (−2h) to 2, and chronic arthritis from days 14 (−2h) to 28. DPPI−/− mice treated with casapase-1 inhibitor showed complete protection against inhibition of chondrocyte PG synthesis, indicating no bioactive IL-1. Data represents the mean±SEM of 5 explants per group. B. Bioactive IL-1. Strong reduction of the bioactive IL-1 in DPPI−/− mice treated with caspase-1 inhibitor, when compared to wild-type mice, wild-type mice with capsase-1 inhibitor or DPPI−/− mice. Data represents the mean±SEM of 5 explants per group. C/D/E. Severe cartilage PG depletion at day 2 of SCW-induced arthritis in Balb/C wild-type, Balb/C wild-type completed with caspase-1 inhibitor or DPPI−/− mice. F. Reduced cartilage damage in DPPI−/− mice treated with caspase-1 inhibitor. Note the reduced cartilage PG-loss. Safranin-O staining, 200×. For details see Figure 1. *P<0.01 compared to wild-type control mice, Mann-Whitney U-test.

Table 1

Histopathology in caspase-1−/−, IL-1β−/−, DPPI−/−, MMP-9−/− and Beige/beige mice.

	Days of arthritis	Cell Influx[#]	Proteoglycan depletion[‡]	Cartilage damage[†]	Bone erosion[*]
Wild type[1](C57/B16)	Day 2	2.0±0.3	0.4±0.2	-	-
Caspase-1−/−	Day 2	1.8±0.4	0.4±0.3	-	-
IL-1β−/−	Day 2	1.7±0.4	0.3±0.1	-	-
Wild type[1](C57/Bl6)	Day 7	0.4±0.3	1.8±0.5	-	-
Caspase-1−/−	Day 7	0.2±0.1	**0.8±0.3** ¶	-	-
IL-1β−/−	Day 7	0.1±0.2	**0.1±0.2** ¶	-	-
Wild type[1](C57/Bl6)	Day 28	1.2±0.3	2.5±0.5	1.3±0.3	1.0±0.4
Caspase-1−/−	Day 28	**0.6±0.2** ¶	**1.0±0.3** ¶	**0.7±0.2** ¶	0.5±0.2
IL-1β−/−	Day 28	**0.2±0.1** ¶	**0.0±0.0** ¶	**0.0±0.0** ¶	**0.0±0.0** ¶
Wild type[2](Balb/C)	Day 2	1.2±0.3	1.5±0.2	-	-
Wt[2]/Casp-1 inh.	Day 2	0.8±0.2	1.3±0.3	-	-
DPPI−/−	Day 2	1.9±0.4	1.7±0.3	-	-
DPPI−/−/Casp-1 inh.	Day 2	1.3±0.3	**0.6±0.4** ¶	-	-
Wild type[2](Balb/C)	Day 28	1.2±0.3	1.8±0.5	1.5±0.3	1.0±0.2
Wt[2]/Casp-1 inh.	Day 28	1.0±0.2	1.5±0.4	1.1±0.5	0.6±0.3
DPPI−/−	Day 28	2.1±0.4	2.7±0.3	1.8±0.4	1.3±0.4
DPPI−/−/Casp-1 inh.	Day 28	**1.2±0.3** ¶	**1.4±0.4** ¶	**0.5±0.2** ¶	**0.4±0.2** ¶
Wild type[3]	Day 28	1.4±0.3	2.5±0.5	1.0±0.4	1.0±0.3
MMP-9−/−	Day 28	1.6±0.4	2.0±0.3	1.2±0.4	0.8±0.2
Wild-type[1]	Day 28	1.8±0.3	2.2±0.3	1.0±0.3	0.8±0.4
Beige/Beige	Day 28	1.4±0.2	2.6±0.5	0.9±0.5	0.9±0.3

Histopathology was examined on days 2 or 7 of acute SCW-induced arthritis and day 28 of chronic SCW-induced arthritis. For acute SCW mice were intraarticularly (i.a.) injected only at day 0. For chronic SCW fragments were i.a. injected at days 0, 7, 14 and 21. Data represent the mean±SD score of at least 6 mice per group.

DPPI=Dipeptidyl peptidase I. MMP-9=matrix metalloproteinase 9. Beige/Beige=mice lacking neutrophil elastase and cathepsin G.

[#]Number of inflammatory cells in synovial tissue on a scale from 0–3.

[‡]Proteoglycan depletion reflects the loss of Safranin O staining in the cartilage, ranging from 0 to 3.

† Cartilage damage reflects erosion of the surface and chondrocyte death on a scale of 0–3.

* Bone erosion was graded on a scale from 0–5 ranging from no damage to complete loss of the bone. Patella, tibia and femur were scored for bone erosion.

¶ P<0.01, Mann Whitney U-test

1 compared to the wild type mice (C57/Black6)

2 wild type mice (Balb/C) strain.

3 Wild-type (C57Black/6 × 129Sv).